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Etablierung und Evaluierung der in vivo Isolation von zirkulierenden Tumorzellen für die individualisierte Diagnostik des Prostatakarzinoms

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Die Therapieoptionen für das metastasierte Prostatakarzinom (PCa) werden zunehmend zielgerichteter und müssen individuell abgestimmt werden. Allerdings ist für eine optimale Therapieentscheidung eine wiederholte Beurteilung des Tumors notwendig. Der „Goldstandard" in der Tumordiagnostik ist bisher die konventionelle Nadelbiopsie. Diese setzt aber einen operativen und damit invasiven Eingriff voraus. Darüber hinaus ist eine Probenentnahme nicht immer möglich. Eine Alternative können zirkulierende Tumorzellen (CTCs) als blutbasierte Biomarker bieten. CTCs sind Zellen, denen es gelungen ist, sich vom Tumor zu lösen und in den Blutkreislauf zu gelangen. Sie präsentieren Subklone des Karzinoms zu einem definierten Zeitpunkt, wodurch molekulare Veränderungen im Tumor z.B. unter Therapie festgestellt werden können. Allerdings stellt die geringe Konzentration der CTCs im Blut eine große technische Herausforderung für ihre Detektion dar. Alle bisher bekannten CTC-Isolationstechnologien haben unterschiedliche Limitationen, ihr gemeinsamer Nachteil ist das geringe Blutvolumen (≤ 30 ml), welches für die CTC-Isolation zur Verfügung steht. Aus diesem Grund fokussiert diese Habilitationsschrift die Etablierung und Evaluation des CellCollectors (CC), eine in vivo CTC-Isolationstechnologie bei Patienten mit Prostatakarzinom. Der CC ist ein an der Spitze mit EpCAM-Antikörpern funktionalisierter Edelstahldraht. Während seiner Anwendung verbleibt der Draht 30 min in der Cubitalvene, dadurch können bis zu 600 ml Blut auf CTCs untersucht werden.
Ausgangspunkt war die ex vivo Eignungsprüfung des CCs für die Isolation von CTCs aus Blutproben von Patienten mit Prostatakarzinom. In Abhängigkeit vom Tumorstadium konnte bei lokal (PCa-l), lokal-fortgeschritten (PCa-la) und metastasierten (PCa-m) Patienten eine unterschiedliche Anzahl von CTCs detektiert werden. Weiterführend erfolgte die molekulare CTC-Charakterisierung auf mRNA-Ebene, wobei die Transkripte vom Epidermal Growth Factor Receptor oder dem Prostata-Spezifisches-Membran-Antigen in den CTCs von PCa-m Patienten nachgewiesen werden konnten.
Die ersten in vivo Anwendungen des CCs zeigten in unserem Studienkollektiv eine sehr gute Verträglichkeit. Signifikant unterschiedliche CTC-Zahlen wurden in den Gruppen der lokal und metastasierten PCa-Patienten isoliert. Darüber hinaus konnte ein Unterschied im Gesamtüberleben (OS) aufgrund der CTC-Zahleinteilung ≥ 5 CTCs und < 5 CTCs detektiert werden. Eine Bewertung der in vivo CTC-Isolation erfolgte durch den Vergleich mit dem FDA zertifizierten CellSearch-System. Der CC erreichte eine 78,4 %ige und das CellSearch-System eine 67 %ige Detektionsrate bei den PCa-m Patienten, was auf eine höhere Sensitivität des CCs in der CTC-Isolation hinweist. Beide CTCs-Isolationstechnologien zeigen in der ROC-Kurvenanalyse eine vergleichbare diagnostische Genauigkeit der CTC-Zahl. Nur auf die Gruppe der Patienten mit einem metastasierten kastrationsresistenten Prostatakarzinom (CRPC) begrenzt erreicht der CC eine höhere Sensitivität im Nachweis von CTCs, jedoch isolierte das CellSearch-System die höhere CTC-Zahl. Weiterführend wurde in dieser Arbeit eine PCa-spezifische Funktionalisierung des CCs entwickelt, welche eine sensitive und spezifische Isolierung von CTCs aus Blutproben von PCa-m Patienten erreichte. Im Vergleich mit weiteren Isolationstechnologien zeigte die PCa-spezifische Funktionalisierung des CCs die höchste Detektionsrate. Kultivierungsfähige CTCs konnten aber ausschließlich mit einem antiköperunabhängigen System isoliert werden.
Aus den durch diese Arbeit gewonnenen Erkenntnissen resultiert, dass der CC eine sensitive Methode für die in vivo Isolation von CTCs bei Patienten mit PCa ist. In der mCRPC-Patientengruppe hat das CellSearch-System eine höhere diagnostische Genauigkeit. Darüber hinaus zeigt sich, dass die optimalste Isolationstechnik nicht auf eine Eigenschaft der CTCs beschränkt sein darf. CTCs bieten die Möglichkeit die Patientenheterogenität zwischen identischen Tumorentitäten zu erkennen, die dynamische molekulare Veränderung der Erkrankung zu überwachen und so die personalisierte Therapie zu unterstützen.

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II Abkürzungsverzeichnis

ADT	Androgen Deprivation Therapie
ALK	Alkalische-Phosphatase-Konzentration
AR	Androgenrezeptor
AR-V7	Androgenrezeptor-Spleißvariante-Variante7
ARS	Androgenrezeptorsignalweg
BPH	Benigne Prostatahyperplasie
BfArM	Bundesinstitut für Arzneimittel und Medizinprodukte
bzw.	beziehungsweise
ca.	circa
CC	CellCollector
CD	Cluster of Differentiation
CK	Cytokeratin
CTCs	Circulating Tumor Cells/zirkulierende Tumorzellen
DAPI	4', 6-Diamidino-2-phenylindol-dihydrochlorid
DNA	Deoxyribonucleic Acid
DTC	Disseminierte Tumorzellen
DRU	Digital-rektale Untersuchung
DKK-1	Dickkopf-1
EGFR	Epidermal Growth Factor Receptor
EBRT	External Beam Radiotherapy/perkutane Bestrahlung
EMT	Epithelial-mesenchymale Transition
EpCAM	Epithelial cell adhesion molecule/epitheliales Adhäsionsmolekül
FDA	Food and Drug Administration
HER2	Human Epidermal Growth Factor Receptor 2
HIFU	Hochintensiver Fokussierter Ultraschall
HR	Hazard Ratio
ISUP	International Society of Urological Pathology
IL	Interleukin
KI	Konfidenzintervall
KLK3	Kallikrein-3
LDH	Laktatdehydrogenase
LNCaP	Lymph Node Carcinoma of the Prostate
LHRH	Luteinisierendes Hormon Releasing Hormon
LK	Lymphknoten
MGG	May-Grünwald-Giemsa

mRNA	messenger Ribonukleinsäure/ Ribonucleic Acid
MET	Mesenchymale-epithelial Transition
MW	Mittelwert
Mo.	Monat
OS	Overall Survival/Gesamtüberleben
o.g.	oben genannt
PARP	Poly-ADP-Ribose-Polymerase
PCa-l	lokales Prostatakarzinom
PCa-la	lokal fortgeschrittenes Prostatakarzinom
PCa-m	metastasiertes Prostatakarzinom
mCRPC	metastasiertes kastrationsresistentes Prostatakarzinom
PCa	Prostatakarzinom
RT-PCR	Reverse-Transkriptase-Polymerase-Kettenreaktion
RNA	Ribonucleic Acid
PSA	Prostataspezifisches Antigen
PSCA	Prostata-Stammzell-Antigen
PSMA	Prostataspezifisches Membranantigen
ROC	Receiver-Operating-Characteristics
RP	Radikale Prostatektomie
SC	ScreenCell
TNM	Tumor, Node and Metastasis
sHER2	soluble Human Epidermal Growth Factor Receptor 2
TGF-β	Transforming Growth Factor β
TUR-P	Transurethrale Resektion der Prostata
UICC	Union for International Cancer Control
u.a.	unter anderem
VEGF	Vascular Endothelial Growth Factor
vs.	versus
z.B.	zum Beispiel

1 Einleitung

1.1 Prostatakarzinom

Das Prostatakarzinom (PCa) wurde im Jahr 2020 bei 1,4 Millionen Männern weltweit diagnostiziert. Mit einem Anteil von rund 6,8 % steht es an fünfter Stelle bei den karzinombedingten Todesfällen [1]. Für Deutschland lag die Inzidenz gemäß Robert-Koch-Institut im Jahr 2018 bei etwa 65.200 Neuerkrankungen und mit 24,9 % ist es die häufigste Krebserkrankung beim Mann. Bei den zum Tode führenden Krebserkrankungen belegt das Prostatakarzinom den zweiten Platz mit 14.963 verstorbenen Patienten [2]. Die 10-Jahres-Prävalenz liegt bei 470.000 Patienten [3]. Autopsie-Daten zeigten eine Prävalenz von 21 % über alle Altersgruppen (20-90 Jahre), wobei meist kleine (4 cm^3) und gut differenzierte Adenokarzinome nachgewiesen wurden. Jedoch verdoppelt sich der Nachweis von schlecht differenzierten PCa mit jeder Lebensdekade [4]. Das Risiko für einen 75-jährigen Mann in den nächsten zehn Jahren an einem PCa zu erkranken liegt bei 6 %. Hingegen liegt es bei einem 35-jährigen Mann unter 0,1 %. Diese altersabhängige Inzidenz ist der wichtigste Risikofaktor für Männer an einem Prostatakarzinom zu erkranken. Im Vergleich zu anderen Tumorentitäten ist das mittlere Erkrankungsalter hoch und liegt bei 71 Jahren, im Mittel versterben die Patienten neun Jahre nach Diagnose [5]. Die absehbare demographische Entwicklung in Deutschland hat eine Erhöhung des Anteils der Älteren zur Folge, was mit einer deutlich steigenden Prävalenz und Inzidenz des Prostatakarzinoms verbunden sein wird [2]. Weitere etablierte Risikofaktoren sind die Ethnie und eine positive Familienanamnese, welche das relative Risiko für das Auftreten der Erkrankung bei erstgradigen Verwandten um 2,5-4,3 % erhöht [5].

1.1.1 Klinik und Diagnostik

Das Prostatakarzinom ist im lokalen auf die Prostata begrenzten Stadium meist asymptomatisch. Im fortgeschrittenen Stadium wird der Tumor evident und es kann zu Miktionsbeschwerden, erektiler Dysfunktion und Hämaturie kommen. Dies weist auf ein bereits lokal-infiltratives Wachstum in den Blasenhals, die Urethra und das umliegende neurovaskuläre Gewebe hin. Das metastasierte Stadium kann Symptome wie Anämie, Knochenschmerzen und periphere Lymphödeme verursachen [5]. Dies waren es die ersten klinischen Symptome, welche häufig zur Diagnose des Prostatakarzinoms führten. Infolgedessen ist es wichtig, dass im Rahmen der Früherkennung Karzinome im lokalen Stadium detektiert werden, damit eine kurative Behandlung des Patienten erfolgen kann.
Aktuell wird die Prostatakarzinomfrüherkennung in Deutschland für Männer ab 45 Jahren einmal jährlich empfohlen und beinhaltet die Untersuchung der äußeren Geschlechtsorgane, die digital-rektale Tastuntersuchung der Prostata sowie Lymphknotenpalpation. Außerdem

kann nach einer ergebnisoffenen Aufklärung über die Vor- und Nachteile des Testergebnisses eine Bestimmung des prostataspezifischen Antigens (PSA) im Serum angeboten und mit zur Früherkennung herangezogen werden [4]. Die Bestimmung des PSA-Wertes im Serum wurde als organspezifischer Tumormarker für das Prostatakarzinom von der Food and Drug Administration (FDA) 1986 zugelassen. PSA ist auch als humanes Kallikrein 3 (KLK3) bekannt und gehört zur Familie der Serinproteasen. Es ist eine Androgen-regulierte Protease und wird von Prostataepithelzellen produziert. Nur ein geringer Teil wird ins Serum sezerniert [6]. Die Transkription wird durch Androgene induziert, wobei der aktivierte Androgenrezeptor (AR) als Transkriptionsfaktor für das KLK3-Gen fungiert, mit dem Resultat einer vermehrten Synthese von PSA [7]. Die Expression von PSA ist im Wesentlichen auf das Prostatagewebe begrenzt und sowohl bei malignen als auch bei benignen Prostatazellveränderungen erhöht. Der PSA-Wert ist somit ein organspezifischer, aber kein tumorspezifischer Wert. Erhöhte Konzentrationen im Serum können temporär durch benigne Prostatahyperplasie (BPH), Prostatitiden, Harnverhalt oder Manipulationen der Prostata verursacht werden. Des Weiteren kann der PSA-Wert durch verschiedene Medikamente (Finasteride, Antiandrogene, LHRH-Analoga) beeinflusst werden [5]. Maligne Prostatazellen exprimieren jedoch deutlich mehr PSA als normales oder hyperplastisches Prostatagewebe.

Die Diagnose eines PCa`s führt über verschiedene Schritte, dazu gehören die Anamnese, die klinische Untersuchung, das Blut-Laborscreening (PSA-Wert), die histologische Sicherung und bildgebende Verfahren. Der Goldstandard der Diagnostik des Prostatakarzinoms ist die sonographie-gestützte, transrektale Prostatastanzbiopsie. Diese wird bei einem mindestens zweifach kontrolliertem PSA-Wert $\geq$ 4 ng/ml, einer auffälligen digital-rektalen Untersuchung oder einem kontinuierlichen PSA-Anstieg empfohlen [5].

1.1.2 Pathologie und Staging

Der größte Teil der Prostatakarzinome (> 90%) sind Adenokarzinome. Selten sind es andere Tumorentitäten wie Plattenepithelkarzinome, Urothelkarzinome oder Sarkome. Zur Klassifikation und zur Unterstützung der Prognoseerstellung des Prostatakarzinoms wird der histologische Differenzierungsgrad, das Gleason-Grading und das lokale Tumorausbereitungsmuster mittels TNM-Klassifikation (Tumor, Node and Metastasis) der Union for International Cancer Control (UICC) genutzt (Tab. 1)[8]. Das Gleason-Grading wurde erstmal 1966 von Donald Gleason veröffentlicht und beschreibt fünf Stadien der Drüsenstrukturveränderung des Prostatakarzinoms [9]. Die Summe aus vorherrschendem und zweithäufigstem Differenzierungsgrad der Drüsenmorphologie wird als Gleason-Score bezeichnet. Das geltende Graduierungssystem wurde 2016 von Epstein et al. [10] aktualisiert und beschreibt die fünf neu evaluierten histologischen Wachstumsmuster der

Drüsenstruktur (Abb. 1). Die Gleason-Muster 1 und 2 entsprechen sehr gut bis gut differenzierten Drüsen, welche sehr selten vorkommen und nicht mehr diagnostiziert werden sollten [11]. Das Gleason-Muster 5 zeigt keine glanduläre Differenzierung mehr und weist die höchste maligne Veränderung auf.

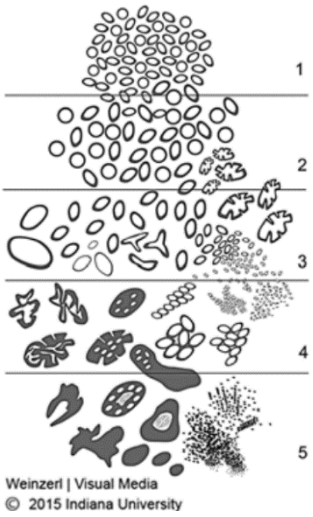

Abb. 1: Histologisches Muster des Prostatakarzinoms nach Epstein et al. [9].

Die TNM-Klassifikation beschreibt die anatomische Ausbreitung des Tumors, wobei die lokale Ausdehnung des Primärtumors (T), das Fehlen oder Vorhandensein von regionären Lymphknotenmetastasen (N) sowie das Vorhandensein von Fernmetastasen (M) bezeichnet wird (Tab. 1). Anhand dieser einheitlichen Angabe zur Tumorausbreitung erfolgt eine Stadieneinteilung und die damit verbundene wesentliche Prognoseabschätzung [8]. Das lokal begrenzte Prostatakarzinom umfasst die Stadien T1-T2 N0 M0 und das lokal fortgeschrittene Prostatakarzinom die Stadien T3-T4 N0 M0. Sollten die Patienten einen positiven N-Status und/oder M-Status aufweisen, liegt ein fortgeschrittenes bzw. metastasiertes Stadium des Prostatakarzinoms vor.

Tab. 1: TNM-Klassifikation der UICC für das Prostatakarzinom (2017) [8]

TNM	T: Primärtumor, N: Lymphknotenstatus, M: Metastasierungsstatus.
TX	**Primärtumor nicht beurteilbar**
T0	**Kein Anhalt für Primärtumor**
T1	**Klinisch nicht erkennbarer Tumor (weder tastbar noch bildgebend sichtbar)** **T1a:** Tumor zufälliger histologischer Befund in maximal 5 % des resezierten Gewebes **T1b:** Tumor zufälliger histologischer Befund in mehr als 5 % des resezierten Gewebes **T1c:** Tumor durch Nadelbiopsie bei erhöhtem PSA-Wert diagnostiziert
T2	**Tumor auf Prostata begrenzt** **T2a:** Tumor befällt maximal 50 % eines Prostatalappens **T2b:** Tumor befällt mehr als 50 % eines Prostatalappens **T2c:** Tumor in beiden Lappen
T3	**Tumor durchbricht die Prostatakapsel** **T3a:** Extrakapsuläre Ausbreitung (ein- oder beidseitig), Samenblase tumorfrei **T3b:** Tumor infiltriert Samenblasen
T4	Tumor ist fixiert oder hat sich auf benachbarte Strukturen (außer der Samenblase) wie Schließmuskeln, Rektum und/oder Beckenboden ausgebreitet
N	**Regionäre Lymphknotenmetastasen** **NX:** Regionäre Lymphknoten nicht beurteilbar **N0:** Kein Anhalt für Lymphknotenmetastasierung **N1:** Regionäre Lymphknotenmetastasen
M	**Fernmetastasen** **MX:** Fernmetastasen nicht beurteilbar **M0:** Kein Anhalt für Fernmetastasen **M1:** Fernmetastasen **M1a:** Nichtregionäre(r) Lymphknoten **M1b:** Knochen **M1c:** Andere Lokalisation(en)

1.1.3 Therapie des Prostatakarzinoms

Der klinische Verlauf des Prostatakarzinoms kann sehr unterschiedlich sein. Die lokal begrenzten Prostatakarzinome (klinisch M0) sind potenziell einer kurativen Therapie zugänglich. Innerhalb dieser Gruppe erfolgt eine Differenzierung nach dem Progressionsrisiko. Unter Berücksichtigung des initialen PSA-Wertes, des Gleason-Scores und des T-Stadiums erfolgt die Unterteilung in drei Risikogruppen nach D´Amico [12], (Tab. 2). Therapieformen sind die radikale Prostatektomie (RP), die Brachytherapie oder die perkutane Radiotherapie (EBRT). Primäre Therapieoptionen sind die RP und die EBRT, welche für alle Risikogruppen geeignet sind. Die Brachytherapie ist nur für Patienten mit einem niedrigen Risikoprofil gemäß S3-Leitlinie geeignet [5]. Eine vierte Therapieoption ist

die aktive Überwachung (Active Surveillance) für Patienten mit einem niedrigen Rezidivrisiko. Hier erfolgt keine Therapie, sondern engmaschige Kontrollen mit Rebiopsie.

Tabelle 2: Einteilung des lokal begrenzten Prostatakarzinoms bezüglich der Entwicklung eines Rezidivs in Risikogruppen nach D´Amico [12, 13]

Risiko	PSA	Gleason-Score	T-Stadium
niedriges Risiko	≤ 10 ng/ml	6	1c, 2a
intermediäres Risiko	> 10-20 ng/ml	7	2b
hohes Risiko	> 20 ng/ml	≥ 8	2c

Der „Goldstandard" im fernmetastasierten Stadium ist die medikamentöse Therapie. Das metastasierte Prostatakarzinom kann sich im hormonsensitiven oder im kastrationsresistenten Stadium befinden. Eine kurative Therapie ist in diesen Stadien nicht mehr möglich [5]. Neuere Daten zeigen, dass Patienten mit einem hormonsensitiven PCa von einer frühen Chemotherapie oder von einer Kombinationstherapie aus Androgendeprivationstherapie (ADT) mit einer neueren Generation der Hormonpräparate Abirateron, Apalutamid, Enzalutamid, Doralutamid profitieren [5, 14-16]. Die bevorzugten Chemotherapeutika sind Docetaxel und Cabazitaxel aus der Gruppe der Taxane.

Trotz Ansprechens auf ADT entwickeln ca. 90 % der Patienten innerhalb von ein bis drei Jahren ein kastrationsresistentes Prostatakarzinom (CRPC) [17]. Die Kriterien für das Vorliegen eines CRPC sind eine Testosteronkonzentration im Serum < 50 ng/dl unter Androgendeprivation, ein biochemischer Progress (3 konsekutive PSA-Wert-Anstiege um mehr als 50% des PSA-Nadirs, PSA > 2 ng/ml) oder mindestens zwei neue Knochenmetastasen oder eine neue Weichteilmetastase, radiologisch nachweisbar [18]. Die ADT sollte dennoch weiter erfolgen und durch eine systemische Chemotherapie mit Docetaxel (Zweitlinie Cabazitaxel) oder durch eine Therapie mit den Androgenrezeptor-Antagonisten der neuen Generation (androgen receptor axis-targeted agents (ARTAs) ergänzt werden [5].

Nach Ausschluss viszeraler Metastasierung ist eine Therapie mit Radium-223 möglich. Ein Therapieversuch mit Lutetium-177-PSMA kann erst nach Ausschöpfen aller o.g. Therapieoptionen erfolgen [5]. Als lokal symptomatische Therapie kann z.B. eine palliative transurethrale Resektion der Prostata (TUR-P) oder die Bestrahlung von einzelnen Metastasen zur Schmerzlinderung erfolgen [5].

In den letzten zwei Jahrzehnten haben die Ergebnisse von wegweisenden Phase 3 Studien zur Zulassung neuer Therapeutika mit unterschiedlichen Wirkmechanismen und damit zu einer mehr personalisierten Systemtherapie geführt. Dazu gehören Substanzen aus den Gruppen der Target-Therapie (Androgen-Rezeptor), der Immuntherapeutika, der Radionuklidtherapie und der Gruppe der PARP (Poly-ADP-Ribose-Polymerase)-

Inhibitoren [19, 20]. Für diese zielgerichteten Therapien sind dringend prädiktive Biomarker notwendig, damit ein individuelles Therapieansprechen vorhergesagt werden kann. Die Herausforderung für den behandelnden Arzt liegt hier in der Wahl der besten Therapiesequenz für den richtigen Patienten zum optimalen Zeitpunkt.

1.1.4 Metastasierung

Karzinome sind solide Tumore, bestehend aus einem Zellverband epithelialen Ursprungs. Das Prostatakarzinom geht aus dem drüsenbildenden Gewebe der Vorsteherdrüse hervor. Charakteristisch für den Zellverband des Karzinoms sind Zell-Zell- und Zell-Extrazelluläre Matrix-Kontakte über Adhäsionsmoleküle. Metastasierende Karzinome generieren Zellen oder Zellcluster, welche sich vom Zellverband lösen, in das Gefäßsystem infiltrieren und in entfernten Organen Metastasen bilden.

Die Mehrzahl der karzinombedingten Todesfälle (ca. 90 %) werden durch Metastasen verursacht, was u.a. auf die noch unzureichend verstandene multiple Metastasierungskaskade zurückzuführen ist [21]. Im Jahr 2000 und 2001 publizierten Hanahan und Weinberg acht Charakteristika von Tumorzellen als „Hallmarks of Cancer", welche für den vielschichtigen Prozess der Tumorgenese entscheidend sind. Für eine erfolgreiche Bildung einer Metastase in einem entfernt liegenden Organ müssen Tumorzellen folgende Fähigkeiten aufweisen: unabhängige Proliferation von Wachstumsfaktoren, uneingeschränkte Replikation des Genoms, Vermeidung einer Immunreaktion, Deregulation des zellulären Metabolismus, Apoptoseresistenz, Tumorangiogenese, Infiltration in fremde Gewebe und Ausbildung von Metastasen [22, 23]. Aktuell wurden weitere potentielle „Hallmarks of Cancer" für Tumorzellen von Hanahan publiziert, dazu zählen die uneingeschränkte phänotypische Plastizität, die nicht mutationsbedingte epigenetische Reprogrammierung, ein polymorphes Mikromileu des Tumors und die Seneszenz der Tumorzelle [24].

Am Anfang der Metastasierungskaskade steht die Infiltration der Tumorzellen in das lokale Stromagewebe. Die Prozesse, welche die Migration und Invasion der Zellen fördern, sind noch nicht vollständig geklärt. Tumore können sich mit Hilfe der Vascular Endothelial Growth Factor (VEGF)-induzierte Angiogenese während des Wachstums mit Nährstoffen und Sauerstoff versorgen. Überdies ermöglicht diese Neubildung von Blutgefäßen den Kontakt zwischen Tumorzelle und Gefäßendothel [25]. Diese Blutgefäße sind meist durchlässiger, was aus Fehlern in der Zellverbindung resultiert. So können Tumorzellen durch Tumorwachstum oder mechanische Kräfte in das Gefäßsystem gelangen [26]. Dieser Zustand ermöglicht dem Tumor die hämatogene Metastasierung. Tumorzellen, die das Blutgefäßsystem erreicht haben, bezeichnet man als zirkulierende Tumorzellen (CTCs).

Einzelne Epithelzellen im Blut unterliegen aufgrund von fehlenden Zell-Zell-Kontakten dem programmierten Zelltod (Anoikis). In der Blutzirkulation überleben nur CTCs, welche

widerstandsfähig gegenüber der Immunabwehr, dem Wegfall Stroma-bedingter Einflüsse des Primärtumors und dem Scherstress sind [27]. Zum Prozess der Invasion in das Blutgefäßsystem trägt die phänotypische Plastizität der Tumorzelle bei, welche eine dauernde Anpassung an permanent wechselnde Milieubedingungen gestattet. Der reversible Prozess der Epithelial-mesenchymale Transition (EMT) ist ein Bestandteil dieser Plastizität. In CTCs werden EMT-assoziierte Prozesse durch parakrine Signale, wie z.B. Interleukin-6 (IL-6) und TGF-β (transforming growth factor-β), induziert. Es kommt zu einem Zytoskelett-Umbau und zum Verlust der epithelialen Zelleigenschaften. Durch die Herabregulation der Expression von epithelialen Adhäsionsmolekülen (z.B. E-Cadherin, EpCAM) kommt es u.a. zum Verlust der apikalen-basalen Polarität und führt zur Auflösung des Zell-Zell- und des Zell-extrazellulären Matrix-Kontakts. Die erhöhte Expression von mesenchymalen Markern (z.B. Vimentin) führt zur Bildung eines mesenchymalen Phänotyps, welcher den CTCs im Blutsystem einen Überlebensvorteil durch bessere Migrations- und Invasionseigenschaften verschafft. Zusätzlich führt dieser Umbau zu einer Ausbildung von Stammzelleigenschaften, schützt vor Apoptose und irreversibler Seneszenz [28-30]. Der EMT-Prozess wird nicht immer vollständig von den Tumorzellen durchlaufen. Das führt zu partiellen mesenchymalen/epithelialen CTC-Phänotypen (M/E-Hybrid-Phänotyp) und damit zu einer heterogenen CTC-Population [21, 31], (Abb. 2). Der Hybrid-Phänotyp besitzt im Vergleich zu den CTCs, welche am Ende des EMT-Prozesses sind, ein größeres Metastasierungspotential und Stammzelleigenschaften [32].

Während der Zirkulation im Blut kommt es zum Arrest von Tumorzellen, z.B. durch die Interaktion mit Thrombozyten und Leukozyten. Diese Verbindung schützt die CTCs vor Scherkräften und oxidativem Stress in den venösen und arteriellen Systemen [31] (Abb. 2). Der so entstandene Mikroembolus ermöglicht die Umgehung der im Blutgefäßsystem vorhandenen Barrieren [20].

Die letzten Schritte der Metastasierungskaskade sind die Infiltration der Tumorzellen in das Parenchymgewebe entfernt liegender Organe (Extravasation) und die Bildung von Mikro- und Makrometastasen. Für diese Immigration in entfernt liegende Organe müssen die Tumorzellen die Gefäßwand wieder durchdringen (Abb. 2), [33, 34]. Notwendig dafür ist die Wiedererlangung des anfänglichen epithelialen Phänotyps durch Mesenchymal-epitheliale Transition (MET) [29]. Eine weitere Austrittsmöglichkeit aus dem Gefäßsystem ist der Tumorembolus, welcher zu einer Gefäßruptur führen kann. CTCs, welche aus der Zirkulation in Organe einwandern, werden als disseminierte Tumorzellen (DTC) bezeichnet. Die Ansiedlung von einzelnen DTC und die Bildung von Metastasen werden nicht allein von den neoplastischen Zellen, sondern auch vom Mikromilieu des Ansiedlungsortes beeinflusst [35]. Bereits im Jahr 1889 postulierte Stephen Paget in seiner „Seed-and-Soil"- Theorie, dass

Tumorzellen (seed) sich nur in Organen (soil) ansiedeln, welche einen fruchtbaren Boden dafür bieten [36].

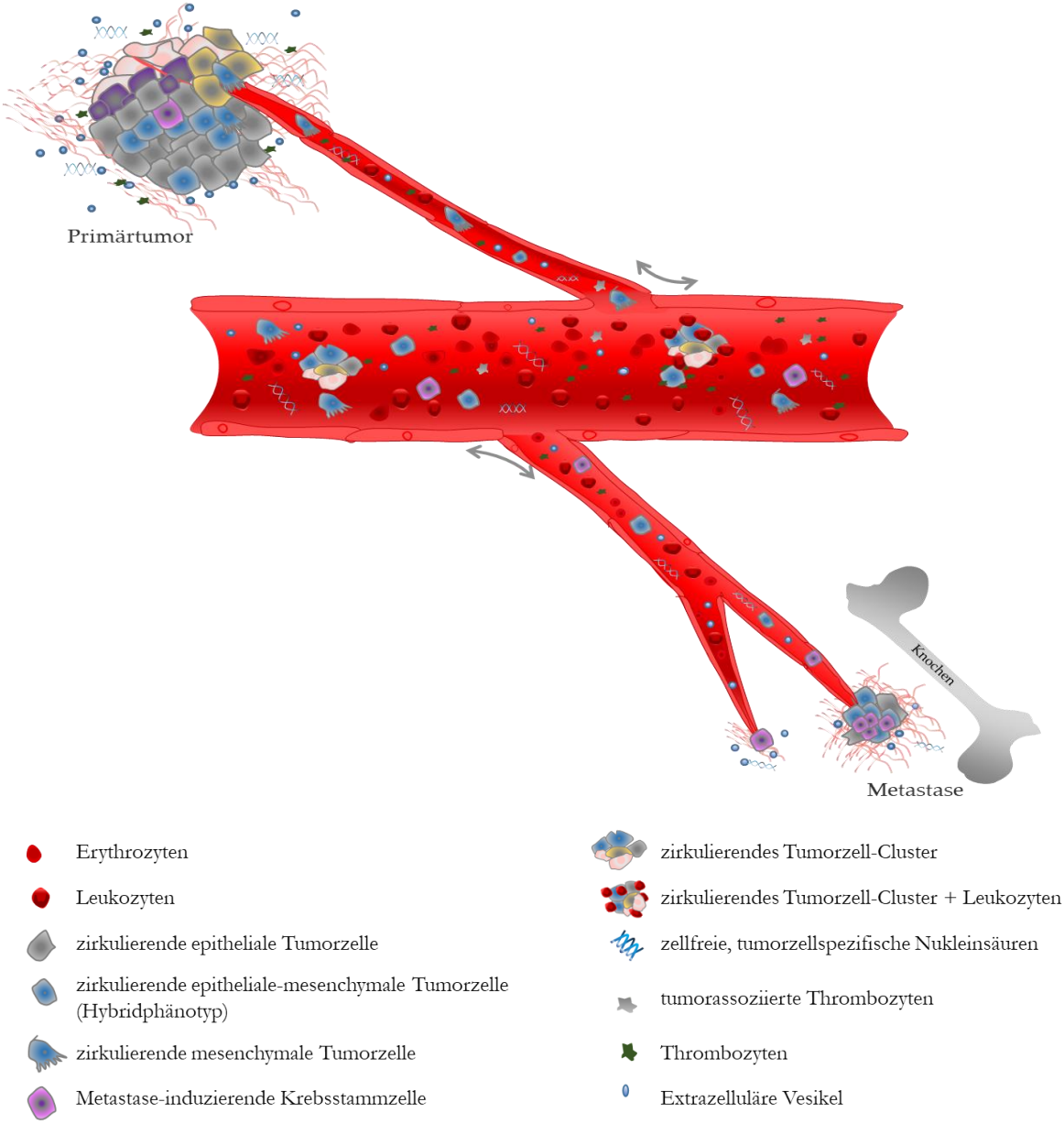

Abb. 2: Schematische Darstellung der Invasion-Metastasierungs-Kaskade.
Systemische Aussaat von zirkulierenden Tumorzellen (CTCs)/Tumorzell-Cluster ins Blut bis zum finalen metastasierten Wachstum. Die Vielfalt der molekularen Mechanismen, die zur Metastasierung führen, spiegelt sich in der heterogenen CTC-Population wider. Es gibt mehrere Eintrittsstellen für CTCs in das Blut (Primärtumor, Lymphknoten, Metastasen) und molekulare Mechanismen, die an der Invasion beteiligt sind sowie die reversiblen Prozesse der Epithelial-mesenchymale-Transition und Mesenchymal-epitheliale-Transition. Das Überleben von CTCs im Blut wird u.a. durch die CTC-Clusterbildung und die Interaktion mit Leukozyten und Thrombozyten ermöglicht. Zur Extravasation in entfernt liegende Organe (z.B. Knochen) und zur Bildung von Metastasen ist nicht jede CTCs fähig. Tumorzellen, die von Mikrometastasen oder Metastasen stammen, können auch zurück in den Blutkreislauf zirkulieren; inwieweit diese „Rezirkulation“ zur weiteren Metastasierung beiträgt, ist noch nicht geklärt (verändert nach Keller et al. [33]).

