Clinical relevance of circulating epithelial tumor cells (CETCs) and circulating cancer stem cells (cCSCs) during different therapies of breast cancer patients

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„Man braucht nichts im Leben zu fürchten, man muss nur alles verstehen.“

Marie Curie

(1867-1934)

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Abkürzungsverzeichnis

ALDH1	Aldehyd-Dehydrogenase I
AML	akute, myeloische Leukämie
APC	*antigen presenting cell*
AR	Androgenrezeptor
AUC	*area under curve*
BCS	Brust-erhaltende Operation
DNA	Desoxyribonukleinsäure
CAM	Chorioallantoismembran
cCSCs	zirkulierende Krebsstammzellen
CETCs	zirkulierende epitheliale Tumorzellen
CSCs	Krebsstammzellen
CTCs	zirkulierende Tumorzellen
ctDNA	*circulating tumor DNA*
CTLA-4	*cytotoxic T-lymphocyte-associated protein 4*
EDTA	Ethylendiamintetraazetat
EpCAM	*epithelial cell adhesion molecule*
EMT	*epithelial-mesenchymal transition*
ER	Östrogenrezeptor
FISH	*fluorescence in-situ hybridization*
FITC	*fluorescein-isothiocyanate*
HER2	humaner epidermaler Wachstumsfaktorrezeptor 2
MCF-7	*Michigan Cancer Foundation-7*
NK	natürliche Killerzellen
NOD/SCID mice	*nonobese diabetic/severe combied immunodeficient mice*
pCR	pathologische komplette Remission
PCR	*polymerase chain reaction*
PD-1	*programmed cell death receptor 1*
PD-L1	*programmed cell death ligand 1*
PD-L2	*programmed cell death ligand 2*

PE	Phycoerythrin
PI	Propidiumiodid
PR	Progesteronrezeptor
RNA	Ribonukleinsäure
ROC	*receiver operating curve*

Zusammenfassung

Der schwerwiegendste Aspekt maligner Tumore ist ihre Fähigkeit Fernmetastasen in lebenswichtigen Organen zu bilden. Bei der Mehrheit neudiagnostizierter solider maligner Tumore liegen zum Zeitpunkt der Diagnose keine Metastasen vor. Dennoch erleiden bis zu 50% der Patienten ein Rezidiv. Dies beruht auf der Disseminierung von Tumorzellen, die dann Metastasen im Sekundärgewebe bilden können. Jedoch können nicht alle disseminierten Tumorzellen zu Metastasen in distanten Organen heranwachsen. Eine kleine Subpopulation von Zellen, die ein hohes Selbsterneuerungspotential besitzt, scheint für die Entstehung von Tumoren und Rezidive verantwortlich zu sein. Diese Subpopulation wird als Krebsstammzellen oder Tumor-initiierende Zellen bezeichnet.

Im ersten Teil der vorliegenden Arbeit konnte mit einem neuartigen 3D-Zellkultursystem die aggressivste Subpopulation der CETCs, die zirkulierenden Krebsstammzellen, die *in-vitro* zu Tumorsphären heranwachsen, identifiziert und weiter charakterisiert werden. Immunfluorimetrische, als auch genetische Untersuchungen konnten zeigen, dass sie Stammzelleigenschaften besitzen, die typisch für das Mammakarzinom sind. Die Anzahl an cCSCs spiegelte direkt die Aggressivität des Tumors wider und konnte das Risiko für Fernmetastasen bestimmen. Die Entwicklung eines *patient-derived* Xenograft Modells konnte zudem die Tumorigenität der *in-vitro* kultivierten Tumorsphären erstmals beweisen. Nach ihrer Applikation auf die Chorioallantoismembran des Hühnerembryos konnten sie innerhalb kürzester Zeit einen Tumor generieren, der pathologisch dem Primärtumor entsprach. Diese neu entwickelten Modelle konnten die Existenz der Krebsstammzellen nicht nur im Tumorgewebe sondern auch im peripheren Blut der Krebspatienten bestätigen und deren klinische Relevanz verdeutlichen. Dies eröffnet einen Weg Tumor-initiierende Zellen in unterschiedlichen Tumorentitäten zu identifizieren, molekular zu charakterisieren und zielgerichtete Therapien zu entwickeln.