Es gibt Hinweise, dass sich z.B. PCa-Stammzellen in homöostatischen Stammzellnischen im Knochenmark ansiedeln [37]. In einer solchen prämetastatischen Nische können sich die DTCs kolonisieren und Mikrometastasen bilden oder in einen Ruhezustand (Tumor

Dormancy) gelangen. Der Ruhezustand der DTCs kann Wochen, Monate oder sogar Jahre andauern [21]. Eine außergewöhnliche Fähigkeit der DTC ist die Rezirkulation in das Blutgefäßsystem, womit der Pool an zirkulierenden Tumorzellen zusätzlich gefüllt werden kann. Die Bedeutung dieser Rezirkulation ist für die Metastasierung noch nicht geklärt [33]. Mutationsanalysen zeigten für das Prostatakarzinom, dass das Genom von Fernmetastasen in Lunge und Leber, bezüglich der klonalen Herkunft, von Low-grade-Anteilen und nicht von High-grade-Anteilen des Primärtumors stammt [38]. Ferner können einzelne Tumorherde aus klonal unterschiedlichen Läsionen ohne gemeinsame Treibermutationen entstehen und so können mehrere genomische und phänotypisch heterogene primäre Prostatakarzinome bei einem einzelnen Patienten vorhanden sein [39]. Das verschiedene Tumorzellklone im Primärtumor für eine Metastasierung verantwortlich sind und das Tumorzellen zwischen den einzelnen Metastasen ausgetauscht werden können, zeigten auch Gesamtgenomsequenzierungsdaten von Primärtumoren und Metastasen von Patienten mit einem kastrationsrefraktären Prostatakarzinom. So ist es möglich, dass Metastasen den Ursprung von neuen Metastasen darstellen [40].
Insgesamt ist diese komplexe und heterogene Konstellation molekularer Veränderungen ein Hindernis für die Diagnose und Behandlung von Prostatakarzinompatienten [39].

1.2 Zirkulierende Tumorzellen

Wie im Kapitel 1.1.4 beschrieben, können sich zirkulierende Tumorzellen aus einem soliden Tumor oder von Metastasen lösen und frei im Blut zirkulieren (Fig. 2). Die Konzentration der CTCs im Blut eines metastasierten Patienten liegt bei ca. 1-10 CTC/10^8 ml Blutzellen. Die vermutete Halbwertszeit im Blut beträgt nur wenige Stunden [35]. Erstmalig im Jahr 1869 beschrieb der Pathologe Thomas Ashworth Zellen (CTCs) im Blut eines verstorbenen metastasierten Patienten, welche denen des Tumors glichen [41]. Inzwischen hat sich bestätigt, dass CTCs ein essentieller Faktor in der hämatogenen Metastasierung sind und sie das aktuelle Tumorgeschehen im Patienten in Form einer „Real Time Biopsie“ reflektieren [42]. Mit Hilfe der Isolation und Charakterisierung von CTCs als Bestandteil der „Liquid Biopsy“, kann die Informationslücke im klinischen Alltag hinsichtlich der molekularen Veränderung von fortgeschrittenen Tumoren geschlossen werden. Eine serielle Probenentnahme durch die konventionelle Nadelbiopsie den „Goldstandard“ in der Tumordiagnostik ist mit einem invasiveren Eingriff verbunden und eine Biopsie von Tumor oder Metastase ist nicht immer möglich.
Die geringe Anzahl der CTCs im Blut im Vergleich zu der hohen Anzahl von Erythrozyten und Leukozyten und der heterogene CTC-Phänotyp sind jedoch elementare Herausforderungen an die Detektion und Charakterisierung von CTCs.

Mit Zulassung des CellSearch-Systems für die CTC-Zahlbestimmung im Blut von Prostatakarzinom- [43], Mammakarzinom- [44] und kolorektalen Karzinom- [45] Patienten durch die FDA wurde der Nutzen von CTCs als Biomarker für die klinische Diagnostik bekräftigt. In diesem Zusammenhang wurde eine klare CTC-Definition („klassische Definition“) festgelegt. CTCs müssen ein sichtbares Zytoplasma aufweisen, das Kern/Zytoplasma-Verhältnis muss zugunsten des Zellkerns verschoben sein und eine Größe von ≥ 4 µm aufweisen. Sie müssen sowohl EpCAM als auch Zytokeratine (CK) 8, 18 und 19 exprimieren und negativ für den Pan-Leukozytenmarker CD45 sein [46]. Nachteil dieser Definition ist, dass der heterogene Phänotyp der CTCs, welcher z.B. unter EMT entsteht, nicht berücksichtigt wird.

Es existieren über 45 verschiedene CTC-Isolationstechnologien, wobei die Anreicherung der CTCs über die biologischen oder physikalischen Eigenschaften der Zellen erfolgen kann [47]. Alle Methoden haben die gleichen Herausforderungen zu lösen. Von besonderer Bedeutung ist der hoch spezifische, sensitive Nachweis der verschiedenen CTC-Phänotypen, die Kosteneffektivität, die Zeit bis zur Befunderstellung und - sehr wichtig - die präzise molekulare Charakterisierung für den Einsatz in der personalisierten Onkologie [48]. Aufgrund der Fülle der Techniken wird im folgenden Abschnitt nur auf die aus meiner Sicht wichtigsten Methoden eingegangen.

Die physikalischen Eigenschaften der CTCs wie Dichte, Zellgröße, elektrische Ladung und Verformbarkeit ermöglichen die Separation zwischen Tumorzelle und Blutbestandteilen. So können CTCs unabhängig vom Expressionsprofil isoliert werden. Eine, die Zellmorphologie erhaltende Isolationsmethode ist die Dichtegradientenzentrifugation (Ficoll-Paque) [49, 50]. Basierend auf der Dichtediskrepanz zwischen verschiedenen Blutzellpopulationen reichern sich die CTCs in der sogenannten Interphase zusammen mit den Lymphozyten an. Weitere Anreicherungsverfahren selektieren CTCs anhand ihrer Größe mit Hilfe eines Einmalfilters. Dazu gehören u.a. die ISET-Technik (Isolation by Size of Epithelial Tumor cells [ISET]) [51] und das ScreenCell-Verfahren [52]. Die Porengröße der Filter beider Systeme ist 8 µm, d.h. „nicht-klassische“ kleine CTCs (< 4 µm) gehen verloren. Ein großer Vorteil ist, dass CTCs ebenfalls morphologisch intakt und ohne Antikörperbindung isoliert werden können. Des Weiteren ermöglichen beide Methoden die Kultivierung von CTCs.

Die Epic-Plattform verwendet zur „CTC-Isolation“ weder physikalische noch biologische Eigenschaften der CTCs. Durch Erythrozytenlyse verbleiben alle kernhaltigen Zellen in der zu untersuchenden Probe. Anschließend werden sie auf zehn bis zwölf Objektträger mit einer Konzentration von $3x10^6$ kernhaltige Zellen pro Objektträger ausgestrichen und bis zur molekularen oder immunzytochemischen Analyse bei -80 °C gelagert [53].

Die Anreicherung der CTCs über die biologischen Eigenschaften erfolgt über die Expression von tumor-assoziiertem Antigen (positive Selektion) oder dem Pan-Leukozytenmarker CD45 (negative Selektion). Etabliert hat sich das EpCAM-Signal der Zellen, welches von gesunden und malignen epithelialen Zellen aber nicht von mesenchymalen Leukozyten exprimiert wird. Für das Prostatakarzinom konnte nachgewiesen werden, dass EpCAM bereits im primären Tumorgewebe und in Metastasen überexprimiert ist [54].

Die gegenwärtig am häufigsten verwendete sowie standardisierte Methode für die Anreicherung und den Nachweis von CTCs, ist das CellSearch-System. Das halbautomatische System nutzt EpCAM als Zielmolekül und reichert die Zellen immunomagnetisch über EpCAM-Antikörper funktionalisierte Ferrofluide (Beads) an. Für die Bildung eines Tumorzell-Antikörper-Komplexes wird der Antikörper-Bead-Komplex mit 7,5 ml Blut inkubiert und nachfolgend mit Hilfe von Magneten aus dem Blut separiert. Die so isolierten Zellen werden immunzytochemisch auf die o.g. CTC-Kriterien durch die Analyse an einem halbautomatischen Fluoreszenzmikroskop geprüft [44].

Weitere Systeme, welche die immunomagnetische Anreicherung über die EpCAM-Expression der Tumorzelle nutzen, sind u.a. der AdnaTest [55] und das MagSweeper-Verfahren [56]. Die isolierten CTCs werden auf mRNA-Ebene charakterisiert, wobei der AdnaTest speziell für das Prostatakarzinom PSA-, EGFR- und PSMA-Transkripte mit Hilfe einer Multiplex-PCR detektiert und damit eine karzinomspezifische CTC-Charakterisierung erlaubt [55]. Das MagSweeper-Verfahren ermöglicht, zusätzlich zur RT-PCR-Analyse von ausgewählten Genen, eine immunzytochemische Charakterisierung auf CK- und CD45-Expression in den CTCs [56]. Die Mikrofluidic-Chip- Technologie (CTC-iChip) kombiniert die Antikörper-vermittelte CTC-Isolation mit einer mechanischen Filterung der Blutprobe [57]. Der CTC-iChip ermöglicht aber auch eine negative Selektion über anti-CD45 funktionalisierte Mikrosäulen und damit eine antigenunabhängige CTC-Anreicherung [58].

Alle CTC-Isolierungstechniken, welche bisher erwähnt wurden, haben eine gemeinsame Limitation: das geringe Blutvolumen ($\leq$ 30 ml).

In der vorliegenden Habilitationsschrift wird ein neues System für die Anreicherung von CTCs direkt aus dem Blutgefäßsystem evaluiert, welches diese Limitation umgehen kann.

Der CellCollector (CC) ist ein EpCAM-Antikörper funktionalisierter medizinischer Draht, welcher direkt über eine in der Cubitalvene liegende Venenverweilkanüle eingebracht wird (Abb. 3). Die Antikörperbindung erfolgt kovalent über eine Hydrogelschicht, welche an einer 2 µm dicken Goldschicht haftet. Die Funktionalisierung des Drahtes betrifft die 2 cm lange Spitze des 16 cm langen Drahtes. Der CellCollector verbleibt 30 min in der Cubitalvene, dadurch können bis zu 600 ml Blut für die Detektion von CTCs analysiert werden. Die isolierten CTCs werden nach den obengenannten CTC-Kriterien identifiziert. Zu Beginn

dieser Arbeit lagen für den CC nur in vivo Daten für eine kleine Gruppe von Mammakarzinompatientinnen (n = 12) und Lungenkarzinompatienten (n = 12) vor [59].

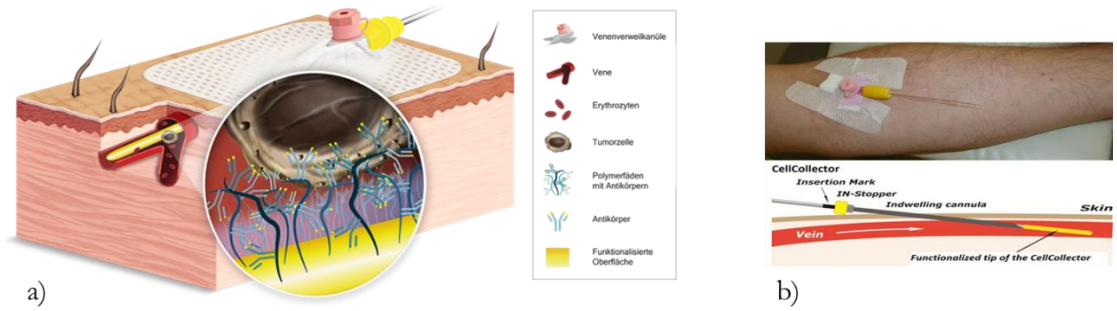

Abb. 3: Schematische Darstellung der CellColletor-Anwendung a) in der Cubitalvene eines Probanden b) [60]

1.2.1 Klinische Relevanz der zirkulierenden Tumorzellen

Die klinische Bedeutung von CTCs bei Patienten mit lokalisiertem Prostatakarzinom wurde bisher nur in wenigen Studien analysiert [48].

Für das metastasierte Prostatakarzinom konnten zahlreiche Studien mit dem FDA zugelassenen CellSearch-System nachweisen, dass die CTC-Zahl (< 5 CTC vs. ≥ 5 CTC) eine prognostische Aussage für das progressionsfreie (progression-free survival, [PFS]) und das Gesamtüberleben (overall survival, [OS]) hat und die Abnahme der CTC-Zahl unter systemischer Therapie mit einem verbesserten Outcome assoziiert ist [43, 61-64]. Darüber hinaus korreliert eine höhere CTC-Zahl mit progredienten Knochenschmerzen, PSA-Wert-Anstieg, niedrigerem Hämoglobinwert und einer erhöhten Alkalische-Phosphatase-Konzentration im Blut von mCRPC-Patienten [62].

Heller et al. [65] analysierten retrospektiv die CTC-Zahl und integrierten diese in das konventionelle Prognose-Modell „ALPHA“ (Albumin, Laktatdehydrogenase, PSA, Hämoglobin und ALK). Ihr Ziel war die genauere Quantifizierung der Prognose zur Vorhersage des kurzfristigen vs. langfristigen Überlebens für Patienten mit einem mCRPC. Zu diesem Zweck wurden Daten von Patienten, die in die Phase-III-Zulassungsstudie Abirateronacetat (AA) plus Prednison (COU-AA-301; NCT00638690) und die Registrierungsstudie mit einem ähnlichen Design zur Bewertung von Orteronel plus Prednison (ELM-PC4; NCT01193244) aufgenommen wurden, verwendet. Die Ergebnisse zeigen, dass mit der Einbeziehung der CTC-Messung in das Prognose-Modell „ALPHA“ eine genauere Einschätzung des Risikos hinsichtlich der Baseline- und Post-Baseline-Prognose für Patienten mit mCRPC möglich ist.

Die Bedeutung der CTC-Zahl als Prognosefaktor bekräftigt die Prostate Cancer Working Group (PCWG) in ihrer Leitlinie (Version 3) und empfiehlt, die CTC-Zahl als einen

Endpunkt in klinischen Studien (unter Verwendung des CellSearch-Systems) festzulegen [66].

Die molekulare Charakterisierung von gepoolten oder einzelnen CTCs auf DNA-, RNA- oder Proteineigenschaften spiegeln den Echtzeit-Phänotyp des Primärtumors oder der Metastase wider [33]. Dies schafft eine Verbindung zwischen Diagnostik und Therapie. Ein therapeutisch-relevanter Marker ist die Androgenrezeptor-Splice-Variante-7 (AR-V7), eine Variante des Androgenrezeptors, dem die ligandenbindene Domäne fehlt. Dieser Bereich des ARs ist jedoch essentiell für die Wirksamkeit von Abirateron und Enzalutamid, zwei Inhibitoren des Androgenrezeptorsignalweges (ARS). In CTCs konnte die AR-V7 Variante auf mRNA-Ebene bei mCRPC-Patienten, welche mit Enzalutamid- oder Abirateronacetat vorbehandelt wurden, nachgewiesen werden. Es zeigte sich, dass das Auftreten der AR-V7 in CTCs mit einer primären Therapieresistenz gegenüber den beiden Medikamenten korreliert [67]. Darüber hinaus demonstriert die nukleare Lokalisierung der AR-V7 Variante in CTCs ebenfalls einen Überlebensvorteil durch die Behandlung mit Taxanen gegenüber der ARS gerichteten Therapie [68]. Diese Beispiele zeigen den Nutzen der CTCs für die individualisierte Onkologie, welche eine möglichst genaue Patienten- und Therapiestratifizierung benötigt und damit einen Mehrwert gegenüber den bisher klassifizierten Markern bietet.

2 Zielstellung

Zirkulierende Tumorzellen sind Zellen, denen es gelungen ist, sich vom Tumor zu lösen und in die Blutzirkulation einzutreten. Dadurch ist es ihnen möglich, entfernte Organe zu infiltrieren. Als Bestandteil der „Liquid Biopsy" können CTCs in Echtzeit Veränderungen im Tumor reflektieren, was im Rahmen einer einmaligen Tumorbiopsie meist nicht möglich ist. In der individualisierten Onkologie kann mit Hilfe der CTCs eine personalisierte Therapieentscheidung z.B. für adäquate Therapiesequenzen getroffen werden. Die größte Herausforderung in der CTC-Detektion stellt die geringe Konzentration der CTCs im Blut dar, was sensitive und spezifische Isolationstechnologien erfordert. Alle bisher bekannten Isolationstechnologien haben unterschiedliche Limitationen, ihr gemeinsamer Nachteil ist das geringe Blutvolumen (≤ 30 ml), welches für ex vivo CTC-Isolation zur Verfügung steht. Eine zentrale Zielstellung der vorliegenden Arbeit besteht darin, zu prüfen, ob die in vivo Isolation von CTCs durch den neuen CellCollector, der die Untersuchung eines großen Blutvolumens ermöglicht, eine höhere Sensitivität der CTC-Detektion in Prostatakarzinompatienten mit unterschiedlichen Stadien erreicht.

Dazu müssen folgende Fragen beantwortet werden:

- Ist der Einsatz des CellCollectors ex vivo in Blutproben von Prostatakarzinompatienten für die Funktionalitätsprüfungen in der personalisierten Diagnostik möglich? (Originalarbeit 1)
- Ist der CellCollector in vivo bei Prostatakarzinompatienten verschiedener Stadien und Kontrollgruppen anwendbar? Zeigen die CTCs eine prognostische Relevanz? Ist die diagnostische Genauigkeit der in vivo Technologie mit dem CellSearch-System als Referenzsystem vergleichbar? Gibt es eine Assoziation der CTC-Zahl mit Entzündungs- und Biomarkern als möglichen Bestandteil des individualisierten Therapiemonitorings für das Prostatakarzinom? (Originalarbeiten 2 und 3)
- Erlaubt die Entwicklung einer PCa-spezifischen Funktionalisierung des CellCollectors eine sensitivere CTC-Isolation aus dem Blut von PCa-m Patienten? (Originalarbeit 4)
- Wichtig für die personalisierte Therapie sind Kenntnisse zur Art des Tumors, die aus den Eigenschaften der CTCs gewonnen werden können. Die Validierung der hierzu erforderlichen Kultivierung und geeignetsten Separationsmethode der CTCs ist ein weiteres Ziel dieser Arbeit. (Originalarbeit 4)

3 Ergebnisse und Diskussion

3.1 Proof-of-Concept-Studie: Anreicherung von zirkulierenden Tumorzellen aus dem Blut von Patienten mit Prostatakarzinom

Originalarbeit 1: **Theil G,** Fischer K, Weber E, Medek R, Hoda R, Lücke K, Fornara P. **The Use of a New CellCollector to Isolate Circulating Tumor Cells from the Blood of Patients with Different Stages of Prostate Cancer and Clinical Outcomes - A Proof-of-Concept Study.** PLoS One 2016 Vol. 11 Issue 8 Pages e0158354

Das Ziel der vorliegenden Proof-of-Concept-Studie war es, die Eignung des CellCollector (CC) für die Isolierung und Charakterisierung von CTCs aus dem Blut von Prostatakarzinompatienten zu prüfen. Der Schwerpunkt lag dabei auf immunzyotchemischen und auf weiterführenden molekularen Charakterisierungen der isolierten Zellen. Der CC ist eine EpCAM-basierte neue in vivo CTC-Isolationstechnologie, welche unter 1.2 beschrieben wurde. Zum Studienzeitraum lag für die in vivo Anwendung noch keine behördliche Genehmigung vor.

Es wurden 43 PCa-Patienten in das Studienkollektiv eingeschlossen, welches aus 18 Patienten mit lokalisierten (PCa-l), sieben Patienten mit lokal fortgeschrittenen (PCa-la) und 18 Patienten mit metastasierten (PCa-m) Prostatakarzinom bestand. In die Kontrollgruppe wurden elf Patienten mit histopathologisch bestätigtem BPH-Befund und ohne Anzeichen eines PCa`s eingeschlossen. Das mediane Alter betrug 69 (58-77) Jahre für Patienten mit lokalisiertem PCa, 71 (71-81) Jahre für Patienten mit lokal fortgeschrittenem PCa und 71,5 (53-87) Jahre für Patienten mit metastasiertem PCa. Die Kontrollgruppe hatte ein medianes Alter von 67 (58-83) Jahren. Ein Großteil der PCa-l Patienten (88,9%) wurde primär mit RP therapiert. Überraschenderweise hatten alle Patienten der PCa-la Gruppe positive chirurgische Resektionsränder und 85,7 % dieser Patienten erhielten eine ADT. Alle PCa-m Patienten wurden mit ADT behandelt und 65 % der Patienten erhielten eine Erstlinien-Chemotherapie mit Docetaxel (Originalarbeit 1, Tab. 1).

Die Funktionalitätsprüfung des CCs erfolgte in einem präklinischen Schritt mit Hilfe eines fluiddynamischen Systems. Die Parameter dieses Systems waren an die hämodynamischen Parameter des Blutkreislaufes angepasst.

Zunächst wurde die EpCAM-Antikörper-Funktionalisierung des CCs mit Hilfe von Spiking-Experimenten überprüft. Dazu wurde das Blut von gesunden Spendern mit LNCaP-Zellen verschiedener Konzentrationen (50, 200, 500 Zellen/ml) angereichert. Die Detektionsrate der LNCaP-Zellen lag in einem Bereich von 10-31,5 %, in Abhängigkeit von der gespikten Zellzahl.

Unter diesen Voraussetzungen wurde der CC für die Detektion von CTCs aus den Blutproben (15 ml) des Studienkollektivs eingesetzt. Die Ermittlung der CTC-Zahl erfolgte nach den unter 1.2. genannten Kriterien. Ergebnisse zählten als positiv, wenn ≥ 1 CTC nachgewiesen wurde.

Die CTC-Zahlen wurden von 31 der 43 PCa-Patienten und elf Kontrollpatienten ermittelt (Originalarbeit 1, Abb. 3). In der Kontrollgruppe konnten im Median 0 (0-4) CTCs mit einer Detektionsrate von 31,5 % nachgewiesen werden. Die Detektionsrate der PCa-l Patienten lag bei 92,3 % (Median 5, 0-8 CTCs). Bei den Patienten mit einem lokal fortgeschrittenen PCa lag die Detektionsrate bei 100 % (Median 18, 10-25 CTCs). Die CTC-Zahlen der PCa-la und der PCa-l Patienten zeigten keinen signifikanten Unterschied (p = 0,39). Die detektierte CTC-Zahl bei den PCa-l Patienten erlaubt keine krankheitsspezifischen Schlussfolgerungen. Darüber hinaus konnte im Nachbeobachtungszeitraum von 55 Monaten in dieser Patientengruppe kein Einfluss der CTC-Zahl auf das Gesamtüberleben beobachtet werden. Hier könnte eine Kombination der CTC-Zahl mit der molekularen Charakterisierung der CTCs zusätzliche sowie notwenige Informationen für den klinischen Nutzen der CTCs in der lokalisierten PCa-Gruppe bieten. Das Epic System konnte verschiedene CTC-Phänotypen, wie CK-negative, AR-positive CTCs und CTC-Cluster bei Patienten mit lokalem PCa und hohem Rezidivrisiko, aber einem kurativen Behandlungskonzept, in 73 % der untersuchten Blutproben nachweisen. Ein möglicher Nutzen dieser CTC-Phänotypisierung zeigt sich in einer besseren Risikostratifizierung in dieser Gruppe und damit verbunden, ein schnellerer Zugang zu multimodalen Therapieansätzen [69]. Dies zeigt die Notwendigkeit von therapierelevanten spezifischen CTC-Charakterisierungen auf. So konnten Antonarakis et al. darstellen, dass bei Patienten mit fortgeschrittenem PCa der Nachweis von AR-V7 (mRNA) in CTCs mit einer Resistenz gegenüber Enzalutamid und Abirateron assoziiert sein kann [67]. Mit diesem Beispiel wurde der Nutzen von CTCs im klinischen Umfeld, z.B. bei der Behandlung auf eine individuelle Resistenz, verdeutlicht.

Unsere CTC-Detektionsrate von 90,9 %, zusammen mit den hohen CTC-Zahlen im Median 10 (0-98) CTCs in der metastasierten Patientengruppe, gibt einen Hinweis auf ein aktives Tumorgeschehen. Interessanterweise erhielten alle Patienten zum Zeitpunkt der Blutentnahme eine systemische Therapie (100 % ADT und 65 % Chemotherapie). Im Idealfall sollten bei einem Therapieansprechen keine CTCs nachweisbar sein. Eine Detektion von CTCs ist daher ein Hinweis auf einen Tumorprogress unter Therapie. In diesem Zusammenhang weist die moderate Korrelation der steigenden CTC-Zahl mit den klinischen Parametern PSA-Wert (r = 0,345) und Gleason-Score (r = 0,222) ebenfalls auf einen Progress hin. Eine moderate Korrelation zwischen der CTC-Zahl und dem PSA-Wert wurde auch in

anderen Studien dokumentiert. Die Autoren argumentieren, dass die CTC-Zahl ein besserer Prognosemarker für die hämatogene Dissemination als die klassischen Parameter ist [70, 71]. Die CTC-Zahlen unserer PCa-Patienten wurden mit Hilfe einer Kaplan-Meier-Analyse auf die Prognose für ihr Gesamtüberleben (OS) überprüft. Dazu wurde der vom CellSearch-System etablierte Cut-off-Wert für CTCs von < 5 CTCs und ≥ 5 CTCs angewandt [43, 71-73]. Mit dem Ergebnis, dass Patienten mit ≥ 5 CTCs ein signifikant kürzeres OS, HR = 7,0 (95% KI 1,1-29,39) (p = 0,035) hatten. Damit bestätigte sich die CTC-Zahl, welche mit dem CellCollector ex vivo ermittelt wurde, als ein unabhängiger prognostischer Marker für das Gesamtüberleben (Orignialarbeit 1, Abb. 5).

Obwohl die meisten CTC-Analysen mit dem CellSearch-System durchgeführt wurden, ist das geringe Blutvolumen (7,5 ml) ein limitierender Faktor, welcher mit einer reduzierten Sensitivität verbunden ist [74]. Die geringe Reinheit der isolierten Proben gilt ebenfalls als Einschränkung des CellSearch-Systems [75]. Aus den genannten Gründen stellt sich die molekulare Charakterisierung der CTCs bisher als schwierig heraus [76-78]. Im Gegensatz dazu wurden bei den ex vivo Anwendungen des CCs nur geringe Kontaminationen (Leukozyten, Artefakte und Zelltrümmer) an der CC-Oberfläche dokumentiert. Dies war ein erheblicher Vorteil für die immunzytochemische und molekulare Charakterisierung der angereicherten Zellen. Die Bindung von EpCAM-positiven Leukozyten lag in einem konstanten Bereich von 5 bis 10 Leukozyten pro CC. Die Evaluation des CCs, hinsichtlich der molekularen Charakterisierung von isolierten CTCs, erfolgte zunächst in Spiking-Experimenten. Dafür wurden Blutproben (15 ml) mit Zellzahlen von 50, 200 oder 500 LNCaP-Zellen pro Probe angereichert und mit dem CC im fluiddynamischen System inkubiert. Anschließend wurde die Drahtspitze in 5 mm Abschnitte geteilt und mit Hilfe einer Multiplex RT-PCR auf die Expression von EGFR, PSMA und PSA quantitativ überprüft. In Abhängigkeit der gespikten Zellzahl wurden in allen analysierten Proben die oben genannten Transkripte nachgewiesen. Darauffolgend wurde die molekulare Charakterisierung der CTCs aus Blutproben von Patienten (PCa-l, n = 5; PCa-m, n = 7) durchgeführt. Kein Nachweis der zu analysierenden Transkripte erfolgte in den Proben der PCa-l Patienten. In der metastasierten Gruppe konnte eine Sensitivität von 57,1 % für den Nachweis von mindestens eines der oben genannten Transkripte erreicht werden, wobei in den positiven Proben EGFR (3/7) und PSMA (1/7) detektiert wurde (Originalarbeit 1, Tab. 2). Das detektierte EGFR-Signal ist nicht spezifisch für das Prostatakarzinom, weist aber auf eine mögliche Progression hin. EGFR spielt eine zentrale Rolle in der Zellproliferation, der Migration, der Motilität, der Invasion und bei dem Überleben von normalen Zellen sowie von Karzinomzellen. Es ist bekannt, dass die PSMA-Expression mit einer Progression in der kastrationsrefraktären Phase des Karzinoms zunimmt. Ein weiterer Vorteil des PSMA-

Proteins ist seine stabile Expression während des EMT-Prozesses und der damit verbundene Nutzen für CTC-Anreicherung im metastasierten PCa-Stadium [75, 78]. Unser Ergebnis legt nahe, dass die isolierten CTCs einen heterogenen Phänotyp aufweisen, welcher für die epithelialen Karzinome beschrieben wurde [32, 70]. Der EMT-Phänotyp ist ein kritischer Punkt in der CTC-Isolierung bei metastasierten PCa-Patienten, da er mit einer Herunterregulation der EpCAM-Expression einhergeht. Daraus resultiert, dass eine verminderte Expression von epithelialen Markern zu falsch negativen CTC-Zahlen führen kann [29].

Eine Einschränkung unserer Proof-of-Concept-Studie ist natürlich die kleine Studienpopulation. Die 55-monatige Nachbeobachtung der Patienten war jedoch ausreichend, um den prognostischen Wert unserer Methode zu demonstrieren.

Zusammenfassend konnte durch unsere Ergebnisse gezeigt werden, dass ex vivo mit dem CC eine sensitive Isolierung sowie eine molekulare Charakterisierung von CTCs aus den Blutproben von Prostatakarzinompatienten möglich ist. Mit Hilfe dieser Ergebnisse konnte u.a. der Antrag auf Zulassung der in vivo Anwendung des CCs durch das BfArM gestellt werden. CTC-Isolationstechnologien sollten den heterogenen Phänotyp und die geringe Konzentration der CTCs berücksichtigen können. Eine PCa-spezifische Charakterisierung von CTCs ermöglicht zusätzliche Informationen, welche für individuelle Behandlungskonzepte genutzt werden können.

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RESEARCH ARTICLE

The Use of a New CellCollector to Isolate Circulating Tumor Cells from the Blood of Patients with Different Stages of Prostate Cancer and Clinical Outcomes - A Proof-of-Concept Study

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Abstract

Background and Methods

Circulating tumor cells (CTCs) constitute a useful approach for personalized medicine. Nevertheless, the isolation of these cells remains very challenging because they rarely circulate in the blood. Another current problem is the cancer-specific characterization of these cells, which requires a method that allows for the molecular and immunocytochemical profiling of all captured cells. The purpose of our proof of concept study was to investigate the use of a medical wire (CellCollector, GILUPI) to isolate CTCs in the blood of prostate cancer (PCa) patients, which allowed CTCs to be counted and molecularly characterized. Forty-three PCa patients in different stages and 11 control subjects were studied. Some randomized samples were used to detect tumor-associated transcripts, such as prostate-specific membrane antigen (PSMA), prostate-specific antigen (PSA) and epidermal growth factor receptor (EGFR), in the isolated CTCs.

Results

The mean CTC counts were 4.6 CTCs [range, 0–8] in patients with localized PCa, 16.8 CTCs [range, 10–25] in patients with locally advanced PCa, and 26.8 CTCs [range, 0–98] in patients with metastatic PCa. The median follow-up time was 24 months, and there was a significant difference in the cancer-specific survival rates. Patients with CTC counts under 5 CTCs lived significantly longer ($p = 0.035$) than patients with more than 5 CTCs. We also demonstrated that the captured CTCs could be molecularly characterized. We detected

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tumor-associated transcripts of EGFR and PSMA in patients with metastatic PCa in 42.8% and 14.3% of the analyzed samples, respectively.

Conclusion

Our results indicate that the sensitive isolation and molecular characterization of CTCs can be achieved ex vivo using the wire. Patients with more than 5 CTCs had a mortality risk that was 7.0 times greater that of those with fewer than 5 CTCs (hazard ratio 7.0 95%, CI 1.1–29.39). This proof of concept was required for the approval of the use of the CellCollector in a clinical study for the in vivo isolation of CTCs from the blood stream of PCa patients by the Federal Institute for Drugs and Medical devices (Germany, BfArM).

Introduction

Prostate cancer (PCa) is characterized by heterogeneous phenotypes that display a broad range of clinical outcomes from relatively indolent to lethal metastatic disease. The standard diagnostic tools include the level of prostate-specific antigen (PSA) in the blood, transrectal ultrasonography (TRUS), histological Gleason grading of biopsy specimens, and clinical tumor, node, metastasis (TNM) staging, which are all insufficient for an accurate risk stratification of each patient. Given the wide range of clinical outcomes and the heterogeneity of the disease, physicians' main challenge remains distinguishing latent tumors from clinically significant ones. Thus, there is a clear need for additional, improved prognostic markers [1]. Circulating tumor cells (CTCs) might represent a new opportunity for the management and monitoring of disease progression. Recent clinical trials have demonstrated that the presence of CTCs in metastatic breast, prostate, and colorectal cancer patients are frequently proportional to survival [2–4]. The clinical usefulness of these cells include the prediction of clinical tumors, disease prognosis, therapeutic monitoring, and therapeutic outcome. However, they have not been used in the clinical setting.

The most important challenge facing CTC research is the sensitive isolation (enrichment) of these rare cells, which typically present as a single tumor cell against a background of millions of blood cells [5]. Although many approaches for detecting and characterizing CTCs from blood samples exist, the only standardized CTC-isolation platform is the CellSearch® System (Janssen Diagnostics), which has been cleared by the US Food and Drug Administration (FDA) for monitoring metastatic breast, colon and prostate cancers [2,3,6]. This isolation technique is based on the use of magnetic beads coated with antibodies against the epithelial cell adhesion molecule (EpCAM) to capture EpCAM-expressing cells, followed by immunostaining of the captured cells. The cell-enumeration results are always expressed as the number of CTCs per 7.5 ml of blood [7]. The limitation of all current systems is the small blood volume available for CTC enrichment, which is related to the relatively low sensitivity of this approach [8].

Here, we investigated the use of the new CellCollector, which is usually used for the *in vivo* isolation of CTCs. Previously, the wire was only applied *in vivo* through a standard venous cannula into the cubital veins of 12 breast and 12 non-small lung cancer patients, and CTCs were detected in all examined tumor stages, including early-stage cancer [9]. We used the CellCollector to capture EpCAM-positive cells in a very small number of PCa patients to demonstrate the general feasibility of capturing CTCs for this type of cancer.

This proof-of-concept study was urgently needed because the wire could not be used *in vivo* in prostate cancer patients. Therefore, we determined whether the CellCollector could be used *ex vivo* for the isolation of CTCs from blood samples (15 ml) obtained from PCa patients with localized, locally advanced and metastatic cancer and a control group. Captured CTCs were identified based on the intensity of the cytokeratin immunofluorescence signal and the location of Hoechst 33258 nuclear stained cells. The cytomorphological and immunofluorescence staining parameters are identical to those of the FDA cleared CellSearch system.

Additionally, we investigated the application of a molecular analysis technique to the CTCs captured by the wire tip (Fig 1). Using this technique, it was possible to obtain information regarding modifications in intracellular signaling events in response to therapy. This information is urgently needed for the broad implementation of CTCs in clinical practices.

Patients and Methods

Patients and blood sample collection

Our preliminary study was approved by the local ethics committee of Martin Luther University Medical School Halle. Written, informed consent was obtained from all patients and volunteers. Patients were enrolled from our clinic from May 2009 to August 2010. These patients were classified into three groups: the first group had localized prostate cancer (lPCa; stage T1-T2 NO, M0, R1), the second group had locally advanced PCa (laPCa; stage T3-T4 N0, M0, Rx), and the third group consisted of patients with metastatic PCa (mPCa; stage T4 N1-N3/M1). Patients in the mPCa group were required to have documented, computed tomography (CT)-confirmed metastases. The control group consisted of volunteers with benign prostatic hyperplasia (BPH) and no evidence of PCa. Patients with second malignancies were excluded.

The samples were stored for a maximum of 4 h at room temperature before processing. Samples of blood (15 ml) were drawn into ethylenediaminetetraacetic acid (EDTA) tubes, and 4 ml of serum was used for the determination of PSA.

Antibody functionalization of the CellCollector

The CellCollector is a 16-cm-long stainless steel wire commonly used in medicine. The interaction of target CTCs with the wire is mediated by an antibody (chiHEA 125, GILUPI) directed against EpCAM, which is overexpressed in different cancer cell types. This antibody is regularly used in humans.

The tip of the wire was functionalized: a 2-cm portion of the wire was coated with a 0.2-μm-thick layer of gold covered by a 1-5-μm polycarboxylate layer (Fig 2). The functionalization of the wire began with the rehydration of the hydrogel by incubating the wire in sterile distilled water for 15 min. The layer was then activated by incubation in a 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride/N-hydroxysulfosuccinimide (EDC/NHS) solution (Sigma) at 22°C for 20 min. NHS (100 mM) was prepared in 50-mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (Sigma) at pH 5.3. Then, 0.5% of EDC (Sigma) was added to the solution. After activation, the wire tip was rinsed with 5-mM acetic acid (Roth) and functionalized by incubating it with the anti-EpCAM antibody for 1 h at 22°C. The hydrogel was covalently coupled to the anti-EpCAM antibody. Free carboxyl groups on the hydrogel were blocked with 1-M ethanolaminehydrochloride (Sigma) at pH 8.5 (30 min, room temperature), and unbound antibodies were removed from the wire by washing three times with distilled water [9]. The functionalized wires were stored in distilled water at 4°C until use.

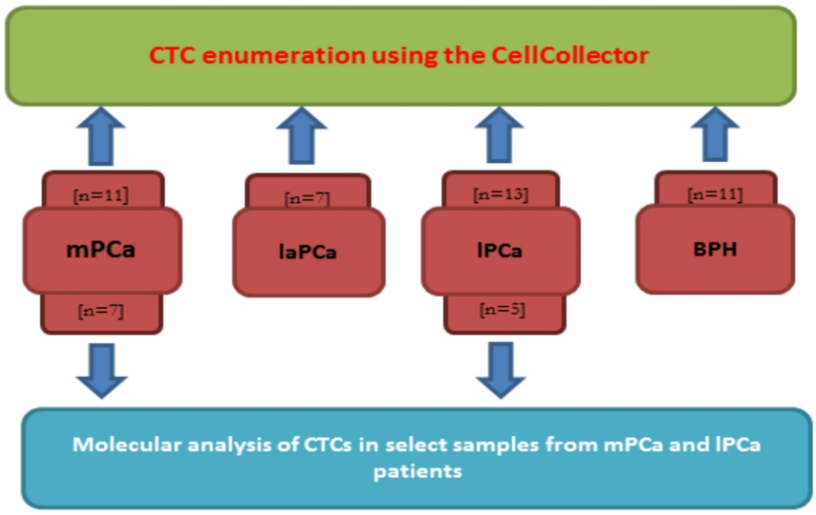

Fig 1. Study design (n = number of blood samples). Localized Prostate Cancer (lPCa), locally advanced Prostate Cancer (laPCa), metastatic Prostate Cancer (mPCa).

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Microfluidic capture of CTCs

This method was first established by experiments with blood that was enriched with cells of the EpCAM-positive PCa cell line LNCapLNCap [10,11], and the performance of the CellCollector was demonstrated with a hemodynamic flow system. The LNCap cells were harvested by trypsin EDTA treatment. To determine cell numbers, an 10 μl-aliquot of cells was placed on a hemocytometer plate, and counts were performed using a conventional inverted microscope.

The parameters of the dynamic model were adapted to the hemodynamic parameters of the peripheral venous blood circulation. The velocity of the blood in the flow system's chamber

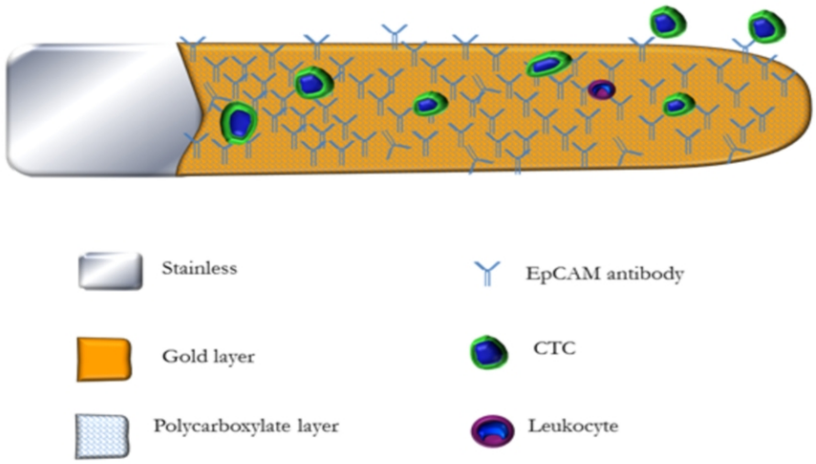

Fig 2. Structural details of the CellCollector.

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(d = 3 mm) was 1.2 cm/s, and the flow speed in a medium-sized human vein is 1–5 cm/s. The functionalized anti-EpCAM wire was inserted into the flow chamber through a septum via a cannula. The flow system was filled with 15 ml of blood, and the blood was pumped through the flow chamber for 14 cycles (30 min). Patient blood samples were subjected to the same conditions.

Immunocytochemical analysis and CTC enumeration

CTCs were identified by immunocytochemical staining and enumerated by a blinded, trained operator. After being incubated with blood in the flow system, the wire was washed three times in phosphate-buffered saline (PBS) (Sigma). Bound cells were fixed with 4% paraformaldehyde (Sigma) for 10 min at room temperature. Subsequently, the cells were blocked with 3% bovine serum albumin (BSA) (PAA) in PBS for 1 h. Primary antibodies, including anti-pan-cytokeratin-fluorescein isothiocyanate (FITC) (CK8, CK18 and CK19, Abcam) and anti-CD45- allophycocyanin (APC, Invitrogen), were added for 1 h. The wire was then rinsed 3 times with 3 ml of PBS, and the nuclei were counterstained with Hoechst 33258 (Invitrogen). CTCs were identified using a Nikon Eclipse E600 fluorescent microscope with a 20x objective. Fluorescent images were recorded with a Vosskühler CCD-1300-QLN-Camera. The images were digitally processed with ImageJ software by altering contrast and brightness in accordance with the Nature Publishing Guidelines. A cell was considered to be a CTC if it was positively stained for pan-cytokeratin, it was negative for CD45, and certain morphological criteria for tumor cells were met: the presence of a nucleus with a round or ellipsoid shape and a cell size ranging from 5 to 50 μm. Leucocytes were defined as nucleated (Hoechst-positive), CD45-positive and pan-cytokeratin-negative cells.

Identification of tumor-specific transcripts in CTCs

First, we performed tumor cell spiking experiments with the PCa cell line LNCap. We enriched EDTA blood samples from healthy donors with a defined cell count (50, 200, or 500 LNCap cells/15 ml blood). Subsequently, the wires were incubated with prepared blood samples in the microfluidic chamber under the conditions described above. The next step was the analysis of 7 blood samples of metastatic PCa patients and 5 localized PCa patients.

After the incubation, the wire was rinsed 3 times with PBS. To isolate mRNA from the captured cells, the functionalized tip of the wire (2 cm) was cut into 5-mm pieces and incubated for 1 min in 100 μl of lysis buffer. The lysis buffer and primers used were components of the commercially available AdnaTest ProstateCancerDetect kit (Adnagen) [12]. Subsequently, the mRNA from lysed cells was recovered by magnetic separation using Oligo (dT) 25 Dynabeads®. The mRNA/bead complex was reverse transcribed using Sensiscript™ Reverse Transcriptase (Qiagen) and RNase inhibitor (Promega). The reverse transcription into cDNA was performed in a one-step reaction (60 min at 37°C min and 5 min at 95°C). The samples were cooled at 4°C and stored at -20°C. The tumor-associated mRNA transcripts from the captured CTCs were analyzed using a multiplex polymerase chain reaction (PCR). The primer mixture allowed for the amplification of three tumor-associated antigens prostate-specific membrane antigen (PSMA), PSA, and epidermal growth factor receptor (EGFR) and the housekeeping gene actin. The primers generated fragments of the following sizes: PSMA, 449 base pairs (bp); PSA, 357 bp; EGFR 163, bp; and actin, 120 bp. In the negative controls, the mRNA and cDNA were replaced with water in the reverse transcription and PCR experiments. The samples were evaluated using the BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) on a DNA 1000 LabChip.

The test was considered CTC-positive for PCa if a PCR fragment of at least one tumor-associated transcript (PSA, PSMA or EGFR) and a fragment of the control gene were clearly detected. The peaks with a concentration of $\geq$ 0.10 ng/μl were considered positive.

Cell lines and culture conditions

The prostatic human tumor cell line LNCap (ATCC), which was established from tumor tissue removed from a metastatic lesion of a man with a prostate carcinoma, was cultured in RPMI 1640 (Invitrogen) medium supplemented with 10% inactivated fetal calf serum (FCS), 2-mM L-glutamine, and 80 g/l gentamycin in a 5% CO_2 humidified incubator at 37°C [10,11].

Statistical design

Statistical analyses were performed and Figs generated using Prism 6 (GraphPad Software, La Jolla, CA). A p-value of $\leq$ 0.05 was considered significant. CTCs were correlated with the PSA level and Gleason sum by determining the Spearman rank correlation coefficients (r). The time-to-event outcome (overall survival [OS]) was evaluated using Kaplan-Meier methods, and the survival-time differences were compared using the log-rank (Mantel-Cox) test.

Results

Patient characteristics

We enrolled 43 PCa patients, and 11 volunteers with BPH served as the control group. Table 1 shows the clinicopathological characteristics of patients with localized PCa (n = 18), locally advanced PCa (n = 7), and metastatic PCa (n = 18). The control group included 11 patients with histopathologically confirmed BPH and no evidence of PCa. The median ages were 69 years (range, 58–77) for patients with localized PCa, 71 years (range, 76–81) for patients with locally advanced disease, and 71.5 years (range, 53–87) for patients with metastatic PCa. The control group had a median age of 67 years (range, 58–83).

In this study, 88.9% of patients with lPCa underwent open or laparoscopic radical prostatectomy. All locally advanced prostate cancer patients had positive surgical margins (PSM), and 85.7% of the patients received androgen treatment. All patients with metastatic PCa underwent androgen-deprivation therapy, and 65% of the patients received docetaxel first-line chemotherapy. Two patients were initially treated with radical retropubic prostatectomy.

Detection of CTCs in different PCa stages

The aim of our proof-of-concept study was to test the functionality of the CellCollector in a preclinical step; therefore, blood samples were studied *ex vivo* and used in the fluid dynamic system, which system provided improved conditions for the *ex vivo* application of the wire. The in vivo application of the CellCollector was not possible at this time point because BfArM approval had not been obtained for this device.

First we evaluated the capture efficiency of the CellCollector with cultured LNCap cells. LNCap cells were spiked into blood of healthy donors. Overall, the percentage of LNCap cells detected after sample processing ranged from 35% at the higher concentration (200 cells/ml) to 10% at the lower LNCap cell concentration (~50 cells/ml). The CTC counts were analyzed in a total of 31 PCa patients and 11 control subjects. Fig 3 shows the scatterplot of all CTC counts.

The control group consisting of BPH patients had a mean of 1.09 CTCs and a median of 0 CTCs (range, 0–4), corresponding to a detection rate of 31.5%. The BPH patients underwent a transurethral resection of the prostate (TURP) to confirm the diagnosis.

Table 1. Baseline demographics and clinicopathological parameters of the study subjects.

	localized	*advanced*	*metastatic*	*control*
Patients (n)	18	7	18	11
Median age, y (range)	**69** (58–77)	**71** (76–81)	**71.5** (53–87)	**67** (58–83)
Race, ethnicity	Caucasian	Caucasian	Caucasian	Caucasian
Gleason score (%)	**2+3** (5.8)	**2+3** (14.3)		
	3+3 (17.6)			
	3+4 (58.8)	**3+4** (28.6)	**3+4** (35)	
	4+3 (17.6)	**4+3** (28.6)	**4+3** (5)	
		4+4 (28.6)	**4+4** (20)	
		4+5 (14.3)	**4+5** (30)	
			5+4 (10)	
			5+5 (5)	
Median baseline PSA ng/ml (range)	**0.254**	**0.023**	**26.7**	**1.75**
	(0.003–10)	(0.04–4.2)	(0.04–1387)	(0.52–15.7)
Primary therapy				
TURP, no (%)				**11** (100)
Surgery (RP), no. (%)	**16** (88.9)	**7** (100)	**2** (11.1)	
Radiation, no. (%)	**5** (27.8)	**3** (42.9)	**10** (55.5)	
Systemic therapy				
Androgen treatment, no. (%)	**3** (16.6)	**6** (85.7)	**18** (100)	
Chemotherapy, no. (%)			**13** (65)	

Control: volunteers with BPH, **RP:** Radical Prostatectomy, **TURP**: Transurethral Resection of the Prostate

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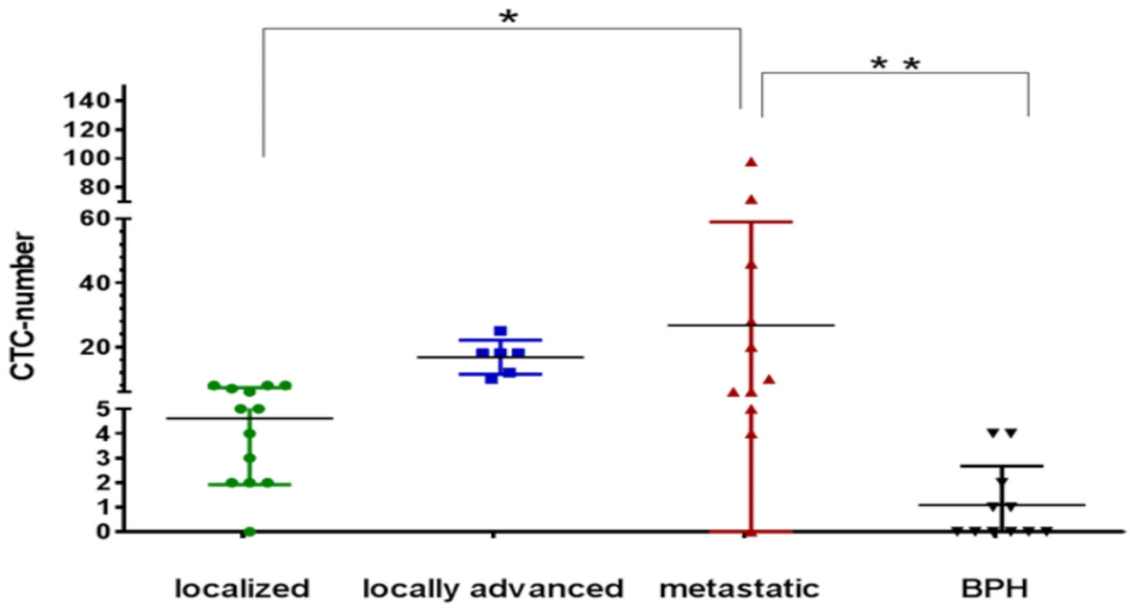
Localized PCa patients displayed a mean of 4.6 CTCs and a median of 5 CTCs (range, 0–8), with a detection rate of 92.3%. These two groups showed no statistically significant difference *($p = 0.615$)* in CTC counts.

Of the 7 patients diagnosed with laPCa, we detected a mean of 16.83 CTCs and a median of 18 CTCs (range, 10–25), with a 100% detection rate. Of these patients, 85.7% underwent androgen treatment, and 100% underwent a radical prostatectomy. The laPCa patients displayed a non-significant difference *($p = 0.39$)* in CTC count compared to the localized PCa group. The CTC number was clearly higher, as in the PCa-l group.

Among the patients with metastatic progression, we detected a median of 10 CTCs and a mean of 26.83 CTCs (range, 0–98), with a 90.9% detection rate. The CTC count of mPCa patients was significantly different than that of patients with localized PCa *($p = 0.01$)* and the control group *($p = 0.0068$)*. Nonetheless, there were no significant differences *($p = 0.55$)* in the CTC counts between the two groups of advanced PCa patients. An important finding of our pilot study was that phenotyping the CTCs on the CellCollector was very user-friendly. The immunocytochemical analysis of the wire demonstrated a clear surface with low contamination by non-specific binding, cell debris and artifacts (Fig 4). The binding of EpCAM-positive leucocytes was very constant in a range of 5 to 10 per wire resulting in a relation of captured CTCs to leukocytes of 50% (5 leucocytes and 5 CTCs) in localized PCa and about 94.6% (5 leucocytes and 89 CTCs) in metastatic PCa patients.

Identification of cancer-specific transcripts in captured CTCs

Initially, we investigated the possibility of performing an mRNA analysis on LNCap cells captured by the wire (50, 200 or 500 cells spiked per 15 ml of blood). In the LNCap cells captured



Number of values	13	6	11	11
Median	5	18	10	0
Mean	4.615	16.83	26.82	1.091

Fig 3. CTC counts in the study population. Each horizontal bar shows the median value. ** $p \leq 0.01$ value and * $p \leq 0.05$.

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by the CellCollector, the mRNA levels of EGFR, PSA and PSMA were detectable in all spiked samples. All analyzed markers and the housekeeping gene actin showed greater mRNA levels as the concentration of added cells increased. The concentrations of cancer-specific transcripts varied, with 0.31 ng/μl of PSMA in 50 spiked LNCap cells and 35.72 ng/μl of PSA in 500 spiked LNCap cells (Table 2).

Next, the molecular profiling of captured CTCs was performed in 5 blood samples obtained from lPCa patients and 7 blood samples obtained from mPCa patients (Table 3). We were able

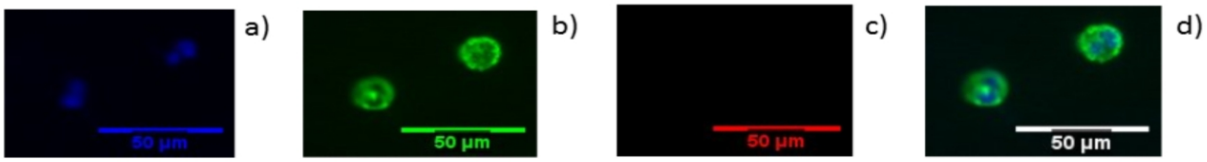

Fig 4. Immunocytochemical analysis of CTCs captured with the CellCollector™ in the blood of prostate cancer patients. The CTCs were identified and enumerated via a) positive nuclear staining (Hoechst), b) positive cytokeratin staining, and c) negative CD45 staining. d) Overlay of all images showing size and morphological characteristics.

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Table 2. Multiplex RT-PCR performed after isolating spiked LNCap cells.

LNCaP Cell Counts	Actin-Signal [ng/μl]	EGFR-Signal [ng/μl]	PSMA-Signal [ng/μl]	PSA-Signal [ng/μl]
50	6.7	2.76	0.31	30.62
200	9.56	4.94	0.66	31.46
500	12.41	13.17	3.21	35.72

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Table 3. Detection of CTCs by multiplex RT-PCR performed after *ex vivo* CTC isolation by the CellCollector and patient characteristics.

Patient No. & stage	Age	PSA (ng/ml)	Gleason	Primary therapy	Systemic therapy	Positive transcripts in CTCs
1 l	**77**	**0.05**	**4+3**	**2, 3**	**1**	**Neg.**
2 m	**75**	**0.06**	**4+5**	**2**	**1, 2**	**EGFR**
3 m	**68**	**7.9**	**4+5**	**1, 3**	**1, 2**	**Neg.**
4 l	**73**	**8.5**	**4+3**	**2**		**Neg.**
5 m	**87**	**82.5**	**4+4**	**3**	**1**	**Neg.**
6 m	**55**	**27.3**	**3+4**	**3**	**1**	**Neg.**
7 m	**70**	**5.1**	**4+5**	**3**	**1**	**PSMA**
8 l	**82**	**0.77**	**3+4**	**2**		**Neg.**
9 l	**53**	**6.1**	**3+4**	**2**		**Neg.**
9 m	**66**	**8.6**	**3+4**	**3**	**1, 2**	**EGFR**
11 l	**58**	**7.3**	**3+4**	**2**		**Neg.**
12 m	**78**	**44.6**	**5+5**	**1, 2, 3**	**1**	**EGFR**

l: localized PCa, **m:** metastatic PCa, **Primary therapy;** TURP [1], Surgery (RP RP = Radical Prostatectomy) [2], Radiation [3], **Systemic therapy:** Androgen treatment [1], Chemotherapy [2]

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to detect cancer-specific transcripts in the CTCs captured by the wire; these transcripts are described in Table 3. The transcripts were identified only in 4 out of 12 (33.3%) blood samples obtained from PCa patients. The classification of blood samples showed that 4 (57.1%) of the blood samples from mPCa patients (n = 7), were positive for one cancer-specific transcript. The EGFR transcript was identified in 3 patients (42.8%) undergoing systemic therapy for metastatic cancer. The PSMA transcript was detected in one patient (14.3%) undergoing androgen-deprivation therapy. The whole blood samples of localized PCa patients were negative for the analyzed cancer-specific transcripts. Thus, mRNA analysis of CTCs captured by the wire is possible.

CTC number and clinical outcomes

For the survival analysis, based on the literature, we used a cut-off point of ≥ 5 CTCs to divide the patients into two groups independent of cancer stage. In the present proof of concept, the Kaplan-Meier analyses demonstrated that patients with >5 CTCs had a mortality risk that was 7.0 times greater than that of patients with ≤ 5 CTCs (hazard ratio (HR) 7.0, 95% confidence interval: 1.1–29.39). There was a significant difference *($p = 0.035$)* in the overall survival (OS) between the two groups. The median OS was undefined for both groups (Fig 5).

Discussion

CTCs have the potential to provide timely information about disease progression and may help to fill the gap between diagnosis and therapy monitoring. Furthermore, CTCs provide the ability to take a "liquid biopsy" and represent a noninvasive tool for obtaining tumor samples at

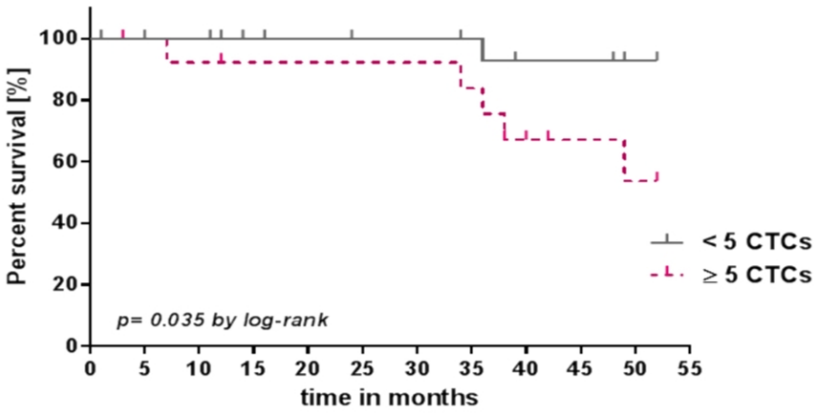

Fig 5. Survival rates (4.5 years) of patients with CTC counts < 5 CTCs and ≥ 5 CTCs. The median survival times are not defined.

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different time points, thereby allowing physicians to validate therapeutic decisions and obtain actual information about the cancer's phenotype [5,13,14].

Our proof-of-concept study reports a new functionalized medical wire (CellCollector) intended for the *in vivo*-and *ex vivo*-isolation of CTCs [9]. The first step in this study was the preliminary application of the wire to blood samples *ex vivo*. This preclinical step was required for the approval of the application of CellCollector *in vivo* by the Federal Institute for Drugs and Medical Devices (BfArM). The wire captured CTCs expressing the cell surface marker EpCAM, which is the same criterion approved by the FDA for the use of the CellSearchSystem in isolating CTCs. We also used the FDA-approved criteria for the identification of CTCs [2]. This definition of CTCs did not allow cancer-specific identification. Our findings first demonstrated that it is possible to use the wire to isolate CTCs *ex vivo* from patients with different stages of PCa. We detected mean numbers of CTCs in the blood of BPH and local PCa patients who were below the 5-CTC threshold. We found that 92.3% of the local PCa group (n = 12) was positive for CTCs. We expect that CTCs are extremely rare and are thus unlikely to be detected in localized PCa patients. The low number of CTCs in BPH and local PCa patients did not allow any disease-specific conclusion. Furthermore, the follow up in the two groups demonstrated that the CTC count had no effect on the survival of local PCa and BPH patients. Therefore, the results of the CellCollector study suggest that a CTC number above or below a disease-specific cut off value is required. A combination of the counts and the genetic characterization of captured cells are urgently needed to achieve better clinical usefulness for CTC parameters for clinicians and patients. Previous studies have identified CTCs in localized PCa patients. Moreno and colleges used FACSCalibur and CellQuest to calculate a mean value of 5.0 CTCs in 7-ml blood samples from lPCa patients [15]. In another pilot study, CTCs were captured using CTC-Chip (anti-EpCAM) and detected in 42% of PCa patients before surgical tumor removal [16]. We confirmed these results, although our results showed no significant differences between the control group and lPCa patients. For this reason, it is necessary to design an additional step for the PCa-specific characterization of the CTCs. This step should induce additional prognostic and therapeutic target information about the captured cells [17,18]. Antonarakis et al. demonstrated that the androgen-receptor splice variant 7 (AR-V7) messenger RNA in CTCs from patients with advanced PCa may be associated with resistance to enzalutamide and abiraterone [19]. These results suggest the benefits and usefulness of

CTCs in the clinical setting. Our metastatic cancer patients displayed a median of 10 CTCs and a mean of 26.8 CTCs. Interestingly, all patients had undergone systemic therapy (100% androgen treatment and 65% chemotherapy) at the time of blood sampling. This could indicate that the cancer was progressing during treatment. In the mPCa group, relating the CTC count with clinical parameters demonstrated only a moderate correlation with the serum PSA level ($r = 0.345$) and the Gleason sum ($r = 0.222$). The other study groups showed no correlations between the CTC count and the clinical or histological parameters. Previous studies have also revealed no or only moderate correlations between the CTC count and other known prognostic parameters [20,21]. In a previous study, the IMMC38 trail confirmed that the PSA titers from patients with progressive, metastatic, castration-resistant PCa were either weakly correlated or not correlated with survival. Those authors summarized that lactate dehydrogenase (LDH) and CTC counts were strongly correlated with survival time [22]. A critical step in CTC isolation in metastatic PCa patients is the epithelial-mesenchymal transition (EMT), which is associated with downregulation of EpCAM expression on the CTC surface. The reduced expression of epithelial markers might therefore result in false negative results [23].

However, we were able to distinguish between the patients' survival prognoses based on their CTC counts. Patients with ≥ 5 CTCs had significantly shorter survival times ($p = 0.035$) independent of the baseline characteristics of the groups. Our preliminary findings confirmed that CTCs are an independent prognostic marker for OS.

Prior studies have verified that CTC enumeration can be used to predict prognoses in patients with metastatic, castration-resistant PCa [2,3,21,22,24]. The largest data sets were obtained with the CellSearchSystem, which has received FDA clearance and undergone broad clinical validation [1,2,21]. However, a limitation of the CellSearchSystem is its required blood volume, and thus, this platform has low sensitivity [25]. Another limitation is the low purity of the isolated cells [8]. Therefore, molecular characterization of the captured CTCs is difficult with this system [26–28]. The clean surface of the CellCollector used in our pilot study constitutes a considerable advantage for the immunocytochemical characterization of captured cells. As a result, immunocytochemical characterization and enumeration are user friendly. Additionally, fewer leukocytes, artifacts and cell debris are captured by the wire surface. Our results show that the molecular phenotyping of PCa-CTCs captured by the wire is possible (Table 3). Initially, we used spiking experiments to check the wire's sensitivity. It is possible to molecularly characterize captured cells, and the spiked cell counts represent the marker concentrations of the cell counts. However, it can be challenging to spike the correct cell count into the blood samples because this technique requires experience in cell counting under a microscope.

Finally, we achieved a sensitivity of 57.1% in the mPCa group and negative results in the lPCa group. We identified the cancer specific transcripts EGFR and PSMA in CTCs. Only one of the 7 patients had decidable PSMA expression. This result suggests that CTCs may have a heterogeneous phenotype. PSMA expression is known to increase in higher-grade PCas, during cancer progression, and following castration. Another advantage of the PSMA protein is its stable expression during the EMT [8,28]. Gleghorn et al. developed a device that allowed for immunocapturing by the PSMA antibody. The geometrically enhanced differential immunocapture (GEDI) microfluidic device captured a median of 54 CTCs from the blood of castration-resistant PCa patients undergoing taxane chemotherapy [29,30].

The EGFR signal in the captured CTCs is not specific for prostate cancer, but this factor plays a central role in cell proliferation, migration, motility, invasion, and survival in normal and malignant cells. Among our analyzed blood samples of PCa-m ($n = 7$), 42.8% ($n = 4$) were positive for EGFR mRNA in CTCs. These results suggest cancer progression and might be a valuable tool in the near future. The limited results from the molecular analysis of captured CTCs may confirm the tumor heterogeneity, which has been described in detail in epithelial

cancer types [20]. These types of analyses are very important because, if specific markers are used, it is feasible to identify which CTCs have aggressive or dormant behaviors. Currently, various different platforms exist for CTC isolation. However, challenges remain, including the need for a robust technology to detect CTCs with high sensitivity and specificity. This technique should reflect the rarity, fragility and heterogeneous phenotypes of the CTCs. Furthermore, we must specifically characterize PCa CTCs to provide more information in addition to the CTC count.

One limitation of our pilot study is, of course, the small study population. However, the 55-month patient follow-up was sufficient to demonstrate the prognostic value of our method. The CellCollector is a CTC-detection system that allows for the molecular characterization of CTCs on the mRNA level and through the immunocytochemical analysis of the captured CTCs.

Conclusion

Our data demonstrated that at different stages of PCa, the sensitive isolation and molecular characterization of CTCs *ex vivo* by the CellCollector are feasible. This proof of concept was required to obtain BfArM approval of the CellCollector in a clinical study for the *in vivo* isolation of CTCs in the blood stream of PCa patients. Clearly, larger prospective trials using the CellCollector are needed to evaluate the method. This exploratory study reveals the opportunity to apply a CTC-isolation technique that allows for the counting and molecular characterization of CTCs.

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Author Contributions

Conceived and designed the experiments: GT KF EW RM RH PF.

Performed the experiments: GT RM KF.

Analyzed the data: GT.

Contributed reagents/materials/analysis tools: GT KF RH PF KL.

Wrote the paper: GT KF PF.

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3.2 In vivo Isolation zirkulierender Tumorzellen bei Prostatakarzinompatienten verschiedener Stadien

Theil G, Boehm C, Fischer K, Bialek J, Hoda R, Weber E, Schönburg S, Kawan F, Fornara P. **In vivo isolation of circulating tumor cells in patients with different stages of prostate cancer.** Oncol Lett 2021 Vol. 21 Issue 5 Pages 357

Die vorliegende Studie validierte den CellCollector (CC) hinsichtlich der in vivo Isolation von CTCs aus dem Blut von Prostatakarzinompatienten verschiedener Stadien und zweier Kontrollgruppen. Dabei lag das Hauptaugenmerk auf der CTC-Zahl im klinischen Verlauf der PCa-Patienten. Eine Bewertung der erhaltenen CTC-Zahlen erfolgte durch einen Methodenvergleich mit dem CellSearch-System als Referenzmethode.