CETCs sind einer Reihe unterschiedlicher physiologischer und immunologischer Hürden ausgesetzt, die deren Überleben im peripheren Blut erschweren. Um sich vor der Eliminierung durch das Immunsystem erfolgreich zu schützen, nutzen die CETCs eine Vielzahl unterschiedlicher Strategien. Im zweiten Teil der vorliegenden Arbeit wurden die unterschiedlichen Mechanismen untersucht, mit denen sich die CETCs vor der Immunüberwachung tarnen können. Im Fokus der Immunonkologie stehen die sogenannten *checkpoints* des Immunsystems, die von den T-Zellen exprimiert werden und normalerweise eine überbordende Immunreaktion verhindern. Tumorzellen können sich

diesen Mechanismus der Immunevasion zu Nutze machen. In dieser Arbeit konnte mit Hilfe immunfluorimetrischer und molekularzytogenetischer Analysen gezeigt werden, dass die CETCs bestimmte *checkpoints* wie PD-L1 und B7-H3 in einem hohen Prozentsatz auf ihrer Zelloberfläche exprimieren. Dies ermöglicht ihr Überleben im peripheren Blut und fördert gleichzeitig die metastatische Ausbreitung des Tumors. Eine Blockade dieser *checkpoints* bereits in frühen Stadien könnte klinisch relevant sein, um die Immunantwort, die sich gegen die Tumorzellen richtet, zu reaktivieren. Das *Monitoring* PD-L1 und B7-H3 positiver CETCs könnte insbesondere nach der operativen Entfernung des Tumors für die Überwachung der Immuntherapie nützlich sein. Ein weiterer Mechanismus, der die CETCs im peripheren Blut vor immunologischen Angriffen, den Scherkräften des Blutes und der Apoptose schützt, ist deren Interaktion mit anderen Komponenten des Blutes. Jüngste Studien deuten darauf hin, dass Thrombozyten eine entscheidende Rolle dabei spielen, das Überleben der CETCs im Blutkreislauf zu fördern. Die Tumorzell-induzierte Thrombozytenaggregation schützt die CETCs vor den Scherkräften des Blutes und gleichzeitig vor einer immunologischen Eliminierung. Dies kann jedoch auch der Grund dafür sein, dass die Antikörper die Oberflächenantigene der Tumorzellen nicht erreichen. In dieser Arbeit konnte die Interaktion zwischen den CETCs und den Thrombozyten zu drei verschiedenen Zeitpunkten nach der Blutentnahme bestätigt werden. Die Tumorzellen bildeten im Blut Aggregate mit den Thrombozyten, wodurch ihr Überleben im peripheren Blut gefördert, jedoch gleichzeitig deren Nachweis unmittelbar nach der Blutentnahme erschwert wurde. Durch die Lagerung der Blutproben über Nacht bei Raumtemperatur kam es zur Ablösung der Thrombozyten von den CETCs, wodurch sie mittels Immunfluoreszenz identifiziert werden konnten. Die Wirkung von Thrombozytenaggregationshemmern könnte einen inhibierenden Einfluss auf den Metastasierungsprozess haben.

CETCs stellen wertvolle tumorspezifische Biomarker dar, die die verbliebene minimale Resterkrankung nach einer scheinbar erfolgreich abgeschlossenen Krebstherapie widerspiegeln. Zudem können sie zeitnah die Effizienz systemischer Therapien abbilden, wodurch sie eine prognostische Relevanz haben. Aufgrund der hohen intra- und intertumoralen Heterogenität, aber auch aufgrund möglicher Veränderungen der Tumorzelleigenschaften im Krankheitsverlauf ist die longitudinale Überwachung der zirkulierenden Tumorzellen von großer Bedeutung. Frühe Resistenzen, bzw. frühzeitige Rezidive lassen sich somit leicht verfolgen. Im dritten Teil der vorliegenden Arbeit konnte mit der maintrac® Methode veranschaulicht werden, dass die Quantifizierung der CETCs