Unsere Studienpopulation bestand aus 14 Patienten mit metastasiertem (PCa-m) und 21 Patienten mit lokalisiertem (PCa-l) Prostatakarzinom. Eine Kontrollgruppe von 16 Männern mit gutartiger Prostatahypertrophie (BPH) und 20 Frauen als zweite Kontrollgruppe wurden ebenfalls eingeschlossen. Die Patienten in der lokalisierten Gruppe und der BPH-Gruppe mussten ein histologisch diagnostiziertes Prostatakarzinom bzw. eine benigne Prostatahyperplasie aufweisen. Die Patienten in der metastasierten Gruppe hatten bildmorphologisch (radiologisch) positive Befunde für Metastasen. Von den 14 PCa-m Patienten wurden 13 (92,8 %) Patienten mit Chemotherapie und zehn Patienten (71,4 %) mit ADT therapiert. Die primäre Therapie für die 21 Patienten mit einem lokalen Karzinom war die radikale Prostatektomie (RP). Fünf Patienten (18,5 %) aus dieser Gruppe erhielten eine postoperative Bestrahlung. Alle Patienten unserer Kontrollgruppe mit benigner Prostatahyperplasie wurden mit einer transurethralen Resektion der Prostata behandelt (Originalarbeit 2, Tab. 1).

Im Studienzeitraum hatten die PCa-m Patienten acht Visiten innerhalb eines Jahres (Visite 1-6: 1.-6. Mo., Visite 7: 8. Mo., Visite 8: 12 Mo.). Die PCa-l Patienten hatten innerhalb eines Jahres 3 Visiten (Visite 1: vor RP, Visite 2: 6 Mo. nach RP, Visite 3: 12 Mo. nach RP). Die BPH-Patienten hatten ebenfalls innerhalb eines Jahres drei Visiten im zeitlichen Abstand von sechs Monaten. Bei allen Patienten erfolgte zum Zeitpunkt der CTC-Isolierung eine PSA-Wert Bestimmung. Die Frauen als zweite Kontrollgruppe hatten nur eine Visite.

Im Rahmen dieser Studie wurde der CellCollector erstmals in vivo in die Armvene von PCa-Patienten und Probanden der Kontrollgruppe eingeführt und somit auch auf seine Verträglichkeit geprüft. Die isolierten Zellen wurden von einem verblindeten, sowie erfahrenen Mitarbeiter, nach den FDA-CTC-Kriterien analysiert. EpCAM-positive Zellen wurden als CTCs gezählt nach den unter 1.2 genannten Kriterien.

Insgesamt konnten 188 CC-Anwendungen in die Auswertung einbezogen werden. Der CC wurde gut vertragen und keine unerwünschten Ereignisse (AEs) oder schwerwiegende unerwünschte Ereignisse (SAEs) wurden beobachtet. In der metastasierten Gruppe waren 78,9 % (n = 57) der 71 Anwendungen positiv für ≥ 1 CTCs. Die mediane CTC-Zahl betrug 4 (0-820) CTCs und der MW betrug 27 CTCs. Im Vergleich zu der 100 % CTC-Detektionsrate in unserer ex vivo CTC-Isolierungsstudie (Originalarbeit 1) ist die oben genannte Detektionsrate leicht reduziert [79]. Eine Erklärung könnten die heterogenen Phänotypen der CTCs bei Patienten mit einem progressiven Karzinomstatus sein sowie die relativ kurze Halbwertszeit der CTCs im Blut [35]. Darüber hinaus wurden im Vergleich zu den ex vivo Anwendungen des CCs mehr Leukozyten und Zellen detektiert, welche positiv für EpCAM, CD45 und pan-CK waren [79]. Dieses Phänomen kann auf eine Aktivierung des Immunsystems hinweisen und eine mögliche therapeutische Reaktion signalisieren [80]. Dennoch bleibt dieses heterogene Bild von gebundenen Zellen an die CC-Oberfläche eine Herausforderung für die CTC-Identifikation. Unserer CTC-Detektionsrate der PCa-m Gruppe zeigte in der Gegenüberstellung zu weiteren Studien vergleichbare Ergebnisse [43, 61, 81, 82].

In der lokalisierten Gruppe waren 45,3 % (n = 24) der 53 CC Anwendungen positiv für ≥ 1 CTCs. Die mediane CTC-Zahl betrug 0 (0–9) CTCs und der MW betrug 1,45 CTCs. Die CTC-Zahlen zwischen der lokalen und der metastasierten Gruppe waren signifikant unterschiedlich ($p < 0{,}0001$). CTCs von Patienten mit lokalem PCa werden als mögliche Marker zur postoperativen Risikostratifizierung diskutiert [83]. In unserem Kollektiv korrelierte die CTC-Zahl vor und nach radikaler Prostatektomie nicht mit den untersuchten klinischen und pathologischen Parametern. Übereinstimmend mit unseren Ergebnissen beobachteten Meyer et al. keine Korrelation der präoperativen CTC-Zahl mit dem Auftreten eines biochemischen Rezidiv in der PCa-l Patientengruppe (n = 152) [84]. Todenhöfer et al. detektierten bei Patienten mit einem lokalen PCa, unter Verwendung eines EpCAM-unabhängigen Isolationssystems, in 50 % der untersuchten Blutproben CTCs. Allerdings zeigt sich auch hier keine Korrelation zwischen CTC-Zahl und den klinischen Parametern wie PSA-Wert, Tumorstadium oder Gleason-Score [85]. Diese Resultate bekräftigen die noch unzureichend geklärte Situation der klinischen Relevanz von CTCs bei Patienten mit lokalem PCa.

In den Kontrollgruppen wurden bei 70,7 % (n = 29) der BPH-Patienten und 85 % (n = 17) der Frauen keine CTCs nachgewiesen. Die mediane CTC-Zahl der BPH-Patienten war 0 (0-13) CTC, die mediane CTC-Zahl der Frauen betrug 0 (0-3) CTCs.

Für einen direkten Methodenvergleich zwischen dem CellCollector und dem CellSearch-System, beides EpCAM-abhängige CTC-Isolationstechnologien, wurden 95 Patienten mit

CellCollector-Anwendungen und mit dem CellSearch-System (7,5 ml Blut) parallel nach identischen Kriterien analysiert. Der CellCollector detektierte bei 46,2 % der PCa-l Patienten im Median 0 (0-9) CTCs und bei 78,4 % der PCa-m im Median 3 (0-820) CTCs. Das CellSearch-System isolierte CTCs bei 10,3 % der PCa-l Patienten im Median 0 (0-1) CTCs und bei 67 % der PCa-m Patienten im Median 3,5 (0-1428) CTCs (Originalarbeit 2, Abb. 4). Die CTC-Zahlen der beiden Systeme zeigten keine Korrelation.

Für die Ermittlung der diagnostischen Genauigkeit des CellCollectors, des CellSearch-Systems sowie der PSA-Werte von PCa-m Patienten wurden ROC-Kurvenanalysen durchgeführt. Bemerkenswerterweise zeigten die Flächen unter den ROC-Kurven (AUC) für alle drei Parameter ähnliche Bereiche: AUC 0,87 (95 % KI 0,8-0,94) für die PSA-Werte, 0,82 (95 % KI 0,74-0,89) für die CTC-Zahlen des CCs und 0,84 (95 % KI 0,76-0,92) für die CTC-Zahlen des CellSearch-Systems (Originalarbeit 2, Abb. 5). Alle untersuchten Parameter hatten damit eine geringe Wahrscheinlichkeit falsch-negative und falsch-positive Ergebnisse zu ermitteln. Die CTC-Zahl und der PSA-Wert haben in unserer Studie eine ähnliche diagnostische Genauigkeit. Goldkorn et al. zeigten in der Studie SWOG S0421 in einer Analyse mit dem CellSearch-System, dass die ROC-Kurven für die CTC-Zahl am Tag 0 (AUC 0,781) eine höhere diagnostische Genauigkeit aufwies als die für den PSA-Wert (AUC 0,665) am Tag 0. Damit wurde die CTC-Zahl am Tag 0 als prognostisch für das OS von mCRPC-Patienten angesehen. Aber auch eine steigende CTC-Zahl nach drei Wochen unter Therapie wurde mit einem signifikant schlechteren OS in Verbindung gebracht [62]. Ein möglicher Grund könnte hierfür sein, dass CTCs im Gegensatz zum PSA nicht direkt von einer Hormonbehandlung beeinflusst werden.

Eine individuelle Therapieüberwachung mit Hilfe von CTC/PSA-Profilen über einen definierten Zeitraum kann dabei helfen eine Therapie neu auszurichten bzw. zu optimieren. Unsere CTC/PSA-Profile wurden nur von PCa-m Patienten mit mehr als drei Visiten erstellt. So zeigte sich als Zeichen des Therapieansprechens ein kurzzeitiger Rückgang des PSA-Wertes und der CTC-Zahl zum Beginn der Hormontherapie bei Patient P072. Bei Patient P022 waren die CTC-Zahlen und der PSA-Werte eindeutig mit zusätzlichen Therapien assoziiert, wie z.B. der palliativen transurethralen Resektion der Prostata und der Entfernung von Hirnmetastasen (Originalarbeit 2, Abb. 6). Das Vorhandensein von CTCs repräsentiert ein aktives Karzinom, welches sich durch die Blutzirkulation ausbreitet. Der Vorteil der CTC-Zahl ist ihre Unabhängigkeit von der AR-Signalübertragung, im Gegensatz zum PSA-Wert. Sinkt sie, so weist das auf einen direkten Rückgang des Metastasierungspotentials hin [86]. Aus den genannten Gründen ist der z.T. konträre PSA-Wert-Verlauf zum Verlauf der CTC-Zahl zu erklären. Wichtig ist, dass die individuellen CTC-Profile die Möglichkeit bieten, die Krankheit zu überwachen, und so eine maßgeschneiderte Behandlungsentscheidung für

einzelne Patienten im metastasierten Stadium zu treffen. Im Bereich der personalisierten Medizin kann ein solches Profil Patienten vor unnötigen Nebenwirkungen einer unwirksamen Behandlung schützen. Unsere Ergebnisse bestätigen das Potenzial von CTCs als pharmakodynamische und intermediäre Endpunkt-Biomarker für das Gesamtüberleben [43, 87]. Leider war diese Option nicht bei allen unseren PCa-m Patienten möglich, was teilweise auf die heterogenen Phänotypen von CTCs in dieser Gruppe zurückzuführen ist [21, 88, 89]. Zur Verbesserung der in vivo CTC-Isolierung bei Patienten mit metastasiertem Prostatakrebs sollte ein EMT-stabiler Marker wie das prostataspezifische Membranantigen [90], zur Funktionalisierung des CCs verwendet werden.

Die Kaplan-Meier-Kurven mit einer medianen Follow-up-Zeit der PCa-Gruppe von 37 Monaten zeigen, dass PCa-Patienten mit ≥ 5 CTCs ein medianes OS von 27,5 Monaten haben, im Vergleich zu 37 Monaten für Patienten mit < 5 CTCs HR 2,6 (95% KI 0,78-8,3). Interessanterweise haben die PCa-m Patienten mit ≥ 5 CTCs zu allen Zeitpunkten und steigenden CTC-Zahlen das kürzeste mediane OS von 25 Monaten, im Vergleich zu 34 Monaten HR 1,9 (95 % KI 0,4-11,6) bei Patienten mit abnehmender CTC-Zahl (Originalarbeit 2, Abb. 7). Die steigende CTC-Zahl ist ein deutliches Signal der Progression des Karzinoms und stellt eine aktive Tumorausbreitung dar.

Zusammenfassend demonstrieren die vorliegenden Ergebnisse, dass die in vivo Detektion von CTCs mit dem CellCollector die Limitation des geringen Blutvolumens anderer diagnostischer CTC-Isolationstechnologien überwinden kann und die Sensitivität der CTC-Detektion erhöht. Das CTC/PSA-Profil eröffnet die Möglichkeit einer personalisierten Therapieüberwachung, die dazu beitragen kann, Patienten vor unnötigen Nebenwirkungen als Folge einer ineffektiven Therapie zu schützen. Der CellCollector wurde gut vertragen und es wurden keine Nebenwirkungen berichtet. Dennoch muss sich der CellCollector in weiteren Studien als robuste Technologie mit hoher Empfindlichkeit und Spezifität beweisen.

Originalarbeit 2

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In vivo isolation of circulating tumor cells in patients with different stages of prostate cancer

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Abstract. Circulating tumor cells (CTCs) provide accurate information on the clinical stage of cancer progression. The present study examined the clinical validity and feasibility of a new medical device for the *in vivo* isolation of CTCs from the blood of patients with prostate cancer (PCa). The GILUPI CellCollector® (DC01) was applied in 188 cases. The CTC/prostate-specific antigen (PSA) profile of each patient was checked for therapeutic monitoring of patients with PCa. The CellCollector, which is a unique *in vivo* approach for the isolation of CTCs, was compared with the CellSearch® system, which is the current standard. Overall survival (OS) and diagnostic performance were evaluated. By *in vivo* isolation, 78.9% (56/71) of patients with metastatic disease (PCa-m) and 46.3% (24/53) of patients with localized disease (PCa-l) had ≥1 captured CTC. Kaplan-Meier analysis revealed that patients with PCa-m that had ≥5 CTCs had a significantly different OS compared with those with <5 CTCs (27.5 months vs. 37 months; HR 2.6; 95% CI 0.78-8.3). Patients with a higher number of CTCs at all time-points had the shortest median OS of 25 months (HR 1.9; 95% CI 0.4-11.6). The effectiveness of CTC isolation technologies demonstrated that in 65.7% of the applications, patients with cancer were positive for CTCs using the CellCollector. By contrast, the CellSearch system detected CTCs in 44.4% of applications. *In vivo* isolation of CTCs demonstrated the clinical viability of the CellCollector, related to the current standard for the isolation of CTCs from patients with PCa. The advantage of the *in vivo* device is that it overcomes the blood volume limitations of other CTC assays. Furthermore, the present study revealed that the CellCollector was well tolerated, and no adverse events (AEs) or serious AEs were reported.

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Key words: CellCollector®, circulating tumor cells, *in vivo* isolation, prostate cancer, liquid biopsy

Introduction

Prostate cancer (PCa) is the fifth leading cause of death in men, with 359,500 deaths worldwide in 2018 (1). The disease phenotypes varied from indolent to aggressive. The local stage is potentially curable with local therapy and shows a 5-year survival rate of nearly 100%, compared with 29.8% for metastatic cases (1). One challenge for clinicians is to determine the optimal sequencing therapies for patients who present intermediate, high-risk localized, locally advanced or metastatic prostate cancer (mPCa) to minimize overtreatment and improve outcomes. Thus, early and precise detection of cancer is important for decreasing patient mortality. In addition, the current therapeutic landscape offers the patient an individualized treatment approach. Nevertheless, the treatment of mPCa is becoming increasingly complex (2). The risk of overdiagnosis and overtreatment remains and has a negative impact on the quality of life of men with PCa (3). One of the greatest challenges in the current management of PCa is adequate assessment of the response to treatment. Prostate-specific antigen (PSA) as a tumor marker for prostate cancer has limitations as a surrogate for survival end points because of insufficient sensitivity and specificity (4). Additionally, PSA determination is not an adequate marker for the evaluation of treatment response. However, personalizing PCa treatment with a biomarker, such as circulating tumor cells (CTCs), offers the possibility to create risk-adapted strategies to optimize patient care. CTCs represent a minimally invasive source of spreading tumor cells and provide important clinical information for the individual patient's treatment in terms of monitoring metastasis, evaluating the efficacy of treatment, and/or facilitating the early detection of treatment resistance (5-7). In recent reports, detection of androgen-receptor splice variant 7 (AR-V7) in pooled CTCs of men with progressive metastatic castration-resistant prostate cancer (mCRPC) was associated with resistance to the androgen receptor inhibitors abiraterone and enzalutamide. This finding shows that CTCs can provide insights into drivers of tumor growth in patients and into the pharmacodynamics effects of targeted therapies (4,8).

However, it remains difficult to isolate and characterize CTCs because of their rarity (1-10 CTCs per ml blood) and

heterogeneous phenotype (9). Additionally, CTCs are present in a large background of hematopoietic cells. In 2004, the CellSearch® system was the first US Food and Drug Administration (FDA)-authorized system for the enumeration of CTCs in 7.5 ml of blood. Clinical studies demonstrated that CTCs captured with the CellSearch system were clearly associated with poor patient outcomes (10,11). At present, several platforms have been developed to detect CTCs. CTC detection can be achieved based on physical and biological properties (12,13).

In a previous study, we evaluated the CellCollector® (GILUPI CellCollector), an *in vivo* approach initially *ex vivo* (*in vivo* was not allowed at this time) in blood samples from PCa patients. Our results showed that the CellCollector could be applied for the sensitive isolation and molecular characterization of CTCs *ex vivo* (14). To date, other study has evaluated the CellCollector in single-center trails in patients with breast, lung, high-risk PCa and neuroendocrine tumors in small cohorts (15-17).

In the present study, we validated the CellCollector, which allowed *in vivo* isolation of CTCs directly from the cubital vein in a cohort of prostate cancer patients in different clinical stages and control groups. This included monitoring prostate cancer patients during treatment for a clinical response correlated to CTC counts and comparison of CellCollector results to those from the CellSearch System.

Materials and methods

Study population and clinical information. The patients provided written informed consent and were enrolled at University Clinic and Outpatient Clinic for Urology, Medical Faculty of Martin Luther University Halle-Wittenberg from February 2011 to March 2012. The medical faculty ethics committee of Martin Luther University Halle-Wittenberg approved the study protocol. Furthermore, we obtained a permit from the Federal Institute for Drug and Medical Devices (Germany, BfArM).

The study population consisted of 14 metastasized (PCa-m) and 21 localized (PCa-l) PCa patients. A control group of 16 men with benign prostate hypertrophy (BPH) and a second control group of 20 women were also included (Table I). The patients were required to have histologically proven prostate cancer in the localized group and documented metastases, as confirmed by computed tomography (CT), in the metastasized group. All patients had PSA levels determined at every time point of CTC isolation.

In vivo CTC isolation. The CellCollector consists of a 160-mm sterile steel wire with a 20-mm functionalized tip containing epithelial-cell adhesion molecule (EpCAM) antibodies on its surface. The antibodies are covalently bonded to a hydrogel that is linked to a gold layer (Fig. 1). The wire was inserted into the cubital vein through a 20G cannula and remained in place for 30 min. Then, the CellCollector was washed three times with phosphate-buffered saline (PBS), and captured cells were fixed with 100% acetone for 10 min at room temperature and blocked with 3% bovine serum albumin/PBS for 30 min. The captured cells were identified by immunofluorescence staining, and the CellCollector was examined for fixed cells using a Nikon microscope (TE2000-E) at 20x magnification. The images were digitally processed with ImageJ software by altering the contrast and brightness in accordance with Nature Publishing Guidelines.

CellSearch system. Blood samples were collected into 7.5 ml CellSave tubes. These samples remained stable for 96 h at room temperature and were sent overnight to the University Medical Center Hamburg-Eppendorf. CTCs were isolated using EpCAM-functionalized immunomagnetic beads with a semiautomated workflow that included enrichment, fluorescent labeling/characterization and automated fluorescence imaging of the rare cell population (9,18,19).

CTC enumeration and morphology. CTC enumeration and identification for both isolation technologies were based on identical criteria. Isolated cells and/or clusters of immunostained cells of interest were examined by a blinded experienced researcher. EpCAM-positive cells were defined as CTCs with the following cytology-based FDA definition: i) size ≥4 μm, ii) visible cytoplasm, iii) high nuclear/cytoplasm ratio, iv) positive fluorescent staining of CK 8, 18, and 19 with negative staining of CD45, and v) 50% of the nucleus contained within the CK border (20).

Statistical evaluation. Since limited data regarding the CellCollector were available at the time of the study design, no formal sample size calculations were performed. Therefore, our analyses were exploratory in nature. We compared the CTC counts between the control group and the PCa-l and PCa-m groups using Kruskal-Wallis test followed by Dunn's multiple comparison test. The overall survival rates were calculated using CTCs value at baseline and follow up visits. The log-rank test was used for comparing the Kaplan-Meier survival curves. For all analyses, $P<0.05$ was considered statistically significant. The accuracy of the CTC counts and PSA level were evaluated by receiver operating characteristic (ROC) analysis. Analyses were performed using GraphPad Prism version 6.

Results

Study population and clinical information. The baseline characteristics and clinical parameters of the different study groups are summarized in Table I. In total, 71 study subjects were enrolled in our trial, and 92.8% of the 14 PCa-m patients received chemotherapy. Ten prostate cancer patients (71.4%) were treated with androgen deprivation therapy (ADT). The primary therapy for all 21 PCa-l patients was radical prostatectomy (RP). Five patients (18.5%) received postoperative radiation. All patients with benign prostatic hyperplasia in our control group were treated with transurethral resection of the prostate. The second control group consisted of healthy women. A schedule of the *in vivo* application and the comparison method is presented in Fig. 2.

In vivo CTC isolation. Overall, 188 CellCollector applications were included in the analysis (Fig. 3). The CellCollector was well tolerated, and no adverse events (AEs) or serious adverse events (SAEs) were reported. In the metastatic group, 78.9% (n=57) of the 71 applications were positive for ≥1 CTCs. Among

Table I. Study population demographics.

	Prostate cancer		Control group	
Characteristics	Metastatic	Localized	BPH	Women
Patients, n	14	21	16	20
Median age (range), years	52 (53-79)	59 (56-72)	67 (58-83)	25.5 (19-38)
Ethnicity	Caucasian	Caucasian	Caucasian	Caucasian
Gleason score at diagnosis				
≤7, n (%)	2 (14.30)	18 (85.7)		
>7, n (%)	12 (85.70)	3 (14.3)		
Median PSA at baseline, ng/ml (range)	23.6 (3.5-1120)	7.91 (1.8-39.5)	1.9 (0.41-15.9)	
Median PSA, ng/ml (range)	21.7 (0.04-1120)	0.04 (0.04-3.3)	0.4 (0.04-3.1)	
Primary therapy				
TURP, n (%)			16 (100)	
Surgery (RP), n (%)	2 (13.3)	21 (100)		
Radiation, n (%)	9 (60.0)	5 (18.5)		
Systemic therapy				
Androgen treatment, n (%)	10 (71.4)	2 (7.1)		
Chemotherapy, n (%)	13 (92.8)			
Site of metastatic disease				
Bone, n (%)	12 (86.7)			
Lymph node, n (%)	6 (42.9)			
Other soft tissue, n (%)	3 (21.4)			

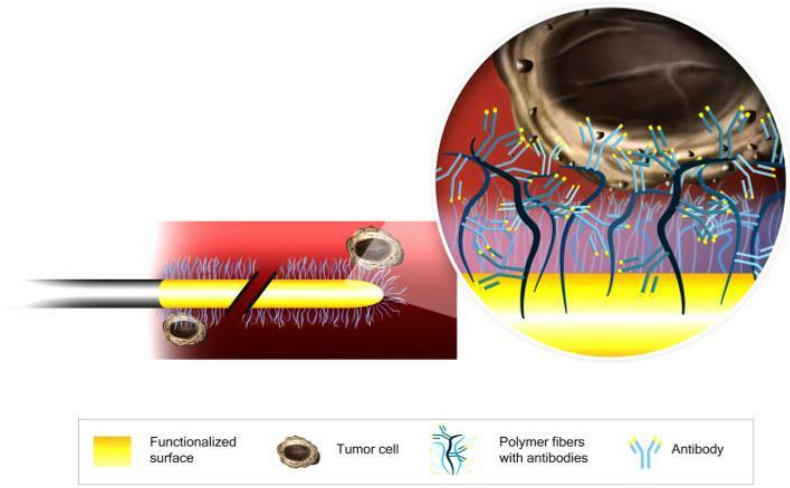


Figure 1. Schematic of the CellCollector®. CTC capture process is achieved by using an antibody against EpCAM protein. CTC, circulating tumor cells; EpCAM, epithelial-cell adhesion molecule.

the metastatic patients with detectable CTCs, the median CTC count was 4 (range, 0-820), and the mean CTC count was 27. In the localized group, 45.3% (n=24) of the 53 CellCollector applications were positive for CTCs. Most of the identified CTCs were single cells, and cell clusters were rarely present. Among the CTC-positive localized PCa patients, the CTC median count was 0 (range, 0-9.0) and the mean CTC count was 1.45, and the CTC count was significantly different (P<0.0001) between the cancer groups (Fig. 3). A total of 70.7% (n=29) of the BPH patients and 85% (n=17) of the women in our control group were negative for CTCs. The median CTC count of the BPH patients was 0 (range, 0-13); a median CTC value of 0 (range, 0-3) was

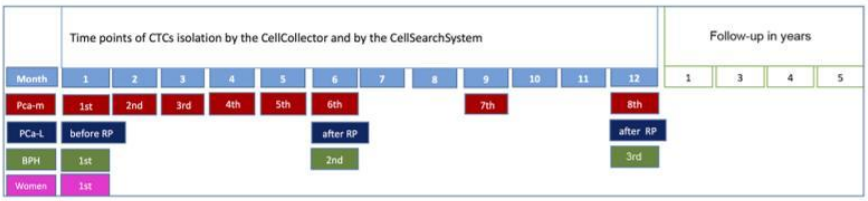


Figure 2. Study design. RP, radical prostatectomy; BPH, benign prostatic hyperplasia; PCa-m, prostate cancer metastasized patient; PCa-l, prostate cancer localized.

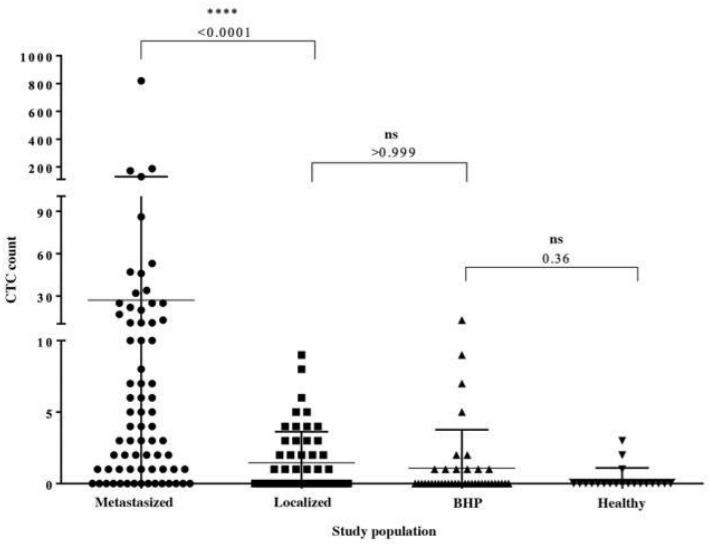


Figure 3. CTC enumeration in metastasized and localized PCa patients and the control groups as evaluated with the CellCollector®. Data are shown as the median and interquartile range. Dunn's multiple comparisons test was used to calculate the P-value. PCa, prostate cancer.

also detected in the female controls. With the exception of the female control group, one wire per group from the PCa-m, PCa-l and BPH groups could not be evaluated.

Effectivity of CTC isolation technologies. For direct comparison of the CTC isolation technology, 95 analyzable blood samples (7.5 ml CellSave) were taken prior to the CellCollector applications and detected by the CellSearch system. As shown in Fig. 4, the CellCollector captured *in vivo* CTCs in 18 of 39 PCa-l patients (46.2%) with a median (range) of 0 (0-9); in PCa-m patients, the CellCollector detected CTCs in 47 of 60 patients (78.4%) with a median (range) of 3 (0-820) CTCs.

The CellSearch system isolated CTCs in 4 of 39 of the PCa-l patients (10.3%) with a median (range) of 0 (0-1) and showed positive results for CTCs in 40 of 60 of the PCa-m patients (67%) with a median (range) of 3.5 (0-1,428) (Fig. 4). Both systems demonstrated no correlation between CTC counts.

To investigate the diagnostic accuracy of the CellCollector, we compared the PSA level and CTCs detected using the CellSearch system in PCa-m patients, BPH patients and healthy donors. Notably, the ROC curves for these three parameters showed similar areas under the curve (AUCs): 0.87 (95% CI, 0.8-0.94) for PSA, 0.82 (95% CI, 0.74-0.89) for the CellCollector and 0.84 (95% CI, 0.76-0.92) for the CellSearch system (Fig. 5). These values indicated a low likelihood of false-negative and false-positive results.

Individual patient CTC/PSA profiles. Regarding the individual CTC/PSA profiles for therapy monitoring in the PCa-m group, we included only metastasized patients with more than three visits. The CTC/PSA profiles over time are illustrated for 4 patients in Fig. 6A-D. The onset of hormonal therapy in patient P072 led to a short decline in the PSA level and CTC count. Approximately one month before visit 8, chemotherapy with docetaxel was started. This patient presented radiologic and PSA progression and a rising CTC count (Fig. 6B). As observed in patient P022, the CTC number and the PSA level were clearly associated with additional therapies, such as palliative transurethral resection of the prostate (TURP) and removal of brain metastases (Fig. 6D). However, the PSA and CTC profiles could be related to the need for additional therapy in only 4 of the 10 patients with metastatic disease (Fig. 6A-D).

Correlation of in vivo CTC count with clinical outcome and CTC kinetics. The median follow-up time in the cancer group was 37 months. The Kaplan-Meier curves, according to the CTC count for metastatic prostate patients, showed that a CTC count of 5 or more has a lower median OS of

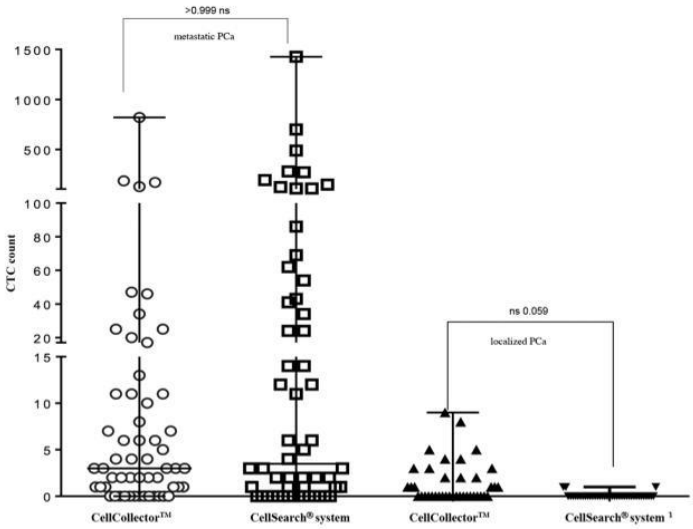
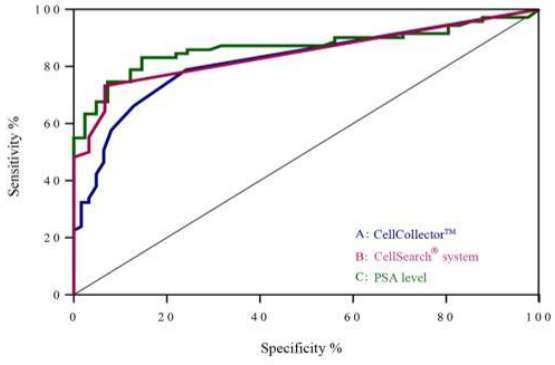


Figure 4. Comparison of CTC isolation technologies (CellCollector® and CellSearch® system). The results of CTC enumeration are summarized for PCa patients after a 12-month follow-up period. A median of 3 CTCs in PCa-m and a median of 0 CTCs in PCa-l were detected with the CellCollector. The CellSearch system detected a median of 3.5 CTCs in PCa-m and a median of 0 in PCa-l, respectively. Data are shown as the median and interquartile range. PCa, prostate cancer; CTCs, circulating tumor cells; PCa-l, prostate cancer localized; PCa-m, prostate cancer metastasized.



	AUC	95% CI	P***
A: PSA level (ng/ml)	0.87	0.80-0.94	<0.0001
B: CellCollector™	0.85	0.76-0.92	<0.0001
C: CellSearch® system	0.82	0.74-0.89	<0.0001

Figure 5. ROC curves illustrating diagnostic accuracy of CTCs count detected with the CellCollector® (blue line), with CellSearch® system (pink line) and the PSA level (green line) of metastasized prostate cancer patients to control group. Results of ROC analysis curves for (A) CellCollector, (B) CellSearch system, and (C) PSA level (ng/ml). ROC, receiver operating characteristic; CTCs, circulating tumor cells; PSA, prostate specific antigen.

27.5 months compared to 37 months for patients with less than 5 CTCs (HR 2.6, 95% CI, 0.78-8.3) (Fig. 7A). Interestingly, PCa-m patients with more than 5 CTCs at all time points and increasing CTC counts showed the shortest median OS of 25 months compared to 34 months (HR 1.9, 95% CI 0.4-11.6) for patients with declining CTC counts (Fig. 7B).

In a match analysis of localized PCa patients before and after RP, 6 of 14 patients (43%) showed at least one CTC. The 6- and 12-month visits after surgery included 8 (58%) and 5 (36%) patients who were CTC-positive. In the follow-up of PCa-l patients after RP, we observed no significant variation in the CTC number and no prognosis of OS. Furthermore, the presence of CTCs did not correlate with the PSA level, Gleason score or tumor, node, metastasis (TNM) classification in any PCa group.

Discussion

The development of sensitive and specific assays for the detection, isolation and characterization of CTCs in the blood of cancer patients is still in progress. At this time, only one assay (CellSearch system) has achieved the level of validity for approval by the FDA (11).

Our objective was to prove the clinical feasibility of the *in vivo* isolation of CTCs in PCa patients. The CellCollector was inserted into the vein of the patient six times per year per patients with metastatic prostate cancer. The wire was well tolerated in patients and the control group.