und vor allem der cCSCs ein geeignetes Stadien-unabhängiges Verfahren darstellt, um die Effizienz der aktuellen Therapie, wie der Immuntherapie, der Radiotherapie und der endokrinen Therapie zu überwachen. Patientinnen mit einem erhöhten Risiko für eine Therapieresistenz und ein Rezidiv könnten somit frühzeitig identifiziert und die Behandlungsstrategie angepasst werden. Die molekularbiologische Charakterisierung der Zellen hinsichtlich therapierelevanter Marker wie PD-L1, B7-H3 und AR bzw. ER könnte bei der Patientenstratifizierung für eine personalisierte Therapie behilflich sein.

Summary

The most pernicious aspect of malignant tumors is their ability to form distant metastases in vital organs. In the majority of newly diagnosed solid malignant tumors, no metastases are present at the time of diagnosis. Nevertheless, up to 50% of patients suffer a recurrence. This is due to the dissemination of tumor cells, which can form metastases in secondary tissue. However, not all disseminated tumor cells can grow into metastases in distant organs. A small subpopulation of cells with a high self-renewal potential seems to be responsible for the development of tumors and recurrences. This subpopulation is referred to as cancer stem cells or tumor-initiating cells.

In the first part of the present work, a novel 3D cell culture system was used to identify and further characterize this most aggressive subpopulation of CETCs, the circulating cancer stem cells that can grow into tumorspheres in vitro. Immunohistochemical as well as genetic analyses have shown that they express stem cell characteristics typical for breast carcinoma. The number of cCSCs directly reflected the aggressiveness of the tumor and determined the risk of distant metastases. The development of a patient-derived xenograft model demonstrated the tumorigenicity of tumorspheres cultured in-vitro from patient blood. After their application to the chick embryo chorioallantoic membrane they were able to generate a tumor pathologically equivalent to the primary tumor within a very short time. These newly developed models were able to confirm the existence of cancer stem cells not only in tumor tissue but also in the peripheral blood of cancer patients. This opens a way to identify tumor-initiating cells in different tumor entities, characterize them molecularly and develop targeted therapies.

CETCs are exposed to a number of different physiological and immunological hurdles that impede their survival in peripheral blood. To successfully evade elimination by the immune system, CETCs use a variety of different strategies. In the second part of the present work, the different mechanisms by which CETCs succeed in protecting themselves from elimination by the immune system were investigated. Immuno-oncology focuses on the so-called checkpoints of the immune system, which are expressed by T cells and normally prevent an exuberant immune response. Tumor cells can succeed in exploiting this mechanism to evade the activated immune system. In this work, immunofluorimetric and molecular cytogenetic analyses demonstrated that CETCs express certain checkpoints such as PD-L1 and B7-H3 in a high percentage on their cell surface. This enables their survival in the peripheral blood while promoting metastatic spread of the tumor. Blocking

these checkpoints at early stages could be clinically relevant to reactivate the immune response directed against tumor cells. Monitoring PD-L1 and B7-H3 positive CETCs could be useful for monitoring immunotherapy especially after surgical removal of the tumor. Another mechanism that protects CETCs in the peripheral blood from immunological attack, blood shear forces, and apoptosis is their interaction with other components of the blood. Recent studies suggest that platelets play a crucial role in promoting the survival of CETCs in the bloodstream. Tumor cell-induced platelet aggregation protects the CETCs not only from the shear forces of the blood but simultaneously from immunological elimination. This protective shield may also be the reason why the antibodies do not reach the surface antigens of the tumor cells. In this work, the interaction between CETCs and platelets was confirmed at three different time points after blood collection. The tumor cells formed aggregates with the platelets in the blood, promoting their survival in the peripheral blood, but at the same time making their detection more difficult immediately after blood collection. Storage of blood samples overnight at room temperature resulted in detachment of platelets from CETCs, allowing their identification by immunohistochemistry. The effect of platelet aggregation inhibitors could have an inhibitory influence on the metastasis process.