We demonstrated a significant difference between *in vivo*-captured CTCs in localized (median=0 CTCs) and metastasized PCa (median, 4 CTCs) patients (Fig. 3). According to our data, CTC counts relative to the criterion threshold of five-displayed individual association with overall survival in metastasized PCa patients (Fig. 7A). This threshold status of more than five CTCs was validated in mPCa patients by the CellSearch system (11). Metastasized prostate cancer patients who showed rising CTC counts at all-time points demonstrated an OS period of 25 months compared to 34 months for those who showed declining CTCs (Fig. 7B). Our data demonstrated that a rinsing CTC count may be involved in a cancer progression resulting in active tumor spreading.

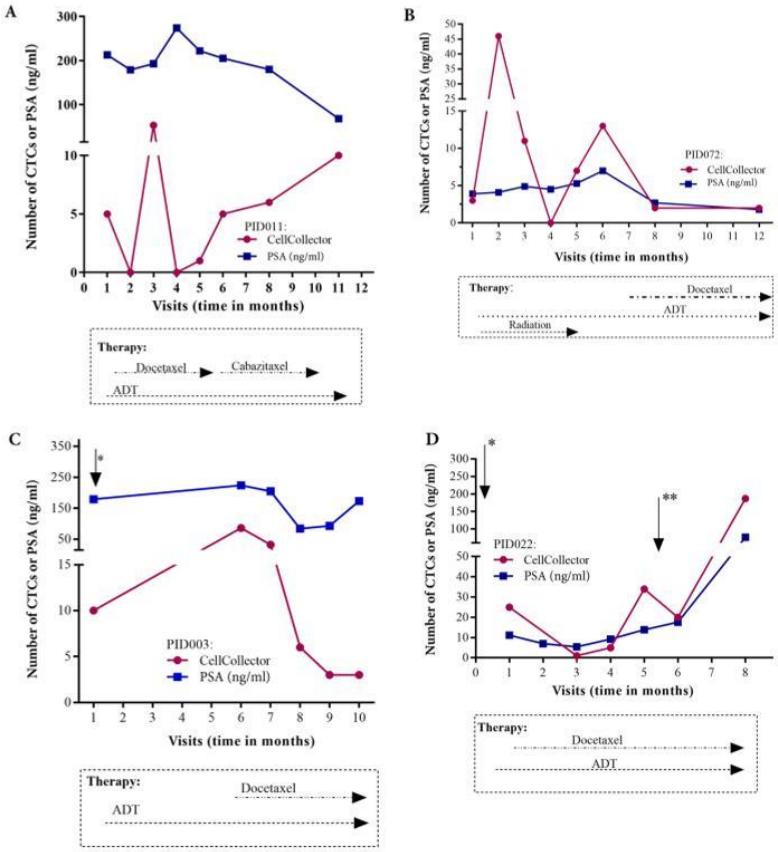


Figure 6. Treatment history and CTC/PSA profiles of 4 metastasized PCa patients, which fulfill the inclusion criteria, up to 12 months. (A) Patient enrolled in the study 4 months after palliative TURP (B) Patient with highly differentiated adenocarcinoma 18 years after prostatectomy. (C) Patient included one month after palliative TURP (*) and showing continuous hormone therapy and disease progression. (D) Only 6 days after study inclusion, palliative TURP (*) was performed with continuous hormone therapy and radiotherapy and removal of a brain metastasis between months 5 and 6 (**). CTCs, circulating tumor cells; PSA, prostate specific antigen; PCa, prostate cancer; TURP, transurethral resection of the prostate.

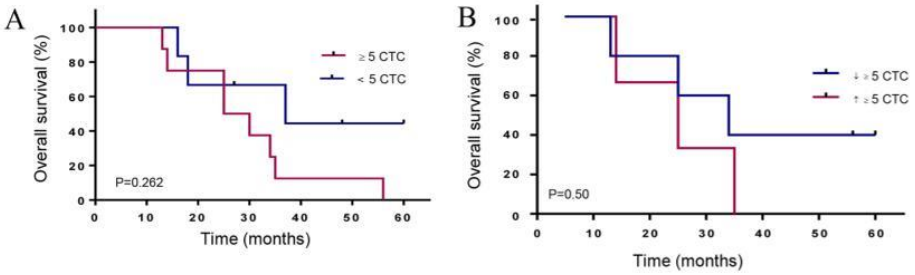


Figure 7. Kaplan-Meier overall survival according to CTCs. (A) Patients with metastatic prostate cancer (PCa-m) with ≥5 CTCs and <5 CTCs showed differences in OS (27.5 months vs. 37 months [HR 2.6, 95% CI, 0.78-8.3]). (B) CTC kinetics for PCa-m patients with ≥5 CTCs at all-time points; the median OS differed between those with declining CTC (↓≥5 CTCs) counts and those with increasing CTC (↑≥5 CTCs) counts (25 months vs. 34 months, HR 1.9, 95% CI, 0.4-11.6). HR, hazard ratio; OS, overall survival; CTCs, circulating tumor cells.

In the current investigation, we further revealed that the area under the curve (AUC) value did not differ significantly between the *in vivo* captured CTC and PSA levels and *ex vivo* captured CTCs (Fig. 5). This indicates a similar diagnostic performance of the CTC count and PSA level. In contrast, Goldkorn *et al* (21) demonstrated in the SWOGS0421 trial that the ROC curves for the day-0 CTC count had considerably higher AUCs than those for the day-0 PSA level. One possible reason could be that CTCs, in contrast to PSA, are not directly affected by hormonal treatment.

Furthermore, we were able to measure the CTC/PSA profile in metastatic prostate cancer patients during therapeutic layering. Importantly, the individual profiles showed promise for therapeutic decision-making in this patient group (Fig. 6). This offers the opportunity for disease monitoring and for making a tailored treatment decision in individual metastatic prostate cancer patients. In the area of personalized medicine, such profiling allows to protect patients from unnecessary side effects of ineffective treatment. Our results confirm the potential of CTCs as pharmacodynamics and potential intermediate endpoint biomarkers for overall survival (7,11). Unfortunately, this option is not possible in all our PCa-m patients, partially due to the heterogeneous phenotypes of CTCs in PCa patients. These phenotypes reflect the epithelial-mesenchymal transition (EMT) process, which plays a critical role in cancer metastasis. Indeed, CTCs undergo phenotypic changes from epithelial to more mesenchymal transitional states during the metastatic transition (22-24). An enhancement of the *in vivo* CTC isolation in metastasized prostate cancer patients should be considered to use an EMT stable marker such as Prostate-Specific Membrane Antigen (PSMA) (25) for functionalizing the CellCollector.

Interestingly, in the metastasized group, the detection rate of 79.2% was slightly reduced compared to the 100% rate of *ex vivo* CTC isolation in our previous study (14). An explanation (as described above) could be the heterogeneous phenotypes of CTCs in patients with progressive cancer status. Furthermore, we detected more leucocytes and cells positive for EpCAM, CD45 and pan-CK (data not shown) in comparison to the *ex vivo* application of the wire (14). It remains challenging to examine and analyze CTCs, as this phenomenon indicates activation of the immune system and may signal a therapeutic response (9). The results of our trial are in general agreement with previous results of other CTC trials in PCa-m patients (11,26-28).

The role of CTCs in PCa-l has been proposed as an option for postoperative risk stratification. We isolated CTCs in 45.3% of the wire applications in PCa-l patients. The monitoring of the CTC count (>1 CTC) before and after radical prostatectomy demonstrated no correlation with clinical and pathological parameters. Meyer *et al* (29) analyzed 152 localized patients preoperatively for CTCs and reported an 11% (n=17) CTC-positive rate. They showed no difference in biochemical recurrence in patients with or without CTCs. Todenhofer *et al* (30) used an EpCAM-independent isolation system and detected CTCs in 50% of PCa-l patients. They also revealed no correlation between CTC detection and PSA level, tumor stage or Gleason score (30). Taken together, our results did not verify a significant association of CTC positivity in patients undergoing RP for preoperative localized PCa. Our control groups also demonstrated a median CTC of 0, which was similar to the median CTC of the local PCa group. We demonstrated in Fig. 5 a good diagnostic accuracy of the CellCollector for CTC capturing comparable to the CellSearch System and the PSA level. The *in vivo* CTC isolation system allows to detect the low number of cells. From the other hand, it create the risk of the false-positive results. The localized prostate cancer group consist of 85.7% patients with a low Gleason score ≤7. It is very unlikely that such tumors release high invasive CTCs. False-positive results in control groups (BPH and healthy women) can have different causes. One reason may be the used characterization of CTCs, which does not distinguish between possible different CTCs phenotypes. CTCs can infiltrate normal tissue and form metastases or, which is more likely, be attacked by immune cells and killed. It is also possible to misinterpret cells, like larger CD45 negative leucocytes or low number of epithelial cells, which potentially enter in blood by the peripheral intravenous cannulation. Castle *et al* (31) discussed also such subtype of cells, which remained to be characterized. In our previous *in vitro* study, we demonstrated that 54% of the BPH patients' blood samples are positive for CTCs (14). In the present trail, only 20.3% of the BPH patients were positive for CTCs. This might suggest a better diagnostic accuracy of the *in vivo* CTC isolation compared the *ex vivo* CellCollector application (14). Our findings suggest that a cutoff value of *in vivo* captured CTCs must be developed. The role of CTCs as prognostic markers in localized PCa seems to be more relevant in locally advanced prostate cancer patients, such as risk stratification for additional treatment.

The limitations of our investigation are of course the small number of patients in the cancer groups. The unknown blood volume of the reported CTC counts must also be noted. Thus, future studies are needed to discover the clinical potential of the wire.

In conclusion, our present findings indicate that *in vivo* capture of CTCs by the CellCollector overcomes the blood volume limitations of other diagnostic approaches and thereby increases the diagnostic sensitivity of CTC analysis. The CTC/PSA-Profile reveals the possibility of personalized therapy monitoring which can help to prevent patients for side effects of invalidly treatment. The CellCollector was well tolerated, and no side effects were reported. Thus, future studies are needed to explore this method in the clinic.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GT designed the study, performed the experiments, analyzed the data and wrote the manuscript. GT and PF confirm the authenticity of all the raw data. CB and KF performed the experiments and were involved in drafting the paper. JB performed the analysis and interpretation of data, and was involved in drafting the paper and revised it critically for important intellectual content. RH and EW designed the study. FK and SS performed the analysis and interpretation of data, and gave final approval of the version to be published. The conception and design of the study, as well as drafting and approving the article, is attributed to PF. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Medical Faculty Ethics Committee of MLU Halle-Wittenberg. Furthermore, we obtained a permit from the Federal Institute for Drug and Medical Devices (Germany, BfArM). The patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that GILUPI GmbH supported the present study. We received funding from GILUPI GmbH; they provided the CellCollector and the patients received travel expenses. The sponsor played no role in the study. GILUPI GmbH provided the schematic image of the wire (Fig. 1).

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3.3 Assoziation von zirkulierenden Tumorzellen mit Entzündungs- und Biom**arkern bei Patienten mit metastasiertem kastrationsresistentem Prostatakarzinom**

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Das Ziel der hier vorliegenden Studie war es, die Assoziation der CTC-Zahl mit Entzündungsmarkern (IL-6 und IL-8) und Biomarkern (DKK-1, sHER2, CD44) bei Patienten mit metastasiertem kastrationsresistentem Prostatakarzinom (mCPRC) als Bestandteil des Therapiemonitorings zu prüfen. Darüber hinaus wurde die Sensitivität und Spezifität der CTC-Detektion zwischen dem CellCollector und dem CellSearch-System verglichen.

Insgesamt wurden 28 Patienten (12 mCRPC-Patienten und 16 PCa-l Patienten) eingeschlossen. Das Durchschnittsalter der mCRPC-Patienten betrug 68,5 Jahre und 63 Jahre bei den PCa-l Patienten. Der Gleason-Score zwischen den PCa-l und mCRPC-Patienten war signifikant unterschiedlich ($p < 0{,}0001$). Zehn Patienten (83,3 %) der mCRPC-Gruppe erhielten Docetaxel als Erstlinienbehandlung und drei (25 %) erhielten Cabazitaxel (ein Patient wechselte im Studienzeitraum von Docetaxtel zu Cabazitaxel). 14 PCa-l Patienten (82,2 %) wurden mit einer RP und zwei (11,8 %) wurden mit HIFU behandelt. Weitere demographische Daten sind in der Tabelle 1, Originalarbeit 3 zusammengefasst.

Im Analysezeitraum hatten die mCRPC-Patienten acht Visiten innerhalb eines Jahres (Visite 1-6: 1.-6. Mo., Visite 7-8: 8. Mo., 12. Mo.). Die PCa-l Patienten hatten innerhalb eines Jahres drei Visiten (Visite 1: vor RP, Visite 2: 6 Mo. nach RP, Visite 3: 12 Mo nach RP).

Die CTC-Isolierung erfolgte mit dem CellSearch-System (CTC_CS) und dem CellCollector (CTC_CC) zu den oben genannten Zeiten. In diesem Zusammenhang wurde ebenfalls eine Serumprobe für die Marker-Analyse entnommen.

Beide CTC-Isolationstechnologien -wie zuvor beschrieben- verwenden das EpCAM-Molekül für Anreicherung der CTCs. Ein Unterschied zwischen beiden Systemen besteht in der Menge des zu analysierenden Blutvolumens: das CellSearch-System benötigt 7,5 ml Blut, während der CellCollector ein größeres Volumen ($\leq$ 600 ml) aufgrund der Verweildauer von 30 min in der Cubitalvene zur Verfügung hatte.

Die CTC-Detektionsrate bei den mCPRC-Patienten betrug 84 % mit CTC_CC und 73,5 % CTC_CS. Der CTC-Median unterschied sich bei den mCRPC-Patienten zwischen den beiden Isolationsplattformen nicht signifikant ($p = 0{,}29$). Der CTC_CC detektierte im

Median 4 (0–820) CTCs und das CTC_CS detektierte im Median 8,5 (0–1428) CTCs (Originalarbeit 3 Abb. 1a). Das CellSearch-System isolierte höhere CTC-Zahlen in der Gruppe der mCRPC-Patienten. Im Gegensatz dazu erreichte der CellCollector eine höhere Detektionsrate. In der lokalen PCa-Gruppe konnten zu allen Zeitpunkten im Median 0 CTCs mit beiden Systemen detektiert werden. Allerdings konnten in der PCa-l Patientengruppe zum Zeitpunkt der 1. Visite 0-5 CTCs vom CellCollector und 0-1 CTCs vom CellSearch-System isoliert werden. Zu den Visiten im 6. und 12. Monate nach RP konnten 0-9 CTCs vom CellCollector und 0 CTCs vom CellSearch-System detektiert werden. Dies weist auf eine höhere Sensitivität des CellCollectors bei nichtmetastasierten PCa-Patienten im Vergleich zum CellSearch-System hin. Ein möglicher Grund für diese Ergebnisse sind die unterschiedlichen EpCAM-Antikörper der Systeme, welche eine differente Affinität zum EpCAM-Molekül haben können. Darüber hinaus sind die Veneneigenschaften der PCa-l Patienten z.T. besser für die in vivo Anwendung des CellCollector geeignet als die der mCRPC-Patienten. Auch wenn die Zahl der detektierten CTCs bei PCa-l Patienten gering ist und ihr klinischer Nutzen zurzeit unklar bleibt, wäre die genauere molekulare Charakterisierung der CTCs entscheidend für eine mögliche klinische Anwendung. Chen et al. [91] analysierten Hochrisiko-PCa Patienten ohne Metastasen und beschrieben den CellCollector als eine effiziente CTC-Isolationstechnologie zur Überwachung von Rezidiven sowie für das Therapiemonitoring in dieser Karzinomgruppe.

In einem Vergleich von verschiedenen CTC-Isolationstechnologien (CellCollector, duales Fluor-EPISPOT $^{PSA/FGF2}$Assay, CellSearch-System) waren die CTC-Zahlen des CellSearch-Systems der genaueste Prädiktor für ein metastasierendes PCa (AUC von 0,76 [95 % KI 0,631–0,908]) [92]. Die ROC-Analyse unserer mCRPC-Patientenkohorte zeigte eine AUC von 0,95 (95 % KI 0,83–1,0) für das CellSearch-System, was die hohe Sensitivität und Spezifität dieses Systems bestätigt. Der PSA-Wert, ein klassischer Marker in der blutbasierten Therapieüberwachung von Patienten mit fortgeschrittenem PCa, zeigte ebenfalls mit einer AUC von 0,90 (95 % KI 0,72–1,0) eine hohe Sensitivität und Spezifität (Originalarbeit 3, Abb. 4). Interessanterweise demonstrieren unsere Ergebnisse bei mCRPC-Patienten eine gute Korrelation zwischen dem PSA-Wert und der CTC-Zahl, welche mit dem CellSearch-System detektiert wurde. Es ist bekannt, dass die CTC-Zahl ein prognostischer Faktor für das Gesamtüberleben bei mCRPC-Patienten ist, aber bisher unabhängig vom PSA-Wert [43, 61, 82, 93]. Des Weiteren stellen auch die Kaplan-Meier-Kurven für unsere mCRPC-Patienten mit ≥ 5 CTCs (HR = 4,6; 95 % KI 1,2-17,03 [p = 0,02]) oder einem PSA-Wert > 53 ng/ml (HR = 4,4; 95 % KI 0,9-21 [p = 0,01]) nahezu identische Verläufe dar. Ein großer Vorteil der CTCs-Analysen liegt aber in der Bereitstellung von zusätzlichen

Charakterisierungsmöglichkeiten des Tumorgeschehen auf Protein-, mRNA- und DNA-Ebene hinsichtlich therapierelevanter Informationen [48].

Wir fanden erhöhte Serumspiegel von sHER2, DKK-1, IL-6 und IL-8 bei den mCRPC-Patienten und den PCa-l Patienten, aber keinen signifikanten Unterschied zwischen den Gruppen. Alle analysierten Marker sind aktiv oder passiv an der Umgehung (bypass pathway) der Aktivierung des Androgenrezeptors beteiligt. Die Freisetzung von Entzündungsfaktoren und Biomarkern in den Blutkreislauf kann die Fähigkeit von CTCs zur Interaktion untereinander oder mit Blutzellen beeinflussen bzw. verstärken [33].

Die CTC-Zahlen der mCRPC-Patienten, welche mit dem CellSearch-System bestimmt wurden, korrelierten signifikant mit den Serumkonzentrationen von DKK-1, sHER2 und dem PSA-Wert. Keine signifikanten Korrelationen konnten dagegen mit den CTC-Zahlen, welche der CellCollector detektierte, festgestellt werden (Originalarbeit 3, Abb. 3).

Die mediane DKK-1 Serumkonzentration von 4625 pg/ml war bei den mCRPC-Patienten im Vergleich zu den PCa-l Patienten (3939 pg/ml) leicht erhöht. Diese erhöhte Konzentration kann auf eine mögliche Umstellung des Phänotyps auf den osteoblastischen Metastasierungstyp hinweisen [94]. Dabei wurde im sechsten Monat der systemischen Therapie ebenfalls ein medianer Konzentrationsanstieg von 112 % von DKK-1 beobachtet, ebenso wie ein medianer Konzentrationsanstieg von PSA (157 %), IL-6 (440 %), CTC_CS (200 %) und IL-8 (156 %), was mit der Unterbrechung der Docetaxel-Behandlung in der mCRPC-Patientengruppe übereinstimmte (Originalarbeit 3, Abb. 2). Die Verdoppelung der medianen CTC-Zahl deutet auf ein aktives Tumorgeschehen im Blut hin. Die medianen IL-6 und IL-8 Konzentrationen im Serum der mCRPC-Patienten waren im Vergleich zu denen der PCa-l Patienten deutlich erhöht. Der Anstieg von IL-6 um 440 % im sechsten Monat der Therapie könnte auf EMT-assoziierte Prozesse bei der Tumorprogression hinweisen [30]. Das ein erhöhter IL-6 Serumspiegel mit dem Tumorstadium korreliert und einen negativen Einfluss auf das tumorspezifische Überleben hat, bestätigen die Ergebnisse mehrerer Studien [95, 96]. Die IL-8 Konzentration im Serum der mCRPC-Patienten stieg auf 156 % (Visite 6: 6. Mo.) im Vergleich zum Ausgangswert (100 %) an. Maynard et al. berichteten, dass die hohe Expression von IL-8 in der Mikroumgebung des Tumors mit einem aggressiven Prostatakarzinom und dem Verlust des AR einhergeht [97]. Eine Analyse von IL-8 Konzentrationen im Serum von PCa-l Patienten ergab keine Korrelation mit der Aggressivität des Prostatakarzinoms [98].

Darüber hinaus zeigte die Serumkonzentration von sHER2 eine aber moderate (r_s = 0,41) und signifikante ($p < 0,001$) Korrelation mit der CTC-Zahl des CellSearch-Systems. Wir konnten nachweisen, dass die mediane Konzentration von sHER2 in beiden Gruppen (PCa-l, PCa-m Patienten) gleich waren. Aber der Konzentrationsbereich (0,83-16,46 ng/ml) in der

mCRPC-Gruppe war deutlich größer, was auf eine aktive Sekretion von sHER2 durch die CTCs in den Blutkreislauf hindeuten könnte. Der Markerverlauf einzelner Patienten zeigt einen Anstieg der sHER2-Konzentration im fünften und sechsten Monat, was mit einer Unterbrechung der Chemotherapie vereinbar ist. Josefsson et al. zeigten eine starke Korrelation zwischen der sHER2-Expression in CTCs und der HER2-Expression in Metastasen und betonten das Potenzial von HER2 für eine CTC-Phänotypisierung in der individualisierten Therapie bei PCa-m Patienten [99].

Zusammenfassend lässt sich sagen, dass das CellSearch-System besser für die Anreicherung von CTCs aus dem Blut von mCRPC-Patienten geeignet scheint als der CellCollector, ein in vivo CTC-Isolationssystem. Jedoch isoliert der CellCollector sensitiver CTCs in der PCa-l Patientengruppe. Wir identifizierten eine moderate Korrelation zwischen den CTC-Zahlen des CellSearch-Systems mit den Biomarkern sHER2 und DKK-1 und eine starke Korrelation mit dem PSA-Wert. Interessanterweise hat die CTC-Zahl von ≥ 5 Zellen und ein PSA-Wert > 53 ng/ml annähernd die gleiche diagnostische Genauigkeit in Bezug auf die Sensitivität und Spezifität für das OS bei unseren mCRPC-Patienten. Die Daten zeigen, dass es von entscheidender Bedeutung ist, die Forschung zu erweitern, welche sich auf die CTC-Phänotypisierung sowie auf die Koexistenz von CTCs mit tumorassoziierten Faktoren im Blut konzentriert.

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Association of Circulating Tumor Cells with Inflammatory and Biomarkers in the Blood of Patients with Metastatic Castration-Resistant Prostate Cancer

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Abstract: The identification of specific biomarkers that recognize the functional drivers of heterogeneity in prostate cancer (PCa) and personalized treatment remain challenging in systemic medicine. Liquid biopsy allows for the detection and analysis of personalized predictive biomarkers in single blood samples and specifies the current stage of cancer. The aim of our preliminary study was to investigate the association between an elevated circulating tumor cell (CTC) count and the levels of inflammatory factors (IL-6 and IL-8) and biomarkers (DKK-1, PSA, sHER2, and CD44) in patients with metastasized castration-resistant PCa (mCPRC) under chemotherapy and those with localized PCa. Such an association could be used as a component of cancer progression monitoring. We compared the sensitivity and specificity of two CTC isolation platforms. Twenty-eight patients (12 mCRPC and 16 localized PCa patients) were enrolled. Over the study period, the CTC detection rates were 84% with CellCollector® and 73.5% with CellSearch® System in mCPRC patients. The CTC counts determined by the CellSearch® System (CTC_CS) were correlated significantly with the DKK-1, sHER-2, and PSA concentrations in mCRPC patients. The CTC counts captured by CellCollector® demonstrated no significant association with the concentrations of the tested blood-based biomarkers. The CTC_CS count (AUC = 0.9 (95% CI: 0.72–1.0)) and the PSA level (AUC = 0.95 (95% CI: 0.83–1.0)) presented approximately the same sensitivity and specificity for the overall survival of mCRPC patients. For better personalized characterization, further research on CTC phenotyping and their interactions with tumor-associated blood-released factors is needed.

Keywords: biomarker; circulating tumor cells; prostate cancer

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1. Introduction

Prostate cancer (PCa) is the fifth leading cause of cancer-related death worldwide [1]. The incidence increases with each decade of age, and thus, 59% of men over 79 years of age have PCa [2]. In an aging population, more PCa would be diagnosed. Furthermore, in men aged 75 years and older, the incidence of regional- and distant-stage disease increased from 2013 to 2016 [3]. For 28% of patients with distant metastasis, the estimated survival rate is approximately 5 years [4]. The majority of these patients have multifocal metastatic sites, such as bone and lymph nodes (particularly vertebrae and pelvis) [5]. Moreover, oligometastatic PCa has distinct biological states and harbors different mutations, which result in heterogeneous phenotypes. Metastatic progression requires certain characteristics of cancer cells, such as plasticity, motility, and colonization, as well as systemic physiological conditions, such as inflammation, which are drivers of metastasis and therapeutic resistance in PCa [6]. Drug resistance is a dynamic process in tumor cells, which includes molecular events such as genome modification and the regulation of diverse transcriptional states. Additionally, cancer cells undergo phenotype acquisition in the process of cellular rewiring [7]. In the last decade, innovations in treatments and combination therapeutic

strategies have been developed and have contributed to the therapeutic armamentarium, improving the outcomes from metastatic PCa [8,9].

Nevertheless, the determination of the optimal personalized drug sequence to minimize possible therapeutic resistance remains a challenge [10]. Therefore, personalized biomarkers of these characteristics are needed to determine treatment responses and facilitate decisions on the selection of agents.

Classic clinical factors, such as the blood levels of prostate-specific antigen (PSA), and pathological factors, such as Gleason grading and tumor, node, and metastasis (TNM) staging, are well-known prognostic markers in PCa [11]. However, these methods are often insufficient for accurate risk stratification, and do not adequately describe the metastatic process. One possibility is liquid biopsy, which includes (among others) circulating tumor cells (CTCs). CTCs detach from primary or metastatic tumors to enter the bloodstream, and a small CTC population has the ability to metastasize to multiple organs [12]. They provide characteristics of the current stage of the tumor or potential metastasis and allow for the real-time monitoring of therapeutic responses. CTCs' interplay with blood components is important for their survival and metastatic characteristics [13,14]. They may interact with neutrophils, platelets, leukocytes, monocytes, and macrophages in the circulation, which protect the CTCs from rapid clearance by natural killer cells and the physical shear stress of blood flow. These interactions promote the survival and extravasation of CTCs at distant sites [13].

The cytokines interleukin 8 (IL-8) and IL-6 are associated with inflammation contributing to PCa and progression to treatment resistance. IL-8 is secreted by monocytes, neutrophils, and endothelial cells. Its signaling in PCa cells is involved in regulating the transcriptional activity of the androgen receptor (AR), and substantiates the transition to an androgen independent proliferation of prostate cancer cells [15]. Furthermore, IL-8 overexpression by tumor cells is often induced in response to chemotherapeutic treatment and may be important in the tumor microenvironment [16,17].

IL-6 stimulates proliferation, promotes angiogenesis, and inhibits apoptosis of PCa cells and other tumor cells. These activities are due to the interaction of IL-6 with multiple signaling pathways, such as the Janus tyrosine family kinase (JAK)-signal transducer and activator of transcription (STAT) pathway and the extracellular signal-regulated kinase 1 and 2 (ERK1/2)-mitogen activated protein kinase (MAPK) pathway [18]. Additionally, IL-6 has been identified as a nonsteroidal compound of AR activation (N-terminus of AR), which is different from ligand activation [19,20]. IL-6 is also known to induce human epidermal growth factor receptor-2 (HER2) signaling through the MAPK pathways [21]. HER2 belongs to the epidermal growth receptor family, which regulates processes such as cell differentiation, migration, and survival. The activation of HER2 results in ligand-independence over homodimerization, heterodimerization with other receptors of the HER family, or proteolytic cleavage of the extracellular domain (sHER2 ECD) [22]. HER2 signaling promotes AR signaling through androgen ligand-independent mechanisms and supports the development of castration-resistant PCa (CRPC) [23,24]. Ma et al. [25] demonstrated that CD44 interacted with HER2 promotes DNA damage repair and radioresistance. Moreover, CD44 expression in cancer cells promotes bone metastases by enhancing tumorigenicity, cell migration, and progression [26,27].

CRPC patients mostly have bone metastasis, which results in skeletal-related events such as pathological fractures. Osteoblast function is dependent on Wnt signaling, controlled by the Wnt inhibitors sclerostin and Dickkopf1 (DKK-1) [28]. Furthermore, DKK-1 expression in tumor cells activated Wnt/β-catenin signaling and demonstrated an interaction with AR signaling [29].

The aim of our preliminary study was to investigate the association of an elevated CTC count with inflammatory molecules (IL-6 and IL-8) and biomarkers (DKK-1, sHER2, and CD44) in patients with metastasized CPRC (mCPRC) under chemotherapy and localized PCa (PCa-l). Such an association could be used as a component of cancer progression monitoring.

2. Materials and Methods

2.1. Patient Cohorts

This is a retrospective analysis of a subpopulation of a prospectively planned clinical trial in the University Clinic and Outpatient Clinic for Urology, Medical Faculty of Martin Luther University Halle-Wittenberg [30]. All of the patients provided written informed consent and were enrolled in the study. This included blood sampling (4.5 mL serum) for future research. The protocol was approved by the medical faculty ethics committee of Martin Luther University Halle-Wittenberg (number of ethical approval: FSMW EPCAM-Prostata-M00, 2012-65). The men enrolled in the first group were patients with histologically confirmed prostate adenocarcinoma with progressive disease, despite castration levels of serum testosterone (<50 ng/dL). Only two of the patients achieved the castration-resistant stage in the second month of the study. All 12 patients were examined every month for 6 months, followed by visits in the 8th and 12th months, for a total of eight visits. CTC evaluation with CellCollector® and the CellSearch® System and blood sampling for additional biomarker analysis were taken before starting the chemotherapy or the bone-targeted therapy. The second group included patients with confirmed prostate adenocarcinoma, who had opted for radical prostatectomy (RP) in the observation period and were assessed three times within 12 months. The first visit was before the prostate removal. The next visits were 6 and 12 months after surgery.

2.2. Sample Collection

Additionally, 9 mL of blood serum was collected for the determination of the levels of PSA, C-reactive protein (CRP), and testosterone and for Luminex analysis. Samples were collected at each visit. The serum was processed within one hour after collection through centrifugation at 1300× *g* for 10 min. The samples were stored at −80 °C.

2.3. CTC Isolation

We used two different methods for CTC isolation, the CellSearch® System (Silicon Biosystem, Menarini, Florence, Italy) and CellCollector® (GILUPI GmbH, Potsdam, Germany), at matched times. Both systems used an epithelial cell adhesion molecule (EpCAM) antibody to capture the CTCs, as previously described [30,31].

CellCollector®, a medical wire, was carefully inserted into the patient's cubital vein via a 20G peripheral venous catheter until the tip of the wire (2 cm) was in the bloodstream of the vein. After 30 min, the wire was pulled out of the vein. In the first step, the captured cells were fixed with 100% acetone for 10 min at room temperature, blocked with 3% bovine serum albumin/PBS for 30 min, and then prepared for characterization.

For the CellSearch® System analysis, 7.5 mL of blood was collected in CellSave® Preservative Tubes. These samples remained stable for 96 h at room temperature and were sent overnight to the Department of Tumor Biology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany.

2.4. CTC Characterization

The matched pair analysis requires the same identification criteria as that of the CTCs. The captured cells were stained with fluorescein isothiocyanate (FITC)-labeled antibodies against cytokeratin 8, 18, and 19 (eBioscience, Abcam) for the detection of epidermal cancer cells in the blood. CD45 staining (anti-CD45-A647, Exbio) was performed to exclude leucocytes. Additionally, the cells' nuclei were stained with Hoechst 33342. Cells were defined as CTCs when they met the following cytology-based FDA definition: (i) size ≥4 µm, (ii) visible cytoplasm, (iii) high nuclear/cytoplasm ratio, and (iv) positive fluorescent staining, as described above [32,33].

The images were digitally processed with ImageJ software by altering the contrast and brightness in accordance with Nature Publishing Guidelines [34].

2.5. Detection of Circulating Biomarkers

The serum levels of sHER2, IL-8, IL-6, DKK-1, and CD44 were simultaneously determined by a custom-made configuration of the Luminex Screening Human Magnetic Assay (R&D Systems). The assays were conducted following the manufacturer's instructions and were performed on a Luminex 100^{TM} Qiagen GmbH system (Hilden, Germany). All of the serum samples required a two-fold dilution in calibration diluent. For the analysis, we used a 96-well flat bottom microplate. The measurements for each sample were performed in duplicate, and the average of the two measurements was used. Limits of quantification were determined using the lowest or highest standard point and a percent CV (%CV = 100 × standard deviation/average) of less than 20%. PSA and CRP were determined with Immulite 100 (Siemens Healthcare Diagnostics GmbH, Eschborn, Germany), according to the manufacturer's instructions.

2.6. Statistical Analysis

All of the determined blood-based biomarkers or metabolites were normalized. The values obtained at the first visits were defined as 100%. The relative secretion values are shown in box plots with medians and interquartile ranges (IQRs). Whiskers represent the minimum and maximum values. Furthermore, all of the data were tested for normal distribution using the Shapiro−Wilk test, and the parameters are presented as the median ± range.

Finally, for the identification of possible correlations between the different markers for the different study groups, Spearman's rank correlation coefficient (r_s) was determined and is represented in a heatmap. The reported p-values are two-sided, and $\leq$0.05 was considered significant. The accuracy of the selected biomarker levels was evaluated by receiver operating characteristic (ROC) analysis. For this analysis, we used no cut-offs, but the median was six visits performed for 24 months survival. The optimal cut-off for the Kaplan−Meier analysis of PSA based on the ROC curve was calculated by the largest value of the formula, sensitivity + specificity − 1, from the median PSA level for every mCRPC patient (likelihood ratio). The mean CTC count was determined based on the CTC counts of visits 1–8 (V1–V8). Kaplan−Meier analysis was used to analyze the overall survival (OS) depending on the mean CTC count. The survival estimates in different groups were compared using the log-rank (Mantel−Cox) test. All of the statistical analyses were performed using GraphPad Prism software versions 7 and 9.

3. Results

3.1. Study Design and Patient Data

A total of 28 patients (12 mCRPC patients and16 PCa-l patients) were enrolled in the analysis. All of the study-related applications were identical in the groups. Age (p = 0.09) and body mass index (p = 0.18) were not significantly different between the groups (Mann-Whitney test). The median age was 68.5 years in the mCRPC patients and 63 years in the PCa-l patients. The median BMI was 27.5 in the mCRPC patients and 29.7 in the PCa-l patients. The Gleason score was significantly different ($p < 0.0001$) between the PCa-l and mCRPC groups (Mann-Whitney test). Ten patients (83.3%) received docetaxel in combination with prednisone as the first-line treatment for mCRPC, and three (25%) received cabazitaxel (one patient switched in the study period from docetaxtel to cabazitaxel) in response to resistance to docetaxel. The PCa-l patients were treated after the first visit with laparoscopic RP (82.3%) or high-intensity focused ultrasound (HIFU) (11.76%). The other baseline characteristics are summarized in Table 1.

Table 1. Study population characteristic and demographics.

Characteristics	mCRPC	PCa-l
Patient (*n*)	12	16
Median (range), years	69 (53–72)	63 (56–75)
Median (range) BMI	27.5 (20.8–39)	29.7 (22.5–34.5)
Median PSA (range), ng/mL at baseline	25.6 (35–1200)	8.2 (0.64–38.8)
Median PSA (range), ng/mL at the last visit	44.95 (0.04–903)	0,04 (0.04–0.06)
Median CRP (range), mg/mL at baseline	7.3 (1.8–94.8)	2.3 (1–26.2)
Median HB (range), nmol/L at baseline	7 (6.3–9.5)	9.4 (7.9–10.5)
Gleason sum, n (%)		
≤7	2 (16.67)	11 (64.7)
>7	10 (83.33)	6 (35.3)
Sites of metastasis, *n* (%)		
Bone	12 (100)	
Visceral	4 (33.3)	
Nodal	10 (83.3)	
Prior treatments, n (%)		
TURP	5 (41.7)	
Androgen treatment	12 (100)	
Radiation	9 (75)	
Treatments between baseline and study end, *n* (%)		
TURP		
Surgery (RP)		14 (82.3)
HIFU		2 (11.8)
Radiation	10 (83.3)	
Bone-targeted therapy	12 (100)	
Chemotherapy		
Docetaxel	10 (83.3)	
Cabazitaxel	3 * (25)	

RP—radical prostatectomy; HB—hemoglobin; PSA—prostate-specific antigen; TURP—transurethral resection of the prostate; HIFU—high-intensity focused ultrasound; BMI—body mass index; CRP—C-reactive protein. 3 * one CRPC patient received docetaxel and switched to cabazitaxel during the study period.

3.2. Assessment of Different Serum and Blood Biomarkers

We isolated CTCs with two different EpCAM-based systems from the mCPRC (n = 12) and PCa-l (n = 16) patients. Over the study period, the CTC detection rates were 84% with CellCollector® (CTC_CC) and 73.5% with the CellSearch® system (CTC_CS) in the mCPRC patients. Furthermore, the CTC-median in the mCRPC patients did not differ significantly (p = 0.29) between the two isolation platforms. A median of 4 CTCs (range 0–820) was captured by CellCollector®, and 8.5 CTCs (range 0-1428) by the CellSearch® system (Figure 1a). The baseline CTC count was zero in one mCPRC patient with CellCollector® and in three patients with the CellSearch® system. At the first visit, seven patients (58.8%) had <5 CTCs and three (25%) had ≥5 CTCs, as determined with CellCollector®. When the CellSearch® system was used, one (8.3%) patient had <5 CTCs and nine (75%) patients had ≥5 CTCs.

The PCa-l group had a median of 0 CTCs detected with both platforms at the first visit; 0–5 CTCs were achieved with CellCollector® and 0–1 CTCs were achieved with the CellSearch® system. In addition, in the cured patients, 0 CTCs were detected using the CellSearch® system. However, CellCollector® captured a median of 0 CTCs with a range of 0–9 at visits 6 and 12 months after RP (Figure 1b).

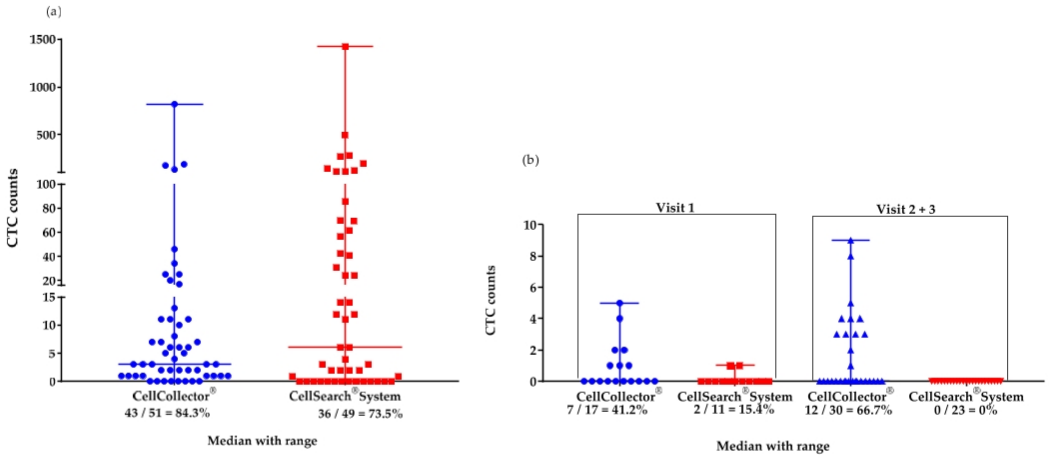

Figure 1. Median (range) values of CTCs isolated with the CellCollector® and CellSearch® Systems. (**a**) mCRPC patients (n = 12) in the study period of 12 months: CellCollector®, 4 CTCs (0–820), and CellSearch® System, 8.5 CTCs (0–1428); (**b**) PCa-l patients (n = 16) at visit 1 (before surgical removal): CellCollector®, 0 CTCs (0-5), and CellSearch® System, 0 CTCs (0–1) and at visits 2 and 3 (6 and 12 months after removal of prostate, respectively): CellCollector®, 0 CTCs (0–9), and CellSearch® System, 0 CTCs (0).

Biomarkers were measured until visit 6 (sixth month) in the study period. Unfortunately, data from visits 7 and 8 could not be included in the analysis because of an insufficient sample size. The serum levels of sHER2, IL-8, IL-6, Dkk-1, and CD44 did not show significant differences between the PCa-l and mCRPC patients (Table 2). The median levels of DKK-1 (4625 pg/mL), IL-6 (11.7 pg/mL), and IL-8 (20 pg/mL) in the mCRPC patients were higher than those in the PCa-l patients (3939 pg/mL, 5.6 pg/mL, and 10.8 pg/mL, respectively). Interestingly, the CD44 level in the mCRPC patients was the lowest in the study population. Moreover, the sHER level demonstrated a decreased concentration over six months in the mCRPC group. The median secretion levels were 3.3 ng/mL in the PCa-l group at visit 1 and 3.5 ng/mL at visits 2 and 3. The mCRPC patients had a median concentration of 3.3 ng/mL, which was approximately equal to the concentrations in the localized cancer stage groups. Interestingly, the sHER concentration had the widest range of 0.3–16.64 ng/mL in the mCRPC group. Significant differences were found for the PSA level ($p < 0.001$), CRP level ($p = 0.03$), and CTC count ($p < 0.001$) between the groups (Table 2).

We investigated the serial secretion of the biomarkers in the treatment follow-up at 6 months in the mCRPC patients (Figure 2). The first values were defined as 100%. The median relative CTC_SC count and the median relative secretion of PSA, IL-6, and IL-8 during the settlement period were the most dynamic markers (Figure 2a,c,e). The CTC count continually changed from 95 to 300% from visit 2 to visit 4. In contrast, the relative CTC_CC counts demonstrated a decreasing level during the period of analysis. The lowest relative CTC_CC count was reduced by 14% at visit 4 (Figure 2b). The median PSA level also showed variations with a range of 36% at visit 3 and 157% at visit 6. The DKK-1 protein showed a relatively constant secretion of 90.1–112.5%. IL-6 secretion remained relatively constant in the range of 126–117% until the fifth month. Interestingly, IL-6 secretion increased 440% in the 6th month. sHER-2 showed variations in a range of 96–66.8%, which revealed a continuous decrease in concentration under therapy. The serial change in IL-8 secretion demonstrated a variation of 66% in the third month to 156% in the 6th month. CD44 secretion was relatively constant over the observation period (104–86%).

Table 2. Serum levels of different biomarkers.

Median (Range)	mCRPC V1–V6	PCa-l V2 + V3	V1	*p*-Value
CD44 (pg/mL)	710 (205.9–4878)	777.1 (230.6–3382)	783.6 (386–2440)	0.70
DKK-1 (pg/mL)	4625 (566.9–8878)	3939 (1632–10937)	3976 (1273–7988)	0.80
sHer2 (ng/mL)	3.3 (0.83–16.46)	3.3 (1.1–7.7)	3.5 (1.27–8.4)	0.39
IL-6 (pg/mL)	11.7 (1.91–180)	5.6 (1.5–587.2)	8.2 (1.0–589)	0.24
IL-8 (pg/mL)	20 (1.98–112.7)	10.8 (4.8–1127)	13.2 (2.6–1216)	0.27
CTC_CC	4 (0–820)	0 (0–5)	0 (0–9)	<0.0001
CTC_CS	8.5 (0–1428)	0 (0–1)	0	<0.0001
PSA (ng/mL)	18.5 (1–1120)	8.2 (0.64–38.8)	0.04 (0.04–1.12)	<0.0001
CRP (ng/mL)	7.3 (1.8–94.8)	2.1 (1–26.4)	n.d.	0.03

CD44—cluster of differentiation 44; DKK-1—Dickkopf1; sHER2—soluble human epidermal growth factor receptor 2; IL-6, -8—interleukin-6, -8; CTC_CC—determined with CellCollector®; CTC_CS—determined with the CellSearch® system; PSA—prostate-specific antigen; CRP—C-reactive protein.

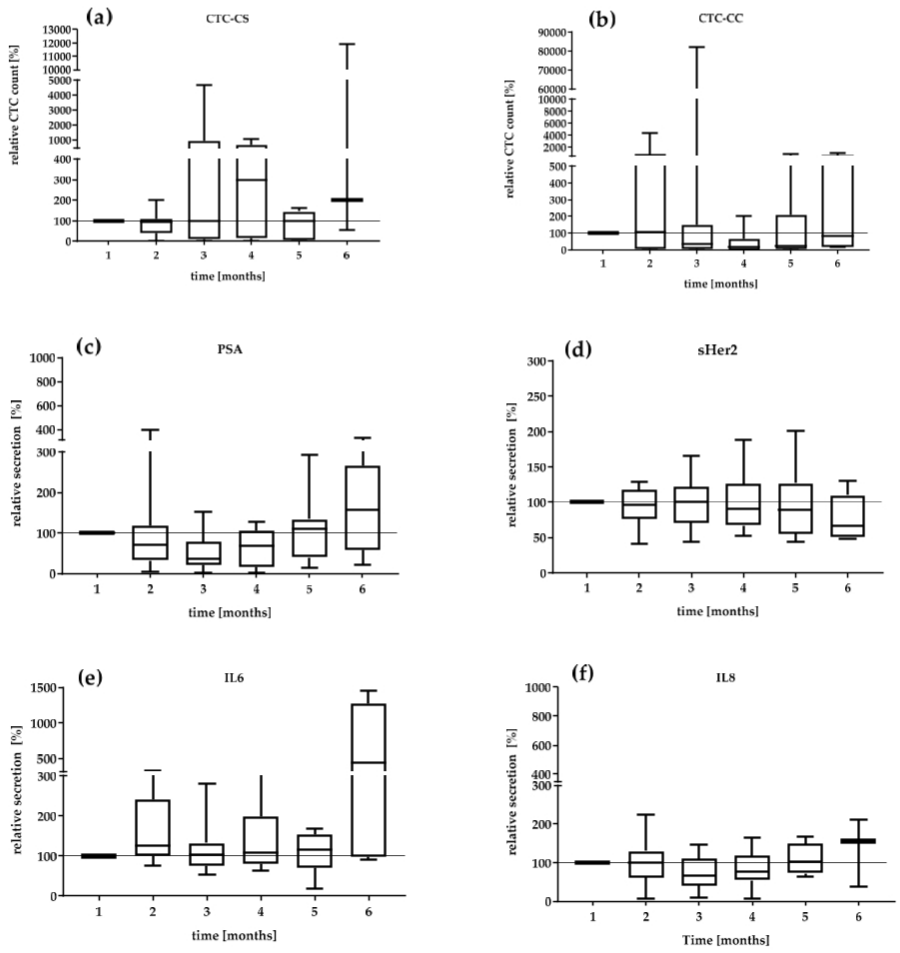

Figure 2. *Cont.*

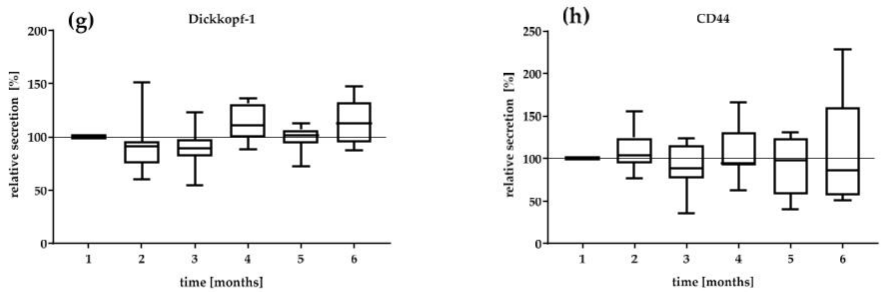

Figure 2. Relative value in percent of (**a**) CTC_CS count, (**b**) CTC_CC count, (**c**) PSA, (**d**) sHER2, (**e**) IL-6, (**f**) IL-8, (**g**) DKK-1, and (**h**) CD44 in the mCRPC patients during 6 months. The median relative secretion with minimum and maximum values. The value of the first visit was defined as 100%.

The correlation of serial CTC secretion between the serial secretion of biomarkers and inflammatory markers is shown in Figure 3. CTC counts determined with the CellSearch® system (CTC_CS) were moderately positively correlated with the concentrations of DKK-1 ($r_s = 0.35$, $p = 0.01$) and sHER-2 ($r_s = 0.41$, $p = 0.004$) in the mCRPC patients. A strong correlation was found between the CTC_CS count and the PSA concentration ($r_s = 0.75$, $p \leq 0.0001$) and the CTC counts of both platforms ($r_s = 0.78$, $p = 0.03$). Within regard to the CTC count captured by CellCollector® (CTC_CC), no significant association was observed with the concentrations of the other blood-based biomarkers. The CRP concentration was strongly positively, but not significantly correlated with the CTC count (CTC_CS $r_s = 0.60$, $p = 0.4$; CTC_CC $r_s = 0.78$, $p = 0.078$). Interestingly, we demonstrated a good correlation between PSA and sHer2 levels ($r_s = 0.55$, $p \leq 0.0001$) and PSA and IL-8 levels ($r_s = 0.47$, $p \leq 0.0001$) in our cohort. For IL-6, a good negative correlation was observed with DKK-1 ($r_s = -0.45$) and CD44 ($r_s = -0.54$).

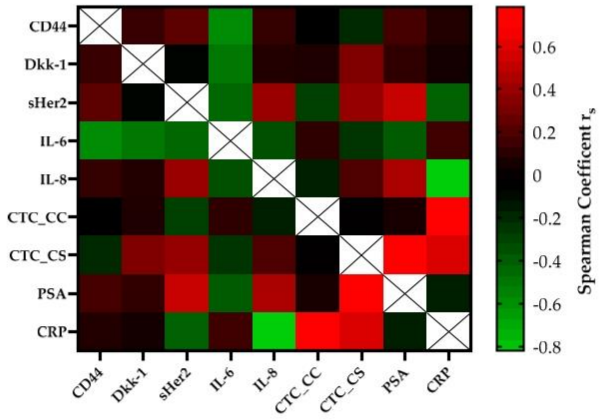

Figure 3. The heatmap of the correlation coefficients (Spearman) among biomarkers of the mCRPC patients. The color-coded correlation is on the left, where red demonstrates a strong positive correlation and light green indicates a strong negative correlation.

In the PCa-l patient group, no significant correlation was found between the CTC counts of either platform and the biomarker levels; however, the levels of markers prior to prostate removal were correlated (Supplementary Figure S1).

The sensitivity, specificity, and area under the curve (AUC) value were determined for CTC_CC, DKK-1, PSA, CTC_CS, and sHER2, which were correlated significantly with the

CTC_SC count. The results demonstrated that for a survival time of 24 months, the AUC values of these markers were 0.63, 0.62, 0.9, 0.95, and 0.79, respectively. The PSA level and the CTC_CS showed the strongest ability to predict survival for 24 months for the mCRPC patients. These results were calculated with the 6-month median level of the evaluated markers for every single mCRPC patient (Figure 4).

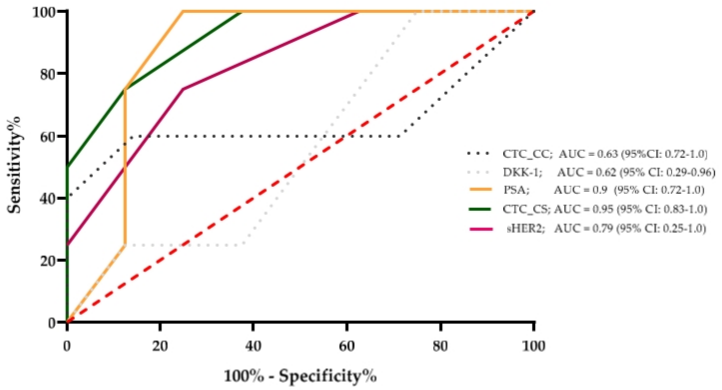

Figure 4. A survival ROC curve was plotted to evaluate the sensitivity, specificity, and AUC of serum concentrations of sHer2, PSA, DKK-1, CTC_CC, and CTC_CS and the 24-month survival.

3.3. The OS Value of CTC Count Versus PSA Level

We reached a follow-up time of 5 years in the study population, and compared the prognostic value of the median CTC count and the PSA concentration. In our analyses, we used the established CTC cutoff values of <5 or ≥5 CTCs [32]. For the PSA level, we used the estimated cutoff value of 53 ng/mL, which was calculated for the mCRPC patients in our study. In this study, the positive likelihood ratio was 8.0 (sensitivity 100%, specificity 87.5) for PSA cutoff values of 53 ng/mL.

Patients (75%) with evaluated CTC counts of <5 cells survived 34 months, with a median of 56 months. Patients (75%) with an evaluated CTC count of ≥5 cells survived 14 months, with a median survival time of 21.5 months. The hazard ratio (HR), referring to <5 or ≥5 CTCs, was 4.6 (95% confidence interval (CI): 1.2–17.03; Figure 5a).

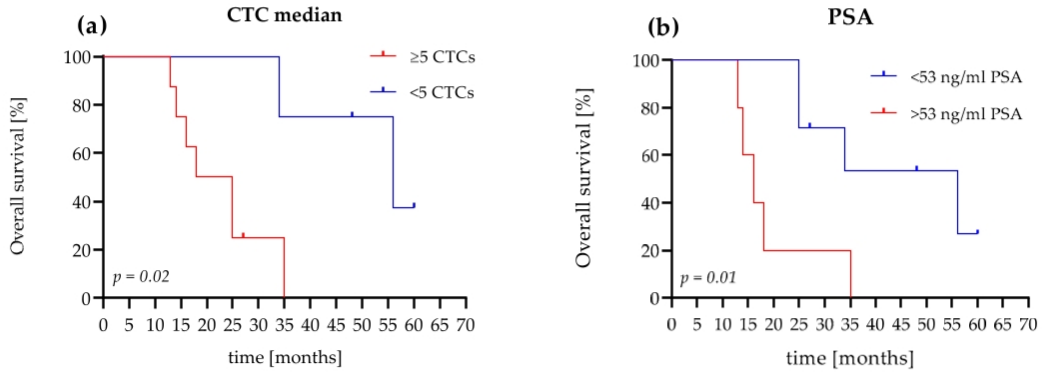

Figure 5. Comparison of Kaplan–Meier curves for OS according to the CTC count and PSA level of the mCRPC patients. (**a**) The patient shows <5 CTCs and a ≥5 CTC difference in OS (56 months versus 21.5 months (HR 4.6, 95% CI, 1.2–17)). (**b**) The patient shows a PSA level <53 ng/mL and a ≥53 ng/mL difference in OS (56 months versus 16 months (HR 4.4, 95% CI, 0.9–21)).

In comparison, with a PSA level <53 ng/mL, 71.5% of the patients survived 25 months with a median of 56 months. Patients (60%) with PSA levels >53 ng/mL survived 14 months and had a median survival time of 16 months. The HR, referred to as <53 ng/mL PSA or >53 ng/mL PSA, was 4.4 (95% CI: 0.9–21; Figure 5b).

4. Discussion

We analyzed the association between inflammatory markers and different biomarkers under therapy in a cohort of patients with PCa-l and mCRPC. Moreover, we compared two CTC isolation platforms for their sensitivity and specificity. There are many CTC isolation platforms; however, all of them have disadvantages and advantages [35,36]. We used CellCollector®, an in vivo CTC isolation system [30,37], and the FDA-approved CellSearch® system [32]. Similarly, in both platforms, CTCs were captured using antibodies against the EpCAM protein and were further characterized. The CellSearch® system required a blood sample of 7.5 mL, while CellCollector® required a larger volume. The CellSearch® system detected a higher CTC count in the mCRPC group, although the detection rate of CellCollector® was 84% compared with 73.5% of the CellSearch® system. Nevertheless, a range of 0–9 CTCs detected using CellCollector® in PCa-l patients compared with a range of 0–1 CTCs detected using the CellSearch® system. These results indicated that CellCollector® might be more useful than the CellSearch® system in nonmetastatic PCa patients because of the higher CTC detection rate. A possible reason for the different results could be the different EpCAM antibodies with differences in the affinity to the EpCam molecule. Furthermore, the veins in localized PCa patients are sometimes better for the in vivo application of the CellCollector® as in mCRPC patients. Even if the number of detected CTCs in indolent localized patients is low and their clinical utility remains unclear, their better specified molecular characterization would be crucial for clinical application. Chen et al. [38] further assessed high-risk nonmetastatic PCa patients and described CellCollector® as an efficient CTC technology for monitoring cancer relapse in localized PCa, as well as for monitoring of the treatment response.

The CTC counts obtained with CellCollector could also be tested in metastatic castration-sensitive prostate cancer patients (mCSPC) as biomarkers for evaluation of the treatment with androgen-receptor-axis-targeted (ARAT) therapy compared with docetaxel to improve the outcome in mCSPC patients [39,40].

However, in a comparison of different CTC platforms (CellCollector®, dual fluoro-EPISPOTPSA/FGF2, and the CellSearch® system), the CellSearch® system was the most accurate predictor of metastatic PCa (AUC 0.76, 95% CI: 0.631–0.908) [41]. Our ROC analysis showed an AUC of 0.95 (95% CI: 0.83–1.0) for the CellSearch® system, which confirmed the high sensitivity and specificity of this system. The PSA level, a classic marker in blood-based therapeutic monitoring of advanced PCa patients, demonstrated a comparatively high sensitivity and specificity with an AUC of 0.90 (95% CI: 0.72–1.0) in our mCRPC patient cohort (Figure 4). Interestingly, our results demonstrated a good correlation between the PSA level and the CTC count determined with the CellSearch® System. CTCs are prognostic parameters in mCRPC patients, but are usually independent of the PSA levels [32,42–44]. In our Kaplan–Meier OS analysis, a CTC count of $\geq$5 cells and >53 ng/mL PSA showed nearly identical HRs (CTC count HR = 4.6, $p = 0.02$ and PSA level HR = 4.4, $p = 0.01$). Our data showed that the CTC count and the PSA value in our cohort of mCRPC patients presented almost identical prognostic values. Nevertheless, CTCs can provide additional cancer-specific characteristics at the protein, mRNA, and DNA levels [35].

Furthermore, we found elevated serum levels of sHER2, DKK-1, IL-6, and IL-8 in the mCRPC patients and the PCa-l patients and found no significant difference between the groups (Table 2). Moreover, all of the analyzed markers were actively or passively involved in the bypassing of the AR signaling and might indicate active signaling in the blood. These factors may also influence the ability of CTCs to enhance inflammatory factors and biomarker release in blood circulation for possible crosstalk with cells.

Moreover, we found that the median DKK-1 serum level of 4625 pg/mL in the mCRPC patients was slightly increased compared with that in the PCa-l patients (3939 pg/mL), which may contribute to the development of osteoblastic metastasis. In addition, the higher DKK-1 concentration could indicate a possible switch in phenotype to the osteoblastic metastasis type [45]. In the serial measurements, a variation of 90.1–112.5% of DKK-1 was observed (Figure 3g). Interestingly, in the sixth month of systemic therapy, an increase of 112% was observed, as well as increases in the levels of PSA (157%), IL-6 (440%), CTC_CS (200%), and IL-8 (156%), which was consistent with the docetaxel treatment interruption (Figure 3). The doubling of the median CTC count suggests active cancer communication or micrometastatic progression. The increased serum level of DKK-1 could be due to the zoledronic acid treatment of the mCRPC patients, as shown by Thiele et al. [46] in an analysis of serum samples at different PCa stages. Our mCRPC cohort was under zoledronic acid treatment.

However, a good negative correlation of -0.45 ($p < 0.0001$) was demonstrated for DKK-1 and IL-6. This effect was described in inflamed joints of rheumatoid arthritis [47]. The median IL-6 and IL-8 concentrations in the serum of the mCRPC patients were substantially increased compared with those in the PCa-l group (Table 2). Culig [48] postulated in his review that serum IL-6 can act as an attractant for tumor cells and is linked to aggressive tumors. The IL-6 concentration of our cohort (11.7 pg/mL in the mCRPC group) was similar to that of the cohort of Nakashima et al. [49], and higher than 7 pg/mL. The increase of 440% in the sixth month of treatment could indicate active signaling pathways in PCa. Our results confirmed the findings from these studies, which concluded that higher IL-6 serum levels were correlated with the tumor stage and were inversely correlated with tumor survival and therapeutic response [18,48]. In the monitoring of mCRPC patients, the IL-8 level increased to 156% (visit at 6 months) compared with the baseline level (100%). Maynard et al. [50] reported that the high expression of IL-8 in the tumor microenvironment is associated with aggressive PCa and with the loss of the AR. Analysis of the IL-8 serum level of PCa-l patients found no correlation with diagnosis and aggressiveness [51]. We also detected lower IL-6 and IL-8 concentrations in the serum of the PCa-l group. In the mCRPC group, we could not demonstrate any significant correlation of interleukins 6 and 8 with the CTC count. One possible explanation could be the CTC status in the blood circulation and current tumor stage, which need to be explored in further studies. It is known that CTCs undergo a phenotype switch from epithelial to mesenchymal transition (EMT), and present a mesenchymal status [52]. Patients with newly diagnosed metastatic castration-sensitive PCa and positive for mesenchymal CTCs show a decline in resistance to androgen deprivation therapy compared with patients who are negative for EMT CTCs [53].

Interestingly the serum level of the sHER2 showed a significant ($p < 0.001$) moderate ($r_s = 0.41$) correlation with the CTC_SC count. Although we could detect sHER2 in the serum, the median concentration was equal in our groups, but the range (0.83–16.46 ng/mL) in the mCRPC group was much wider. This finding suggests that CTCs in the blood circulation express HER2, and that HER2 signaling is activated through the cleavage of sHER2 (ECD). The single patient profile shows an increasing sHER2 concentration in the fifth and sixth months (data not shown), which is consistent with chemotherapy interruption. Josefsson et al. [54] demonstrated a high correlation between HER2 expression in CTCs and metastatic samples, and emphasized the potential for CTC phenotyping for individualized therapy in metastatic PCa. Furthermore, it was demonstrated in 236 PCa patients that HER2 over expression is associated with a low expression of the tumor suppressor gen PTEN (phosphatase and tensin homologue) and reduced the cancer-specific survival [55]. Using the AdnaTest ProstateCancerSelect/Detect kit for CTC isolation from the PCa patients in their study, they captured CTCs with the EpCAM and HER2 protein [54]. The same kit was used for the analysis by Antonarakis et al. [56]. This group detected AR splice variant 7 mRNA (AR-V7) in the CTCs from patients with castration-resistant PCa. The CTCs also express HER2 and AR-V7. This variant of the AR in CTCs has no ligand-binding domain, but via an active HER2 signaling it can bypass the androgen signaling pathway. In

a recently published study, the expressions of AR-V7 and PTEN were determined in CTC. The authors demonstrated that more than two PTEN negative CTCs were associated with a 3.96 hazard ratio for progression or death compared with CRPC patients with less than two PTEN negative CTCs. Moreover, a high CTC AR-V7 positive count (0–20) was associated with a radiographic progression-free survival in ezalutamid-treated patients [57].

We determined the CD44 expression in the serum, but the median concentration in the mCRPC group was only slightly decreased compared with that in the PCa-l patients. However, the concentration range was much wider in this group than in the PCa-l group. Nevertheless, we could not observe any increase in the CD44 concentration after chemotherapy in the mCRPC group. Some patients demonstrated constant levels, and others had decreased levels after chemotherapy. Ma et al. [25] showed the interaction between CD44 and HER2 in PCa cell line, and linked this relationship to potential radio resistance PCa.

In this study, we showed that the CTC count determined with the CellSearch® system (CTC_CS) is more suitable for mCRPC patients than CellCollector, an in vivo isolation system. We identified a moderate correlation between the CTC counts and the biomarkers sHER2 and DKK-1, and a strong correlation with the PSA level. Additionally, we found that a CTC_CS count $\geq$5 cells and a PSA level >53 ng/mL presented approximately the same diagnostic potency with regard to the sensitivity and specificity for OS in our mCRPC patients. Furthermore, for better personalized characterization, it is crucial to expand the research focused on CTC phenotyping, and the interactions of these cells with coexisting, tumor-associated blood-released factors.

The limitations of our preliminary investigations are of course the small number of patients and the heterogeneous group of mCRPC patients (first and second line of chemotherapy). Likewise, the CTC platforms used here capture CTCs with an EpCam antibody but not CTCs with a mesenchymal phenotype. Moreover, we included no independent cohorts such as age match healthy woman or man. A wider characterization might provide additional information about the association between CTC and other biomarkers [13]. Lager studies are needed to further validate our findings.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/life11070664/s1, Figure S1: Heatmap of non-significant correlation coefficients (Spearman) among biomarkers of localized prostate cancer patients.

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3.4 Strategien für die Isolierung und in vitro Kultivierung zirkulierender Tumorzellen bei Patienten mit metastasiertem Prostatakarzinom

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Ein Schwerpunkt der vorliegenden Arbeit war die Entwicklung und Evaluation einer PCa-spezifischen Funktionalisierung des CellCollectors (CC-PCa), welche eine alternative Grundlage für die Isolation von CTCs aus dem Blut von metastasierten PCa-Patienten darstellen soll. In diesem Zusammenhang wurden verschiedene Isolationstechnologien, basierend auf physikalischen und biologischen Eigenschaften der CTCs, hinsichtlich des CTC-Anreicherungspotentials verglichen. Für die Isolationstechnologien, welche die physikalischen Eigenschaften der CTCs ausnützten, wurden der ScreenCell Kit und die Ficoll Dichtegradientenzentrifugation verwendet. Der EpCAM-funktionalisierte CC und der PCa-funktionalisierte CC isolieren die CTCs, basierend auf ihren biologischen Eigenschaften. Des Weiteren sollte das Potential von Isolierungstechnologien auf die Möglichkeit der Kultivierung von CTCs aus dem Blut metastasierter PCa-Patienten geprüft werden.

In diese prospektive Studie wurden zwei unabhängige Patientenkohorten (1. Kohorte [n = 15], 2. Kohorte [n = 12]) von PCa-m Patienten eingeschlossen. Alle Patienten hatten ein histologisch und bildmorphologisch (radiologisch) bestätigtes metastasiertes PCa. Die demografischen Daten unterschieden sich nicht signifikant in beiden Kohorten. Das mediane Alter betrug 74 Jahre (Kohorte 1) und 63,5 Jahre (Kohorte 2) ($p = 0{,}03$). Bei der Erstdiagnose des Prostatakarzinoms hatten zwölf Patienten (80 %) in Kohorte 1 und neun Patienten (90 %) in Kohorte 2 einen Gleason-Score ≥ 7. Allen Patienten wurden zum Zeitpunkt der einmaligen studienbedingten Blutabnahme mit ADT und/oder Chemotherapie behandelt. Der Anteil der Patienten, bei denen bereits Knochenmetastasen vorlagen, betrug $\geq$ 86 % in Kohorte 1 und 100 % in Kohorte 2. Die lymphogene Metastasierung war bei 41,7 % der Patienten von Kohorte 1 und bei 50 % der Patienten von Kohorte 2 bekannt. Weitere klinische und pathologische Parameter der Patienten sind in Tabelle 1 (Originalarbeit 4) zusammengefasst.

Für eine bessere Vergleichbarkeit der Ergebnisse und deren Diskussion wurde in jeder Kohorte mindestens eine CTC-Isolationstechnologie verwendet, welche basierend auf dem EpCAM-Signal CTCs anreichert. Das EpCAM-Signal ist, wie bereits mehrfach erwähnt, der einzige bisher von der FDA für die CTC-Anreicherung zugelassene Zelloberflächenmarker. Die Identifikation der CTCs erfolgte nach den FDA-CTC-Kriterien [46]. CTC-Isolationsergebnisse zählten als positiv, wenn ≥ 1 CTC nachweisbar war.

Im ersten Teil unserer Studie wurde die PCa-spezifische Funktionalisierung des CCs entwickelt. Dazu gehörte die erfolgreiche immunzytologische Evaluation der dafür notwendigen Antikörper, welche die unterschiedlichen prostataspezifischen Proteine wie das prostataspezifische Antigen (PSA), das prostataspezifisches Membran Antigen (PSMA) oder das Prostata-Stammzell-Antigen (PSCA) detektieren. Die etablierte PCa-spezifische Funktionalisierung des CCs bestand aus einer Kombination von vier selektierten Antikörpern (anti-PSMA, anti-PSA, anti-PSCA, anti-EpCAM). Zunächst wurde das Prinzip der PCa-spezifischen Funktionalisierung des CCs im Vergleich zu der EpCAM-Funktionalisierung des CCs mit Hilfe von Spiking-Experimenten (200 LNCaP-Zellen + 15 ml gesundes Spenderblut) überprüft. Die CTC-Detektionsraten der Spiking-Experimente lagen für den CC-PCa bei 48 % und bei 30 % für den CC-EpCAM. In parallelen Blutproben der Kohorte 1 wurden der CC-PCa und der CC-EpCAM analysiert. Dabei zeigte sich, dass der CC-PCa in 86,7 % und der CC-EpCAM in 73,3 % der Proben CTCs detektierte. Die mediane CTC-Zahl von 9 (0-122) CTCs des CC-PCa war signifikant höher ($p = 0{,}002$) als die mediane CTC-Zahl von 3 (0-22) CTCs des CC-EpCAM. Dennoch konnte eine schwache Korrelation ($r_s = 0{,}37$) zwischen den CTC-Zahlen der unterschiedlich funktionalisierten CC-Systeme nachgewiesen werden. Die Differenz der isolierten CTC-Zahlen beruhte alleinig auf der Antikörper-Funktionalisierung der CellCollectoren. Die immunzytochemische Charakterisierung der CTCs war identisch und wurde von einer Person ausgewertet. Diese Ergebnisse bestätigen eine Heterogenität in der CTC-Population aufgrund von unterschiedlichen Zelloberflächenantigenen. Die in dieser Arbeit entwickelte PCa-spezifische Funktionalisierung des CCs ermöglicht die Anreicherung von CTC-Hybridphänotypen, welche epitheliale und mesenchymale Expressionsmuster aufweisen. Wie unter 1.1.4 genannt, ist EMT ein entscheidender Prozess in der Tumorprogression [100]. Die CC-PCa isolierten CTCs mussten zusätzlich zum EpCAM-Signal und/oder PSMA, PSA, PSCA-Signale exprimieren. In anderen Studien lag die Detektion von PSMA-positiven CTCs bei fortgeschrittenen PCa-Patienten zwischen 67 % [101] und 59 % [102]. Im Rahmen dieser Habilitationsschrift (Originalarbeit 1) wurde die PSMA-Expression auf mRNA-Ebene in CTCs nur bei 14,3 % der untersuchten Blutproben von PCa-m Patienten nachgewiesen. Ein Grund dafür könnte sein, dass die CTC-Isolation ausschließlich über das EpCAM-Signal der CTCs erfolgte [79]. Nagaya et al. [102] beobachteten bei mCRPC-Patienten, dass eine erhöhte PSMA-Expression in CTCs mit einem schlechten Therapieansprechen, einem kürzeren PSA-progressionsfreiem Überleben sowie mit einem kürzeren Gesamtüberleben einherging. Im Gegensatz dazu zeigten die CTC-Zahlen der Kohorte 1, welche mit unterschiedlich funktionalisierten CC-Systemen detektiert wurden, keine signifikanten Unterschiede im OS. Jedoch ging das Vorhandensein von ≥ 5 CTCs, welche mit den CC-

EpCAM isoliert wurden, mit nur 0,93 Jahren OS einher. Dagegen ging das Vorhandensein von ≥ 5 CTCs, welche mit dem CC-PCa isoliert wurden, mit 1,5 Jahren OS einher (Originalarbeit 4, Abb. 8). Es ist bekannt, dass PCa-m Patienten eine höhere Anzahl von CTCs sowie eine heterogene CTC-Population im Blut aufweisen, welche den Progress des Karzinoms fördern können [48, 103]. Dennoch ist das EpCAM-Molekül der Karzinomzellen ein essentieller Faktor in der Tumorgenese. Keller et al. [44] bekräftigten in ihrem Review, dass die biologische Funktion des EpCAM-Signals nicht nur auf die interzelluläre Adhäsion beschränkt ist. So sind für den Tumorprogress weitere wichtige Funktionen von Bedeutung, wie Zellproliferation und Krebsstammzell-Eigenschaften. Dies weist auf eine aktive Rolle des EpCAM-Signals bei der Metastasierung hin. Folglich ist es für die CTC-Isolationstechnologien wichtig, dass EpCAM-Molekül nicht vollständig aus den Erfassungsstrategien auszuschließen. Daraus resultiert die Detektion einer möglichst heterogenen CTC-Population [33].

In Kohorte 2 wurden verschiedene CTC-Isolationstechnologien auf der Grundlage der physikalischen CTC-Eigenschaften (Zellgröße, Zelldichte) und damit unabhängig vom CTC-Phänotyp angewandt. Zum Vergleich der CTC-Isolationstechnologien wurde basierend auf den biologischen Eigenschaften, der CC-EpCAM genutzt. Die CTC-Detektionsrate des Ficoll-Systems mit anschließender immunzyotchemischer Charakterisierung lag bei 20 % (2/10). Wurde dagegen histologisch mit May-Grünwald-Giemsa (MMG) gefärbt, lag sie bei 40 % (4/10). Die entsprechenden medianen CTC-Zahlen waren 0 (0-36 CTCs immunzytochemische Färbung), (0-2 CTCs MMG Färbung). Ein großer Vorteil der antikörperunabhängigen Plattformen ist die Isolation von Zellen ohne Antigen-Antikörper-Bindung. Eine antikörpervermittelte Bindung an die CTCs kann die nachgeschalteten Analysen beeinflussen [104]. Die CTC-Detektionsrate für den SreenCell-Filter (SC) mit MGG-Färbung lag bei 80 % (8/10) und die mediane CTC-Zahl war 14 (1-79 CTCs). Bei metastasierten Mammakarzinompatientinnen [105] und Lungenkarzinompatienten [106] detektierte das SC-Filtersystem CTCs in vergleichbaren Bereichen. Die Isolation der CTCs basiert auf der Größe der Zellen CTCs (≥ 8 μm). Erythrozyten (< 8 μm) passieren die Filterporen. Leukozyten (7-12 μm) können zwischen die Poren gelangen, werden aber anhand ihrer Kernmorphologie und Größe identifiziert. Ein möglicher Nachteil dieses Systems ist, dass es zu einer Verstopfung des Filters kommen kann und damit zum Verlust der Blutprobe führt. Eine Ursache dafür könnte die Bildung von kleinen Blutgerinnseln sein, wenn z.B. Blutproben eine hohe Konzentration von CTC-Cluster (> 8 μm) aufweisen. Im Vergleich zu einzelnen CTCs haben CTC-Cluster eine erhöhte Überlebenschance in der Blutzirkulation und eine reduzierte Apoptoseneigung, womit ein höheres metastatisches Potenzial erreicht werden kann [107]. Außerdem können kleinere CTCs (4-8 μm) die

Filterporen passieren. Damit wird die Beurteilung zur morphologischen und phänotypischen Heterogenität in der CTC-Population eingeschränkt [108].

Interessanterweise erreichte CC-EpCAM den zweiten Platz bei den Detektionsraten und den CTC-Zahlen, was die obige Diskussion über die Rolle der EpCAM-basierten CTC-Isolierung bei Patienten mit fortgeschrittenem PCa bestätigt. Der CC-EpCAM detektierte in 50 % (5/10) und im Median 0,65 (8-6) CTCs in den Blutproben.

Für das Wachstum von CTCs in Kultur wurden mit Ficoll (3 ml Blut) und SC-Filter-CTC-Kultivierung (6 ml Blut) von drei mPCa-Patienten (Kohorte 2) parallel verarbeitet und danach analysiert. Lediglich bei einem Patienten konnten CTCs, welche mit dem Ficoll-System erfolgreich isoliert wurden, in der Kultur wachsen. Diese Isolierungsmethode ermöglicht durch minimierten Scherstress der Zellen und fehlende Antikörperinteraktion eine schonende CTC-Anreicherung. Die kultivierungsfähigen CTCs stammen von einem PCa-m Patienten, welcher mit ADT behandelt wurde und sich im Progress seiner Erkrankung (lymphogene, ossäre Metastasierung) befand. Das erfolgreiche Wachstum der CTCs konnte über einen Zeitraum von drei Wochen durch die PSA-Sekretion in das Kulturmedium dokumentiert werden. Die PSA-Konzentration lag in einem Bereich von 0,47 ng/ml in der ersten Woche und 0,02 ng/ml am Ende der dritten Woche (Originalarbeit 4, Abb. 7). Eine mögliche Erklärung für die relativ kurze Kultivierungsphase wäre, dass der PSA-Wert des Patienten in den nachfolgenden zwei Monaten ADT-Behandlung leicht abfiel, was auf die Initiierung des intrinsischen Apoptoseweges in den Tumorzellen und damit auch in den kultivierten CTCs zurückzuführen sein könnte. Dennoch reflektieren die kultivierungsfähigen CTCs bei unserem Patienten eine Dynamik im Tumorprogress zu einem definierten Zeitpunkt. Im Gegensatz zu den Erfahrungen von Koch et al. war die Kultivierung von CTCs auch bei einer geringen CTC-Zahl möglich, wenn auch nur für eine begrenzten Zeitraum [109].

Mittels der ROC-Kurven-Analyse wurde die Sensitivität und Spezifität der verwendeten CTC-Isolationstechnologien (Kohorten 1 und 2) hinsichtlich des Gesamtüberlebens nach 24 Monaten überprüft. Der CTC Cut-off-Wert war 1 ≥ CTC. Die AUC-Werte der CTC-Isolierungstechnologien lagen in einem Bereich von AUC: 0,53 bis 0,79 für 24 Monate OS lagen (Originalarbeit 4, Tab. 2). Interessanterweise wurden die höchsten Sensitivitäten und Spezifitäten vom Ficoll-System mit MGG-Färbung (AUC = 0,79) und vom SC-Filter mit MMG (AUC 0,73) erreicht.

Zusammenfassend zeigen die in dieser Arbeit genutzten CTC-Isolationstechnologien unabhängig von den physikalischen oder biologischen CTC-Eigenschaften einen weiten CTC-Detektionsbereich (20–86,7 %). Die etablierte PCa-spezifische Funktionalisierung des CCs und das SC-Filtersystem erreichten die höchste Sensitivität in der Isolation von CTCs

aus dem Blut von PCa-m Patienten. Dabei zeigten das Ficoll-System und das SC-Filtersystem mit MGG-Färbung die höchste Sensitivität und Spezifität für das Gesamtüberleben von 24 Monaten. In der Kaplan-Meier-Analyse waren ≥ 5 CTCs, welche mit dem CC-EpCAM isoliert wurden, mit dem kürzesten medianen OS von 0,93 Jahren assoziiert. Demnach ist das EpCAM-Molekül für die CTC-Isolierung ein wesentlicher Faktor in Bezug auf das OS bei PCa-m Patienten. Kultivierungsfähige CTCs, welche PSA sekretierten, konnten nur mit dem Ficoll-System isoliert werden. Im Vergleich hinsichtlich Kosten, Arbeitszeit und Anwender-Fähigkeiten der genutzten CTC-Isolationstechnologien zeigten sich ähnliche Anforderungen. Wir kommen zu dem Schluss, dass die am besten geeignete CTC-Isolationstechnik nicht auf eine Eigenschaft der Zellen beschränkt sein darf. Unsere Ergebnisse implizieren, dass es von entscheidender Bedeutung ist, die Forschung zu erweitern, welche sich auf die CTC-Phänotypisierung und auf die Koexistenz von CTCs mit tumorassoziierten Faktoren im Blut konzentriert, um den zielgerichteten Einsatz der CTCs in der klinischen Routine zu ermöglichen.

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Strategies for Isolating and Propagating Circulating Tumor Cells in Men with Metastatic Prostate Cancer

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Abstract: Selecting a well-suited method for isolating/characterizing circulating tumor cells (CTCs) is challenging. Evaluating sensitive and specific markers for prostate cancer (PCa)-specific CTC identification and analysis is crucial. We used the CellCollector EpCAM-functionalized system (CC-EpCAM) and evaluated and developed a PCa-functionalized version (CC-PCa); we then compared CTC isolation techniques that exploit the physical and biological properties of CTCs. We established two cohorts of metastatic PCa patients (mPCa; 15 in cohort 1 and 10 in cohort 2). CTC cultivation experiments were conducted with two capturing methods (Ficoll and ScreenCell). The most sensitive detection rates and highest CTC counts were reached with the CC-PCa and ScreenCell system. Patients with ≥5 CTCs isolated with CC-EpCAM had an overall survival (OS) of 0.93 years, and patients with ≥5 CTCs isolated with CC-PCa had an OS of 1.5 years in cohort 1. Nevertheless, we observed the highest sensitivity and specificity for 24-month survival by the Ficoll with CD45 depletion and ScreenCell system with May-Grunwald Giemsa (MGG) staining. The EpCAM molecule is an essential factor related to OS for CTC isolation based on biological properties in mPCa patients. The best-suited CTC capture system is not limited to one characteristic of cells but adapted to downstream analysis.

Keywords: circulating tumor cells; prostate cancer; isolation platforms

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1. Introduction

Circulating tumor cells (CTCs) have been one of the most discussed biomarkers for monitoring cancer progression in the last decade. They are individual tumor cells or cohorts of cells from primary tumors or metastases that may invade the vasculature in adjacent tissue [1,2]. In the blood of cancer patients, CTCs might reflect important information on current tumor progression and cancer treatment. Furthermore, CTCs represent a heterogeneous population, and only a very small percentage (<<0.01%) of the millions of cells that enter the blood system daily have the ability to form metastases. During dissemination, multiple steps and mechanisms are involved in phenotypic changes in CTCs.

Epithelial–mesenchymal transition (EMT) is a significant mechanism by which CTCs invade the surrounding stroma and blood circulation [3]. In this process, CTCs lose epithelial markers, such as epithelial cell adhesion molecules (EpCAM) and E-cadherin, and show a mesenchymal phenotype. This morphological change is generally reversible via epithelial–mesenchymal transition (MET), which includes the activation of mixed epithelial/mesenchymal CTC hybrid phenotypes [1,4]. These CTC phenotypes are also present in the blood of patients with prostate cancer (PCa) [5].

PCa is the second most diagnosed cancer, with an estimated 1.4 million new cases worldwide in 2020, and it was the fifth leading cause of death in that same year [6]. In the clinic, PCa can be present in a localized indolent or metastatic lethal stage. Individual

patients demonstrate phenotypic and genomic intratumoral heterogeneity, which is challenging for diagnosis and treatment [7,8]. A single tissue biopsy sample may not reflect the current stage of the cancer. Therefore, liquid biopsy as a method that captures heterogeneous tumor material is needed. The isolation and characterization of CTCs provide such opportunity [9,10]. CTC enrichment can be achieved based on physical (size, deformability, density or electrical charge) or biological (cell-surface protein) properties [11]. Unfortunately, the gold standard CellSearch system (Silicon Biosystem, Menarini, Florence, Italy) isolates CTCs with EpCAM-coated ferrofluid beads and identifies an overexpression pattern of cytokeratin (CK) 8, 18 and 19 and the absence of CD45 expression [12,13]. This is the only Food and Drug Administration (FDA)-approved CTC isolation platform for monitoring patients with metastatic prostate (mPCa), breast and colorectal cancers [13–15]. However, EpCAM-based enrichment of CTCs alone does not always correspond to heterogeneity in the CTC phenotype. Furthermore, for PCa, it is crucial to evaluate sensitive and specific markers for cancer-specific CTC identification and analysis.

Prostate-specific membrane antigen (PSMA), a type II transmembrane glycoprotein, has become a clinically validated therapeutic target [16]. PSMA expression in PCa tissue is 100 to 1000 times higher than that in benign tissue [17]. Interestingly, antiandrogen treatment upregulates PSMA expression in patients with castration-resistant prostate cancer (CRPC) [18]. Low levels of PSMA can be expressed in lung cancer, colorectal carcinoma and glioblastoma [19].

Another possible marker for CTC isolation/characterization is prostate stem cell antigen (PSCA), a glycosylphosphatidylinositol (GPI)-linked cell-surface protein that is expressed in >80% of PCa patients [20]. This cell-surface protein shares 30% homology with stem cell antigen type 2 (SCA-2), a surface marker of immature lymphocytes [21]. Furthermore, PSCA expression increases with a higher Gleason grade and progression to androgen independence [22]. PSCA can also be detected in the kidney, urothelium and lung, which has been reviewed by van der Toom et al. [23] 2019.

The most commonly used marker in PCa screening, monitoring and disease progression is prostate-specific antigen (PSA, also known as kallikrein-related peptidase 3). The expression of PSA is specific for PCa, but it also tends to decrease with cancer progression and phenotypic change [7,24]. Moreover, PSA is an androgen-regulated protease, and PCa cell proliferation is highly dependent upon androgen receptor (AR) signaling [25,26]. Androgen deprivation therapy (ADT) results in a decrease in PSA secretion under the detection limit in serum. A rising PSA level is associated with the development of CRPC and the risk of metastasis [27].

Nevertheless, PSMA, PSCA and PSA are potential markers for specific enrichment of CTCs in the blood of patients with metastasized PCa. The aim of our preliminary investigation was to evaluate and develop a PCa-specific CTC isolation method. For this purpose, we used the CellCollector (CC-EpCAM), a medical wire that enables in vivo CTC isolation with a monoclonal antibody directed to the cell-surface-expressed EpCAM of CTCs in the peripheral blood [28–31]. In our trial, we functionalized the new form of the CellCollector with EpCAM antibody or with previously mentioned PCa markers (CC-PCa). In a proof of concept, we analyzed the different CellCollector system ex vivo.

We compared isolation techniques that use the physical (size and the density of cells) and biological (expression of EpCAM, PSMA, PSCA and PSA) properties of CTCs. Additionally, we aimed to determine a suitable CTC isolation method for possible CTC cultivation in advanced PCa patients.

2. Materials and Methods

2.1. Study Collective

This prospective study was planned for the University Clinic and Outpatient Clinic for Urology, Medical Faculty of Martin Luther University Halle-Wittenberg. All patients provided written informed consent before blood collection. The protocol was approved by the medical faculty ethics committee of Martin Luther University Halle-Wittenberg

(2012-65). Men were enrolled with histologically confirmed and metastatic prostate adenocarcinoma. We evaluated differential CTC capture methods in mPCa patients. For this purpose, two cohorts of PCa patients under treatment were prospectively analyzed. Blood samples from cohort 1 (n = 15) were analyzed with two different functionalized and spiralized CellCollector systems (GILUPI GmbH, Potsdam, Germany). In cohort 2 (n = 10), the ScreenCell (SC) kit (ScreenCell SA, Sarcelles, France) for cytological analysis and the SC kit for cell culture and buffy coat analysis accompanied by CD45 depletion were evaluated. The viability of CTCs was checked in cell culture approaches only in cohort 2 (Figure 1). All blood samples (EDTA tubes) were processed within 3 h.

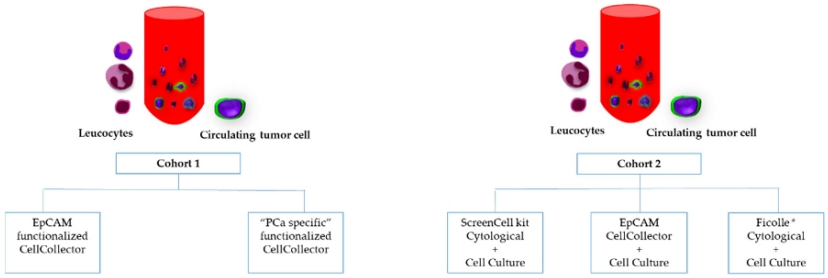

Figure 1. Overview of CTC isolation approaches in the two independent patient cohorts. Abbreviations: Ficoll *: Ficoll + CD45 depletion.

2.2. Antibody Validation for the PCa-Specific Functionalization of the CellCollector System

The PCa cell line LNCaP was purchased from ATCC (www.atcc.org, accessed on 1 April 2018) and routinely maintained in RPMI 1640. The media were supplemented with 10% fetal bovine serum. The cell line was grown on sterile glass slides until it reached a confluence of 70%. The cells were fixed with ROTI-Histofix 4% (Carl Roth, Karlsruhe, Germany) for 15 min, washed with phosphate-buffered saline (PBS) (Sigma/Merck, Darmstadt, Germany), and blocked with 5% milk (Th. Geyer, Berlin, Germany) in PBS. Incubation with anti-PSMA (Cell Signaling Technology, Frankfurt, Germany), anti-PSCA (Abcam, Cambridge, UK) or anti-PSA (Cell Signaling) was performed overnight at 4 °C. The secondary antimouse antibody (DIANOVA GmbH, Hamburg, Germany) was applied the next day for 1 h at RT. Cell nuclei were visualized using Hoechst 33258 (Merck, Darmstadt, Germany). Images (60×) were taken using an inverted fluorescence microscope (Carl Zeiss Microscopy, Jena, Germany).

2.3. CTC Isolation Approaches Based on Biological Properties

CellCollector (CC) system was used to enrich CTC based on expression of the FDA-approved surface marker EpCAM (CC-EpCAM) [13]. Furthermore, we developed a PCa-specific functionalization of the CellCollector system (CC-PCa). This was performed with a combination of 4 different antibodies against PSMA, PSA, PSCA and EpCAM. The concentration of the antibodies was 7.5 µg/mL; in summary, 20 µg antibodies were coupled on the spiral tip.

The single steps of the procedure were performed as described in Theil et al. [32]. We used 16 cm-long spiraled medical stainless steel wires. The 4 cm spiraled tips of the wire were previously covered with a thick (0.2 µm) layer of gold and a polycarboxylate layer (1–5 µm). We incubated them for 15 min in sterile distilled water to rehydrate the hydrogel and then activated them in 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochlo-ride/*N*-hydroxysulfosuccinimide (EDC/NHS) solution (Sigma) for 20 min at 22 °C. Next, a 100 mM solution of NHS in 50 mM 2-(*N*-morpholino)ethane-sulfonic acid (MES) buffer (Sigma), 0.5% EDC (Sigma) was added. Finally, the wire was rinsed using 5 mM acetic acid (Roth) and incubated with EpCAM or PSCA, PSMA, PSA and EpCAM antibodies for 1 h at 22 °C to achieve covalent bonding between the hydrogel and the

antibody. To block the free carboxyl groups, the hydrogel-covered wire was incubated with 1 M ethanolamine hydrochloride (Sigma) at pH 8.5. After washing with distilled water, the wires were stored at 4 °C until use.

In the following step, the performance of CC-EpCAM and CC-PCa was demonstrated in spiking experiments. Single LNCaP cells were spiked into healthy donor blood, and CellCollector wires were placed into the blood in a 7.5 mL EDTA tube, positioned on a rotating platform at room temperature and incubated for 60 min. At the end of the incubation, the wires were immediately placed in PBS and rinsed three times in a tube with new PBS solution. The cells were fixed on the wire with a 10 min acetone treatment and blocked with 3% bovine serum albumin/PBS for 30 min. Patient blood samples were subjected to the same conditions. By in vivo application, the wire would be inserted into the cubital vein through a 20G cannula and remain in place for 30 min. The CC-PCa was not approved for in vivo; for this reason, we compared the different wires ex vivo.

2.4. CTC Isolation Approaches Based on Physical Properties

The ScreenCell Cyto kit (ScreenCell SA, Sarcelles, France) isolates CTCs based on their size (<8 µm) with a filter system. All cells smaller than 8 µm are run through the filter. We performed the analysis using 3 mL of blood according to the manufacturer's instructions (ScreenCell, Sarcelles, France) for cytomorphology characterization of the remaining cells.

Three milliliters of EDTA blood was diluted in 4 mL filtration buffer and incubated for 8 min at room temperature. After incubation, 7 mL of diluted blood was filtered, and the filter device was washed with PBS. The filter was released onto absorbing paper and dried at room temperature for 15 min. After drying, cytomorphology characterization was performed.

The second CTC isolation method was based on the density of the cells and the CD45 depletion step using preconjugated, anti-CD45 magnetic beads (Dynabeads CD45, Life Technologies, Carlsbad, CA, USA). CTC enrichment was succeeded by density gradient separation with Histopaque-1077 (Sigma-Aldrich, Steinheim, Germany). Three milliliters of Histopaque-1077 was transferred to a sterile 15 mL tube and carefully overlayered with 3 mL of blood. This sample was centrifuged for 30 min at 500 g without braking to separate mononuclear cells (including CTCs) located at the interface between the plasma (upper layer) and the Ficoll-Histopaque (bottom). The interface was gently removed to avoid disturbing the layering, transferred to a new sterile tube and washed 3 times with sterile PBS. Recovered cells were resuspended in 500 µL PBS and prepared for microscopical examination (cytospins) by a cytocentrifuge (Epredia Cytospin 4 Zytozentrifuge, ThermoScientific, Waltham MA, United States) at 1000 rpm for 4 min at room temperature. The cytospins (ten slides) dried at room temperature (RT) for a minimum of 30 min and identified by cytomorphological or fluorescent immunohistochemistry characterization.

2.5. Ex Vivo Culture of CTCs

For cultivation with the ScreenCell Kit (ScreenCell Cell Culture Kit, ScreenCell SA, Sarcelles, France), we used 7 mL of diluted blood (6 mL of blood to 1 mL of ScreenCell LC dilution buffer). After incubation for 2 min, 1.6 mL of culture medium was added and homogenized once by inverting the tube, and filtration was started. Subsequently, the filter was released into a tissue culture plate filled with 600 µL culture medium.

CTCs from 3 mL of blood with a Ficoll * gradient were prepared for cultivation, as mentioned above, by adding 500 µL of cultivation medium.

The medium consisted of RPMI Medium 1640 (Life Technologies, Carlsbad, CA, USA), recombinant human epidermal growth factor 20 ng/mL (Life Technologies), recombinant human basic fibroblast growth factor 50 ng/mL (Life Technologies), 1% penicillin–streptomycin mix (Life Technologies) and 1 mL/50 mL B27 supplements, minus vitamin A (Life Technologies). No androgens and glucocorticoids were additionally included.

After gentle mixing, 30 µL of the cell suspension was transferred into wells of a 12-well cultivation plate. Furthermore, 20 µL of cell suspension was added to a well of a 10-well

CELLview slide (Greiner Bio-One, Frickenhausen, Germany). CTCs were cultivated under standard culture conditions (37 °C and 5% atmospheric CO_2) and observed by inverted microscopy. We changed a third of the medium after the 10th, 13th and 22nd days of cultivation and determined the PSA level in the samples.

2.6. Staining an Enumeration of Collected Cells

The cells isolated by the SC filter and Ficoll * were stained with May-Grunwald Giemsa staining. Staining was performed according to the manufacturer's instructions. The filter was incubated for 2 min in May-Grunwald solution and for the next 10 min in Giemsa solution. After that, the filters and slides were washed with distilled water and dried for 15 min at room temperature.

The captured cells were fixed and blocked on the wire surface or on the Ficoll ** slides. The cells were identified as CTCs by immunofluorescence staining using pan-CK- and Hoechst-33258-positive and CD45-negative criteria. The CTCs met the following cytology-based FDA definition: (i) size $\geq$4 µm, (ii) visible cytoplasm, (iii) high nuclear/cytoplasm ratio, (iv) positive fluorescence staining of CK 8, 18 and 19 with negative staining of CD45 and (v) 50% of the nucleus contained within the CK border enumeration [33].

2.7. PSA Measurement

The PSA secretion of the cultivated CTCs in the supernatant was determined with an IMMULITE 1000 Immunoassay System (SIEMENS Healthineers, Erlangen, Germany). We used the third-generation PSA assay, which provides a very low PSA detection limit of 0.005 ng/mL.

2.8. Statistical Analysis

Since the sample size was rather small, we did not perform an extensive statistical analysis. We applied the Mann–Whitney test to compare continuous clinical and demographic parameters in our cohorts. Additionally, several other nonparametric tests were performed as specified in the Results section. Finally, for identification of possible correlations between the different methods, Spearman's rank correlation coefficient (r_s) was determined. The reported p values were two-sided, and $\leq$0.05 was considered significant. The accuracy of the CTC isolation methods was evaluated by receiver operating characteristic (ROC) analysis. Kaplan–Meier analysis was used to analyze overall survival (OS) depending on CTC count [34,35]. All statistical analyses were performed using GraphPad Prism software version 9.

3. Results

3.1. Patient Characteristics and Treatments

All the patients in our two independent cohorts had histologically and radiologically confirmed mPCa. Patients with a second cancer diagnosis were not included in this analysis. We enrolled the patients randomly in a timeline of 6 months for every cohort. Genetic characterization of primary tumor or metastasis were not performed. The clinical and pathological parameters of the patients are summarized in Table 1. A statistically significant difference was observed for only one of the surgical procedures (transurethral resection of the prostate) in the cohorts. The median age of cohort 1 was 74 years and that of cohort 2 was 63.5 years (p = 0.03). Twelve patients (80%) in cohort 1 and nine patients (90%) in cohort 2 had a Gleason score of more than 7 at the time of diagnosis. At the time of CTC sample collection, all patients received ADT and/or chemotherapy. The median PSA level was 13 ng/mL (0.02–353 ng/mL) in cohort 1 and 71.9 (1.7–184 ng/mL) in cohort 2 (p = 0.8). More than 86% of patients in cohort 1 and 100% in cohort 2 had bone metastasis. Lymph node metastasis was present in 41.7% of cohort 1 and in 50% of the patients in cohort 2. One patient in cohort 2 was only under ADT and presented multiple bone and lymph node metastases.

Table 1. Clinical characteristics of the metastatic prostate cancer patients in cohorts 1 and 2.

	Cohort 1	Cohort 2	*p* Value
Patients, *n* (%)	15 (100)	10 (100)	
Median age (range), years	74 (60–84)	63.5 (49–86)	0.03
Ethnicity	Caucasian	Caucasian	
Gleason score at diagnosis, *n* (%)			0.09
≤7	3 (20)	1 (10)	
>7	12 (80)	9 (90)	
PSA at study visit, median ng/mL (range)	13 (0.02–353)	71.9 (1.7–184)	0.8
Primary therapy, *n* (%)			
Surgery	6 (40)	3 (30)	0.69
Radiation	9 (60)	5 (50)	0.69
Current therapy, *n* (%)			
Androgen deprivation	15 (100)	10 (100)	1
TURP	15 (100)	2 (20)	<0.001
Chemotherapy	10 (83.3)	8 (80)	0.66
Site of metastatic disease, *n* (%)			
Bone	12 (86.7)	10 (100)	0.14
Lymph	5 (41.7)	5 (50)	0.68
Brain	1 (8.3)		
Liver	1 (8.3)		

Abbreviations: PSA = prostate-specific antigen, TURP = transurethral resection of the prostate, CTC = circulating tumor cells.

3.2. Identification of Suitable Antibodies for PCa-Specific Functionalization of the CellCollector System

First, to implement CC-PCa for CTC capture, we checked and identified suitable antibodies compatible with the functionalization procedure and effective CTC capture. Immunofluorescence analysis revealed precise patterns of the antibodies used in LNCaP cells. The PSMA (12702S) antibody was used to detect the extracellular domain of the type II transmembrane protein. We confirmed this signal by immunostaining LNCaP cells (Figure 2b). The PSA signal of the antibody (5877S) displayed a strong cytoplasmic portion in the LNCaP cells (Figure 2e). The recombinant amino acid fragment corresponding to amino acid 1 to the C-terminus of human PSCA was used to generate the polyclonal PSCA antibody (ab 220101). We observed predominantly cytoplasmic and marginal cell membrane signals of the PSCA antibody (Figure 2h).

To check the functionality of the prepared CellCollector wires, we spiked LNCaP cells (200 cells) into healthy donor blood and evaluated the detection rate of the CC-PCa and CC-EpCAM systems. These samples (n = 3) were analyzed on one batch. Recovery rates from 48% for CC-PCa (PSMA, PSCA, PSA and EpCAM) and from 30% for CC-EpCAM were observed.

3.3. Comparison of CTC Detection Methods

Different CTC isolation methods were analyzed in the two independent cohorts of mPCa patients (Figure 3). Positive CTC patients in our cohorts were defined as having ≥1 CTC in the blood sample. In cohort 1, we compared the different functionalized CellCollector systems with spiralizer tips (Figure 3a). With CC-PCa, we detected ≥1 CTC in 12 out of 15 (86.7%) patients (Figure 4e–h); with CC-EpCAM, we detected ≥1 CTC in 11 out of 15 (73.3%) patients. The median CTC count detected with CC-PCa was nine (range 0–122) and that with CC-EpCAM was three (range 0–22). Additionally, the Wilcoxon matched-pair analysis demonstrated a median of four CTC differences when comparing CC-PCa to CC-EpCAM (p = 0.002). Interestingly, only a weak correlation was observed between the different functionalized wires (Spearman rank correlation coefficient r_s = 0.37, p = 0.17).

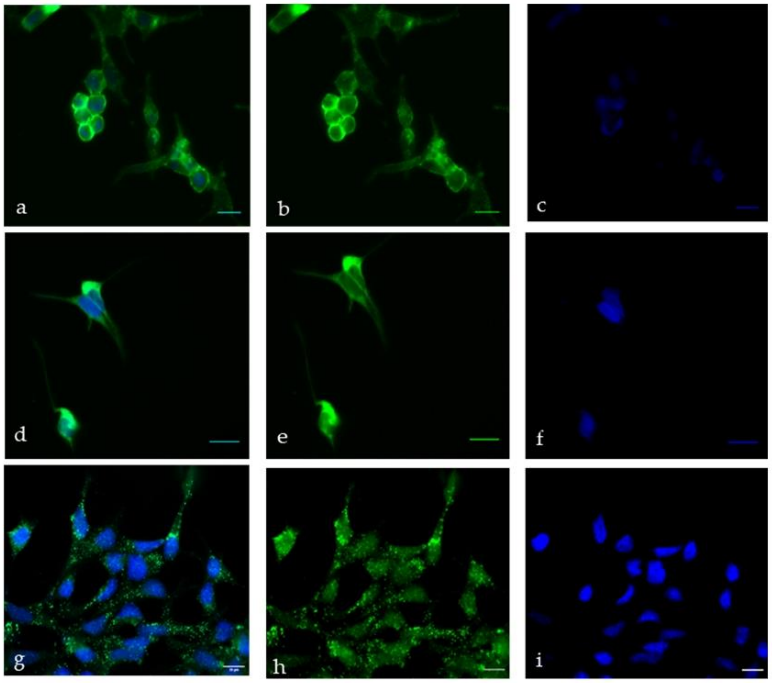

Figure 2. Immunofluorescence staining of LNCaP cells for (**b**) PSMA, (**e**) PSA, (**h**) PSCA and (**c**,**f**,**i**) Hoechst 33258 was used for nuclear counterstaining. Overlay of nuclear staining and marker-specific staining showing (**a**) PSMA, (**d**) PSA and (**g**) PSCA. Scale bares indicate 20 µm.

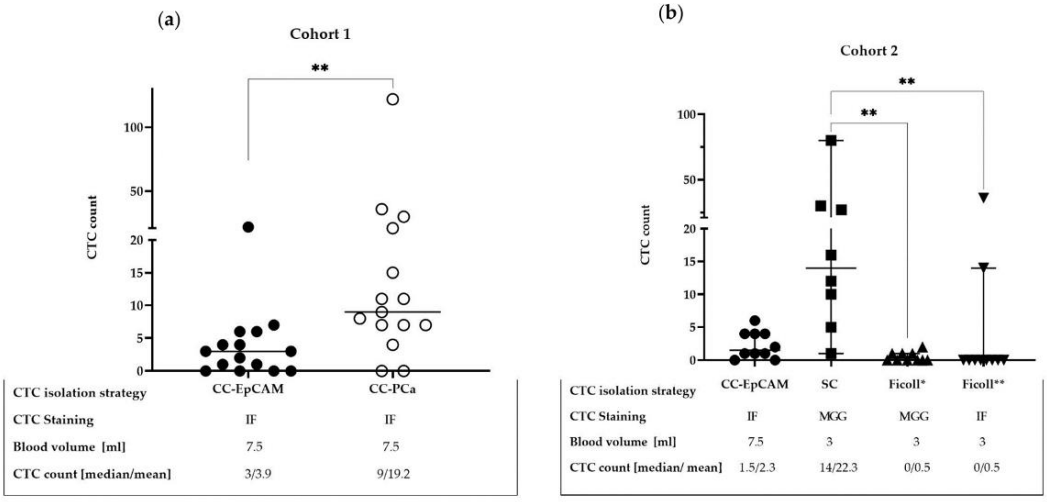

Figure 3. Direct comparison of CTC detection: (**a**) with the CC-EpCAM and CC-PCa systems, and the Wilcoxon matched-pairs signed-rank test was used, p ** 0.002. (**b**) CC-EpCAM, ScreenCell Cyto device (SC) and Ficoll gradient with CD45 depletion Ficoll *-May-Grunwald Giemsa (Ficoll *-MGG) and Ficoll **-immunofluorescence staining (Ficoll **-IF) systems and Dunn's multiple comparison test were used. Ficoll **-IF vs. SC-MGG, p = ** 0.002 and Ficoll *-MGG vs. SC, p = ** 0.002.

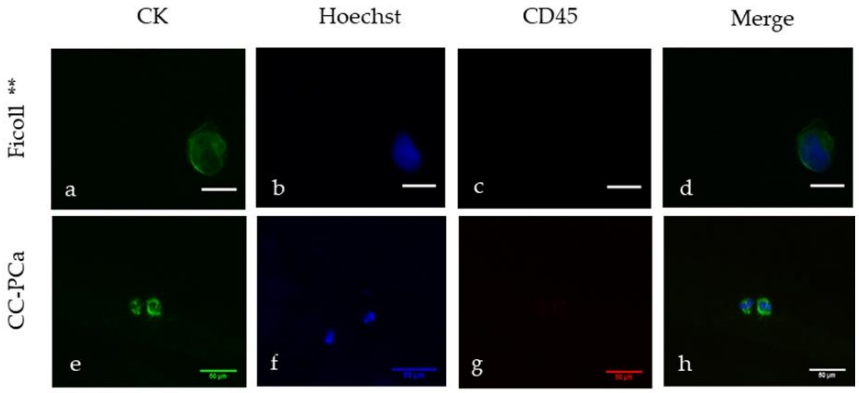

Figure 4. Representative CTC images (**a**,**e**) and negative leukocyte staining (**c**,**g**) from selected patients of our cohorts collected by Ficoll ** (**a**–**d**) and CC-PCa (**e**–**h**). CTCs were defined as cells with positive staining for CK (**a**,**e**) but negative staining for CD45 (**c**,**g**). Hoechst staining showed the presence of cell nucleoli (**b**,**f**). The scale bars indicate 20 µm, and cells were scanned at a magnification of 63.3× (**a**–**d**). The scale bars indicate 50 µm, and the cells were scanned at a magnification of 20× (**e**–**h**). Merge images are presented in (**d**,**h**).

In cohort 2, three different CTC isolation techniques based on the physical and biological CTC characteristics were compared, and two different staining methods for identification were used. The CTC detection rates based on the density (Ficoll *) and CD45 depletion were 2 out of 10 (20%) with immunofluorescence staining (Ficoll **) (Figure 4a–d) and 4 out of 10 (40%) with MGG staining (Ficoll *). The SC filter with MGG staining based on the size of the cells was used to detect CTCs in 8 out of 10 (80%) patient samples (Figure 5). Two of the ten SC probes could not be evaluated because of blood clots on the filters.

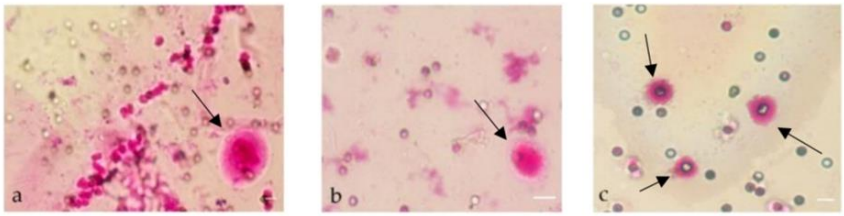

Figure 5. CTCs stained with MGG present in blood samples of PCa patients: (**a**,**b**) Patient #7 current treatment enzalutamide after docetaxel chemotherapy with a PSA level of 105 ng/mL. (**c**) Patient #5 current treatment docetaxel chemotherapy with a PSA level of 5.2 ng/mL. Scale bares indicate 20 µm. Arrows indicate CTCs.

CC-EpCAM detected CTCs per 7 mL in 8 out of 10 (80%) cohort 2 patients (Figure 3b). To better compare all the CTC detection rates (≥1 CTC), we calculated the blood volume needed for CC-PCa to be 3 mL and detected CTCs in 5 out of 10 (50%) blood samples. The median CTC count was 0.65 CTC (range 0–6 CTCs) per 3 mL of blood for CC-EpCAM and 0 CTCs for Ficoll * (range 0–2) and Ficoll ** (range 0–36 CTCs). The SC filter captured a median of 14 CTCs per 3 mL of blood (range 1–79 CTCs) (Figure 3b). The CTC count of the Ficoll * filter correlated moderately with the SC filter, r_s of 0.54, but was not significant ($p = 0.18$). In addition, the CTC count for CC-EpCAM with Ficoll * correlated with a moderate r_s of 0.47 ($p = 0.16$). Significant differences in CTC count were demonstrated between CS-MGG vs. Ficoll * ($p = 0.002$) and CS-MGG vs. Ficoll ** ($p = 0.002$). For the analysis, Dunn's multiple comparison test was used (Figure 3b).

The PSA level demonstrated no significant correlation with the CTC isolation method in either cohort.

To evaluate the sensitivity and specificity of the different isolation methods for predicting survival, we determined the receiver operating characteristic (ROC) and area under the curve (AUC) of the survival models. The CTC cutoff was ≥1 CTC. The results demonstrated that for a survival time of 24 months, the AUC values of the CTC isolation methods ranged from 0.53 to 0.79 (Table 2). Interestingly, the efficacy of CTC isolation with Ficoll * (AUC = 0.79) was higher than that of CC-EpCAM and CC-PCa in cohort 1 and that of CC-EpCAM, SC and Ficoll ** in cohort 2 (AUC = 0.53, 0.67, 0.55, 0.73 and 0.67, respectively, Table 2). Furthermore, the AUCs (0.73 and 0.79) of the CTC isolation methods, which used MGG staining, were the highest (SC and Ficoll *).

Table 2. Receiver operating characteristic curves for the CTC isolation methods.

CTC Isolation Method	AUC	95% CI	*p* Value
Cohort 1: CC-EpCAM	0.53	0.22–0.84	0.86
Cohort 1: CC-PCa	0.67	0.38–0.95	0.29
Cohort 2: CC-EpCAM	0.55	0.14–0.96	0.82
Cohort 2: SC-MMG	0.73	0.36–1.00	0.30
Cohort 2: Ficoll *	0.79	0.50–1.00	0.17
Cohort 2: Ficoll **	0.67	0.30–1.00	0.44

Abbreviations: CC-EpCAM = CellCollector EpCAM functionalized, CC-PCa = CellCollector PCa functionalized, SC-MMG = SC filter with MGG staining. * MGG staining, ** immunofluorescence staining.

Additionally, the isolation methods used required equivalent professional laboratory skills of the staff. The working time per method ranged from >2 h for the SC system to >3 h for the other three systems (Table 3). The SC filter device provided rapid CTC isolation with a single wash step. The CellCollector and the Ficoll gradient systems required more than three wash steps. We observed a higher number (≥500) of contaminating leukocytes when we used the size-based and density-based CTC isolation methods. Lower leukocyte contamination by the Ficoll system was reached by the addition of a CD45 depletion step. However, the CellCollector system had the lowest leukocyte contamination. Of the four isolation methods, only the Ficoll * method was able to isolate CTCs that could be cultivated. Finally, all the systems required trained and experienced observers to identify and image the CTCs.

Table 3. Characteristics of different CTC isolation methods.

Method	CellCollector EpCAM	CellCollector PCa	ScreenCell	Ficoll *
CTC isolation basis	EpCAM	PSMA, PSA, PSCA, EpCAM	Size: <8 µm cells	Density of Cell + CD45
Used marker	CK 8, 18,19, CD45	CK 8, 18, 19, CD45	CK8, 18, 19, CD45 or MGG	CK 8, 18, 19, CD45 or MGG
Test volume (EDTA blood)	7.5 mL	7.5 mL	3 mL	3 mL
Leukocyte contamination	<10	<10	≥2000	≥500
CTC cultivation effort	n.p.	n.p.	no	yes
Hands-on time for the CTC result per test	≥3 h	≥3 h	≥2 h	≥3 h
Cost per test	±300 €	±350 €	±250 €	±200 €

Abbreviations: EpCAM = epithelial cell adhesion molecule, PSMA = prostate-specific membrane antigen, PSA = prostate-specific antigen, PSCA = prostate-specific stem cell antigen, CTC = circulating tumor cell, CD45 = cluster of differentiation 45, MGG = May-Grunwald Giemsa, n.p.

3.4. CTC Cultivation

Blood samples of three mPCa patients in cohort 2 were processed parallel with Ficoll * (3 mL of blood) and CS circular filter CTC cultivation (6 mL of blood). The originating CTCs were directly cultivated in a 24-well cell culture and seeded in CELLview slides. However, the cultivation of CTCs was possible only for one patient, for whom isolation with the Ficoll * system was performed. The patient had bone and lymph node metastasis of the PCa and was treated at the time of blood sampling with ADT. The serum PSA level was 10.7 ng/mL. In addition, he had not received any chemotherapy. After 7 days of culture, we changed a third of the medium, and on the 10th, 13th and 22nd days of cultivation, we determined the PSA level in the collected medium. Furthermore, CTCs were characterized for CK expression (Figure 6a). The cultivated CTCs showed positivity for classical epidermal markers (CK 8, 9 and 10). Single CTCs were also positive for PSA expression by immunostaining (data not shown). The CTCs demonstrated by brightfield imaging mainly adhered to growing cells, and clustering of the cells was observed. The morphologies of the growing CTCs became paler and more elongated in the culture (Figure 6e,f).

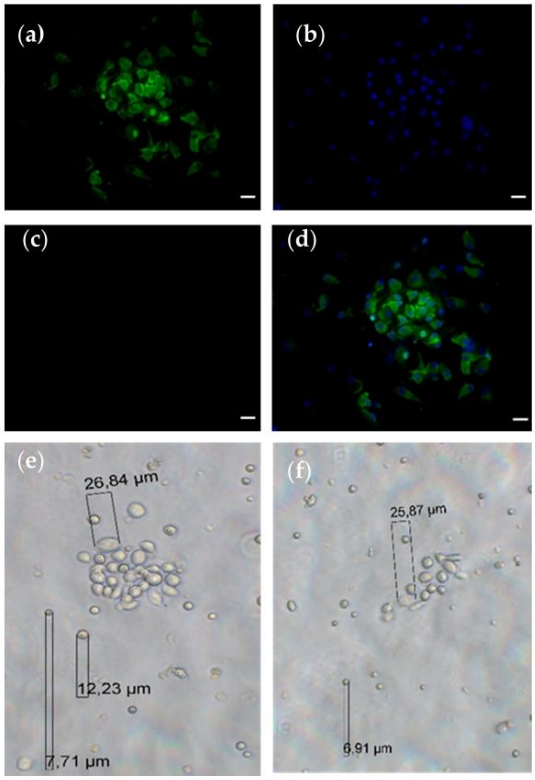

Figure 6. Representative images of cultivated CTCs. The CTCs display positive staining for (**a**) CK 8, 9 and 18, (**b**) Hoechst 33258 staining, (**c**) CD45 negativity and (**d**) overlay of images (**a**–**c**). Scale bar presents 20 µm. Brightfield images of growing CTCs (**e**,**f**).

Furthermore, we analyzed the PSA level in the cell culture wells of the cultivated CTCs. After ten days of cultivation, we determined a mean PSA level of 0.47 ng/mL in the tested wells. Unfortunately, a mean PSA level of only 0.02 ng/mL was detected in the wells over 3 weeks (Figure 7).

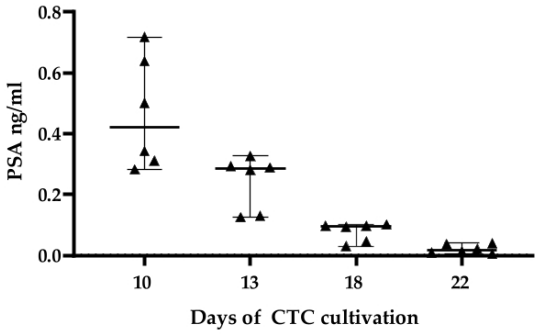

Figure 7. Concentration of PSA in ng/mL of cultivated CTCs in the supernatant displayed as median with range. CTCs were successfully cultivated in different single-cell culture wells of a 24-well plate. CTCs originated from a single patient.

3.5. Prognostic Performance of the CTC Status

Patients of our cohorts were positive for PCa metastasis, which resulted in a slight decrease in general conditions. In study cohort 1, the patients reached a follow-up time of 5 years. We used the established CTC cutoff values of <5 or ≥5 CTCs for the OS analyses [13]. The OS estimated in different groups was compared using the log-rank (Mantel–Cox) test. Patients (73.3%) evaluated with CC-EpCAM and with <5 CTCs survived a median of 2.1 years, and patients (26.7%) with ≥5 CTCs survived a median of 0.93 years. The hazard ratio (HR), referring to <5 CTCs or ≥5 CTCs, was 0.53 (95% confidence interval (CI): 0.13–2.1, Figure 8a).

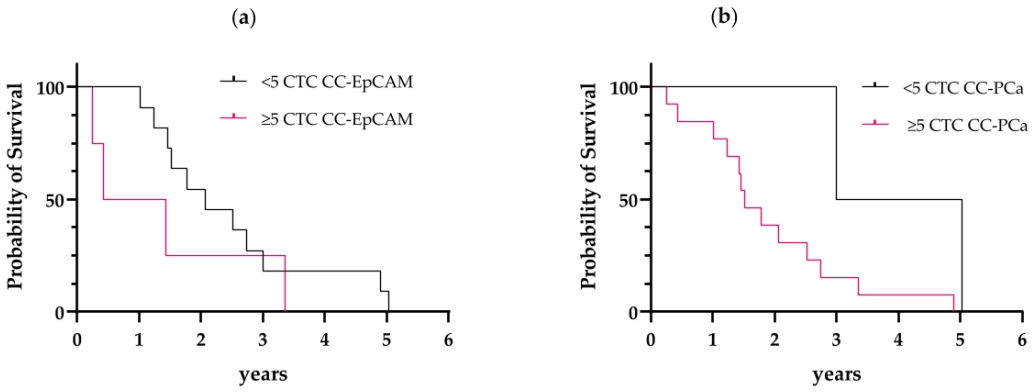

Figure 8. Kaplan–Meier curves of OS according to CTC count determined with (**a**) CC-EpCAM and with (**b**) CC-PCa: (**a**) The patients with <5 CTCs and ≥5 CTCs showed a difference in OS (2.1 years versus 0.93 years (HR 0.53, 95% CI: 0.13–2.1)). (**b**) The patients with <5 CTCs and ≥5 CTCs showed a difference in OS (4.0 years versus 1.5 years (HR 0.33, 95% CI: 0.11–0.97)).

Patients (20%) evaluated with CC-PCa and with <5 CTCs survived a median of 4.0 years, and patients (80%) with ≥5 CTCs survived a median of 1.5 years. The HR, referring to <5 CTCs or ≥5 CTCs, was 0.33 (95% CI: 0.11–0.97, Figure 8b). In cohort 2, we reached a follow-up time of 2 years. Unfortunately, three patients were lost to follow-up. Due to the small study population, we could not perform survival analyses.

4. Discussion

Selecting a well-suited method for isolating/characterizing CTCs remains a challenge. Isolation platforms should be used to detect most heterogeneous CTC phenotypes that survive after drug therapy to identify treatment targets for clinical testing or drug development [4,36,37]. In our study, we evaluated the CTC count with isolation platforms relying on different cell characteristics and laboratory handling. We assessed two cohorts of mPCa patients. The demographical characteristics were not significantly different in our study population. In addition, we used in our cohorts at least one CTC capturing method, based on the EpCAM signal currently recognized at the only FDA-approved surface marker for CTC detection. We performed immunofluorescence staining of the cytology-based FDA CTC criteria. These facts allowed for a more rigorous discussion of the results.

The first step was to develop and evaluate the PCa functionalized CellCollector system as a proof of concept. Our immunofluorescence displayed the classical signals in the analyzed LNCaP cells. The only polyclonal antibody, PSCA, was created against the whole amino acid sequence of the protein. For that reason, this antibody can bind in any position of the protein. This reflects the signals in our analyzed cells. Wang et al. demonstrated in Western blot analyses the expression of PSCA in LNCaP cells [38]. Unspecific capturing of cells in our trial was excluded by the use of the cytology-based FDA definition of CTCs. We revealed in the spiking experiment a moderate recovery rate (30–48%) of the cells with two different CellCollector systems. One reason for this could be the expression of various adhesion molecules of cell line cells that leads to different cell–cell contacts that can potentially influence the recovery rate [39]. Furthermore, in our previous trial, we received with the EpCAM functionalized CellCollector a high recovery rate (35%) of LNCaP (200 cells) and low recovery rate of 10% (50 cells) [32]. This suggested a higher sensitivity for capturing PCa cells with the CC-PCa.

Our data for cohort 1 highlighted the disparities in the detection rate and CTC count in matched blood samples between the different CellCollector systems. These results show detection rates of 86.7% for CC-PCa vs. 73.3% for CC-EpCAM and a significantly higher CTC count for CC-PCa that reflects the spiking experiments. Furthermore, the weak correlation of the CTC counts between the different functionalized CellCollector systems demonstrated the independence of the CTC counts. The only difference between the CellCollector systems was the surface antibody functionalization for capturing CTCs. The immunocytochemistry characterization of the CTCs was identical and performed by the same operator. These results demonstrate the heterogeneity in the CTC population based on different cell-surface antigens. CC-PCa allowed identification of a hybrid population of CTCs, which may reflect epithelial/mesenchymal phenotypes. EMT is a crucial process of tumor progression [40]. In the case of our analysis, in addition to the EpCAM signal, the isolated CTCs expressed PSMA, PSA and/or PSCA. In other studies, the detection rate of PSMA-positive CTCs ranged between 67% [41] and 59% [42] in advanced PCa patients. In our previous clinical trial, we detected a PSMA signal at the mRNA level in CTCs isolated with CC-EpCAM in only 14.3% of mPCa patients [32]. Nagaya et al. [42] observed that increased PSMA expression in CTCs was associated with a poor treatment response and shorter OS and PSA progression-free survival in castration-resistant PCa patients. In contrast, we observed in cohort 1 no significant differences in OS between the different functionalized CellCollector systems. Nevertheless, interestingly, patients with $\geq$5 CTCs isolated with CC-EpCAM had an OS of 0.93 years, and patients with $\geq$5 CTCs isolated with CC-PCa had an OS of 1.5 years in this cohort. Even if advanced PCa patients have more CTCs, these cells can lose the features of epithelial cells and undergo EMT, thereby promoting disease progression [9,43]. However, Keller et al. [44], in 2019, reviewed the biological functions of EpCAM, including the regulation of cell proliferation and cancer stemness, and emphasized the active role of EpCAM in cancer metastasis. Therefore, it is essential for CTC isolation platforms based on biological characteristics to not completely eliminate EpCAM molecules from capturing strategies. This allows the capture of CTCs with mesenchymal and epithelial phenotypes and of various intermediate stages [10].

Markou et al. [31] demonstrated, in a multiplex gene expression profiling of EpCAM-positive CTCs, the detection of stem cell markers (CD133, ALDH1A1 and PSCA) only before surgery or radiotherapy. However, the EMT markers TWIST1, VIM, CDH2 and B2M in isolated CTCs after surgery/radiotherapy in high-risk PCa patients were increased. They concluded that CTCs with an EMT phenotype remain undetected by EpCAM-based isolation methods [31]. CC-PCa, which was developed in our investigation, allowed the capture of such CTCs without a complete exclusion of EpCAM capture.

The Epic Science platform carries out CTC isolation without specific selection, only with red blood cell lysis and deposition of all nucleated cells on 12 glass slides [45]. Scher H. and colleagues quantified 9225 individual CTCs of 179 metastatic CRPC patients isolated with this platform and defined phenotypically distinct cell types [46]. They showed that phenotypic heterogeneity was associated with OS and treatment response. They demonstrated that patients with low heterogeneity in CTC population benefit from treatment with androgen receptor signaling inhibitors, whereas patients with high heterogeneity in CTCs benefit from taxane chemotherapy [46]. These results also confirm the significance and relevance of the CTC heterogeneity evaluation.

In cohort 2, we evaluated different CTC isolation platforms based on physical CTC properties and biological properties based on EpCAM expression. The greatest advantage of antibody-independent platforms is unattached cells. Antibodies or biomolecules for CTC capture may influence downstream analysis [47]. We observed that the CTC detection rates have a broad range independent of the physical or biological CTC properties. The filter system (SC) allowed CTC selection by size and had the highest detection rate in our investigation. Similar results were presented in blood samples of metastatic breast cancer [48] and in lung cancer patients [49] with this filter system. Interestingly, CC-EpCAM reached second place in detection rate and CTC count, which confirms the discussion above of the role of EpCAM-based CTC isolation in advanced PCa patients. The highest CTC count was determined with the SC filter system and MGG staining. The advantage of this system is that CTC selection is based only on the size of the CTCs. Red blood cells (8 µm) pass through the filter. White blood cells (7–12 µm) sometimes fall between pores, but they can be identified on the basis of nuclear morphology and size. The disadvantage of this system is blood clots on the filters, which result in loss of blood samples without any results. A reason for clotting may be a high concentration of CTC clusters (>8 µm), which have a higher metastatic potential through increased cell survival and reduced apoptosis [50]. Furthermore, smaller CTCs (4–8 µm) can run through the filter, which reduces the possibility to analyze the morphological and phenotypical [51] heterogeneity of CTC population.

The lower CTC counts of the Ficoll * systems and the CC-EpCAM system may be the result of the multiple washing and sample transfer steps. The Ficoll * system had the most procedural steps with possible loss of CTCs. However, our research shows the Ficoll * systems had the highest sensitivity and specificity for 24-month survival. On the contrary, the CC-PCa and CC-EpCAM have lower specificity but higher sensitivity of CTC capturing compared to the Ficoll * system. Furthermore, in our previous trial, the EpCAM wire demonstrated in vivo a similar diagnostic accuracy to the FDA-approved CellSearch System [28] which confirmed in vivo application of the CellCollector. Unexpectedly, CTC cultivation was successful only with the Ficoll * system. This isolation method offered nearly untouched CTCs and minimized the shear stress of cells. CTCs were captured from patients who were treated with ADT and had progression of the cancer stage (more than five metastases in bone lymph). The CTC cell line cultivation period was possible for over 3 weeks. The cells secreted PSA at the highest concentration on the 10th day of cultivation. It is possible that the cells changed their PSA expressions under cell culture conditions, and only single cells were positive for PSA signals in immunohistochemistry. CTCs were positive for CK 8, 18 and 19, which confirmed their epithelial state. The PSA level of the patient after 2 months was slightly reduced under ADT, which could be caused

by initiation of the intrinsic apoptotic pathway in CTCs. However, we have not analyzed apoptotic markers.

Koch et al. [52] described, in their investigation of a CTC-derived breast cancer cell line, that a high number of CTCs is necessary for the establishment of cell line analysis. We had experiences that a smaller number of CTCs isolated with Ficoll ** was sufficient for culture, although only for a limited time. This suggests the differences in phenotype and high cell plasticity of both cell lines, which may also represent heterogenic stimuli [53].

The detection of CTC in our patient could indicate the dynamic of tumor growth; however, it reflects only the circumstances of one time point. To describe the correlations between the CTC count and clinical behavior of PCa patients, the monitoring must be continued for a longer time. After we compare the CTC isolation methods, we can plan such studies.

Our study has limitations, including the small sample size. The platform results were obtained by manual enumeration of cells by immunocytochemistry or MGG. Visual identification of CTCs was performed with a higher-level trained operator but can still be subjective.

In summary, the best detection rates and highest CTC counts in our investigation were reached with the CC-PCa and SC systems. Nevertheless, we observed the highest sensitivity and specificity for 24-month survival with the Ficoll * and SC platforms with MGG staining. The EpCAM molecule is an essential factor related to OS for CTC isolation based on biological properties in mPCa patients. We observed the highest purity by the ex vivo use of the CellCollector system. Cultivation of CTCs was only possible with Ficoll *. The cost of the platforms and the required skills of the operator are similar. We used standardized conditions for the isolation and characterization of CTCs and short sample transfer. We conclude that the best-suited CTC capture technique is not limited to one characteristic of the cells and must be adapted to the downstream analysis. Our results imply that the clinical importance of the different CTC phenotypes must be elucidated.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data are not publicly available due to privacy and ethical restrictions.

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5 Thesen

1. Zirkulierende Tumorzellen (CTCs) als Bestandteil der „Liquid Biopsy“ stellen Probenmaterial zur Verfügung, welches in der individualisierten und zielgerichteten onkologischen Therapie benötigt wird.

2. Die geringe CTC-Konzentration im Blut stellt eine große technische Herausforderung dar. Der CellCollector (CC), eine neue Technologie für die in vivo Isolation von CTCs, ermöglicht die Analyse eines größeren Blutvolumens (≤ 600 ml) im Vergleich zu den bisher bekannten CTC-Isolationstechnologien (≤ 30 ml).

3. Die erforderliche ex vivo Eignungsprüfung des CCs zeigte in Blutproben von Prostatakarzinompatienten (PCa) hohe CTC-Detektionsraten (> 90,9 %). Die CTC-Zahl der Patienten mit metastasiertem Prostatakarzinom (PCa-m) bestätigte sich als unabhängiger prognostischer Marker für das Gesamtüberleben (OS). Keine prognostische Relevanz hatte die ex vivo isolierte CTC-Zahl bei Patienten mit einem lokalen PCa.

4. Mittels molekularer Charakterisierung lassen sich tumorassoziierte Transkripte (EGFR, PSMA) in den ex vivo isolierten CTCs bei PCa-m Patienten nachweisen.

5. Die in vivo Anwendungen des CCs demonstriert eine gute Verträglichkeit bei den Studienteilnehmern. Es wurden keine unerwünschten Ereignisse (AEs) oder schwerwiegende unerwünschte Ereignisse (SAEs) dokumentiert.

6. Bei der in vivo CTC-Isolation war das Auftreten von ≥ 5 CTCs über den Untersuchungszeitraum mit einem verkürzten Gesamtüberleben (OS) von 27,5 Monaten assoziiert. Die CTC-Kinetik zeigt, dass Patienten mit ≥ 5 CTCs und einer steigenden CTC-Zahl zu jedem Untersuchungszeitpunkt das kürzeste mediane OS von 25 Monaten haben.

7. Im direkten Methodenvergleich mit dem CellSearch-System erreichte der CellCollector eine höhere Sensitivität. Die CTC-Zahlen der beiden Systeme zeigten keine Korrelation. Die diagnostische Genauigkeit der in vivo Anwendung des CCs ist mit dem FDA-geprüften CellSearch-System vergleichbar.

8. Das CTC/PSA-Profil eröffnet die Möglichkeit einer personalisierten Therapieüberwachung, die dazu beitragen kann, Patienten vor unnötigen Nebenwirkungen als Folge einer ineffektiven Therapie zu schützen.

9. Das CellSearch-System scheint besser für CTC-Anreicherung bei Patienten mit metastasiertem kastrationsresistentem Prostatakarzinom (mCRPC) geeignet zu sein, aufgrund der höheren diagnostischen Sensitivität und Spezifität in dieser Gruppe.

10. Die Korrelation der CTC-Zahl des CellSearch-Systems mit den Biomarkern sHER2 und DKK-1 in der mCRPC-Gruppe weist auf die mögliche Interaktion der CTCs untereinander oder mit Blutzellen hin.

11. Die entwickelte PCa-spezifische Funktionalisierung des CCs zeigt bei metastasierten PCa-Patienten eine höhere Sensitivität in der CTC-Isolation im Vergleich zur EpCAM-Funktionalisierung. Diese spezifische Funktionalisierung ermöglicht die Isolation von unterschiedlichen CTC-Phänotypen sowie Hybridphänotypen und kann so die Tumorheterogenität des Prostatakarzinoms besser reflektieren.

12. Die vergleichende Analyse verschiedener CTC-Isolationstechnologien zeigt, dass der PCa-spezifisch funktionalisierte CC und das ScreenCell-Filtersystem die höchste Sensitivität in der Isolation von CTCs aus den Blutproben von PCa-m Patienten erreichen. Kultivierungsfähige CTCs, welche PSA sekretierten, konnten nur mit dem Ficoll-System isoliert werden.

13. Von entscheidender Bedeutung ist es, die Forschung zu erweitern, welche sich auf die CTC-Phänotypisierung und auf die Koexistenz von CTCs mit tumorassoziierten Faktoren im Blut konzentriert, um den zielgerichteten Einsatz der CTCs in der klinischen Routine zu ermöglichen.

III Erklärungen zur Vorgelegten Habilitationsschrift

Ich, Dr. rer. nat. Gerit Theil, erkläre,

1. dass ich die vorliegende kumulative Habilitationsschrift ohne Fremdhilfe erstellt habe und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt wurden;
2. dass ich keine vorausgegangenen Habilitationsversuche unternommen habe, und dass keine Habilitationsversuche an einer anderen wissenschaftlichen Einrichtung eingereicht wurden;
3. dass die Habilitationsschrift nach den Regeln guter wissenschaftlicher Praxis verfasst wurde.

Ort, Datum Dr. Gerit Theil

IV Lebenslauf

PERSÖNLICHE DATEN

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Seit 11.2018	Leiterin des Urologischen Labors der Martin-Luther-Universität Halle-Wittenberg, Klinik für Urologie
04.2011-10.2018	wissenschaftliche Mitarbeiterin im Urologischen Labor der Martin-Luther-Universität Halle-Wittenberg, Klinik für Urologie Forschung: Liquid Biopsie in der Diagnostik urologischen Tumorpatienten
03.2008-03.2011	wissenschaftliche Mitarbeiterin bei der GILUPI-GmbH, Potsdam Schwerpunkt: Isolierung von zirkulierenden Tumorzellen durch einen Antikörper-beschichteten Nanodetektor; CTC-Diagnostik
11.2006-02.2008	wissenschaftlicher Mitarbeiterin (Doktorandin) am Institut für Physiologische Chemie der Martin-Luther-Universität Halle-Wittenberg Forschung: Entwicklung monoklonaler Antikörper
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