CETCs represent valuable tumor-specific biomarkers that reflect the remaining minimal residual disease after an apparently successfully completed cancer therapy. In addition, they can promptly show the efficacy of systemic therapies, thus having prognostic relevance. Due to the high intra- and intertumoral heterogeneity, but also due to possible changes in tumor cell properties during disease progression, longitudinal monitoring of circulating tumor cells is of high importance. Early resistance, or early recurrence, can thus be easily tracked.

In the third part of the present work, the maintrac® approach was used to demonstrate that quantification of CETCs and especially cCSCs is a suitable stage-independent method to monitor the efficacy of current therapy, such as immunotherapy, radiotherapy and endocrine therapy. Patients at increased risk for treatment resistance and recurrence could be identified early and the treatment strategy adjusted. Molecular characterization of these cells with respect to therapy-relevant markers such as PD-L1, B7-H3, and AR or ER could be useful in patient stratification for personalized therapy.

1 Einleitung

In den vergangen Jahrzehnten wurden große Fortschritte bei der Früherkennung und der personalisierten Krebsbehandlung erzielt, die das Überleben der Krebspatienten signifikant verlängert haben [1]. Bei der Mehrheit der Patienten kann der Tumor in einem frühen Stadium unter lokaler Kontrolle gehalten werden, dennoch ist das Gesamtüberleben bei vielen Krebsarten aufgrund distaler Metastasen verkürzt und die Sterberate mit 90% nach wie vor sehr hoch [2]. Diese hohe Sterberate könnte möglicherweise an dem noch unzureichenden Verständnis der Komplexität der Biologie von Krebserkrankungen und dem Fehlen geeigneter experimenteller Methoden liegen [3]. Zudem zielen die meisten Bemühungen zur Bekämpfung der Krankheit auf den Primärtumor ab. Der größte Teil der Patienten verstirbt aber an den Tumorabsiedlungen, den Metastasen, die erst Jahre nach einer scheinbar erfolgreichen Behandlung des Ausgangstumors auftreten können. Eine effektive Behandlung dieser Metastasen ist die größte Herausforderung bei der Krebstherapie [4–6]. Die meisten metastasierten Patienten werden mit einer systemischen Therapie behandelt, die das Überleben der Patienten verlängert und die Symptome, die mit der Krebserkrankung einhergehen, lindert. In den meisten Fällen ist die Therapie jedoch nicht kurativ [6].

1.1 Klassifizierung und Behandlung des Mammakarzinoms

Brustkrebs ist die am häufigsten diagnostizierte Krebsart bei Frauen weltweit und macht ungefähr 25% aller neu diagnostizierten Krebserkrankungen aus. Man geht davon aus, dass jede achte Frau im Laufe ihres Lebens an Brustkrebs erkrankt [7]. Das mediane Alter beträgt 65 Jahre. Das Stadium der Erkrankung zum Zeitpunkt der Erstdiagnose, die lymphovaskuläre Ausbreitung, der histologische Grad, die biologischen Eigenschaften des Tumors, Begleiterkrankungen, das Alter der Patientinnen und der Menopausenstatus bestimmen die Prognose und die Therapieoptionen. Die Behandlung einer lokalen Erkrankung beinhaltet in den meisten Fällen (wenn möglich) eine Brust-erhaltende Operation (*breast conserving surgery*, BCS), in der Regel gefolgt von einer adjuvanten Radiotherapie. In den meisten Fällen erhalten die Patientinnen zusätzlich dazu eine adjuvante systemische Therapie (Chemotherapie, endokrine Therapie und zielgerichtete Gewebetherapie), die signifikant die Rezidivrate senkt und das Gesamtüberleben erhöht [8,9]. Tabelle 1 zeigt die typischen Therapieoptionen in Abhängigkeit vom Stadium.

Tabelle 1: Typische Therapieoptionen beim Mammakarzinom in Abhängigkeit des Stadiums. (Eigene Darstellung nach Maughan *et al.* 2010. [8])

Table 2. Typical Treatment Options for Breast Cancer by Stage

Cancer stage and type	*Primary treatment*	*Node evaluation*	*Adjuvant therapy: Hormone receptor negative*	*Adjuvant therapy: Hormone receptor positive*	*Adjuvant therapy: ERBB2 overexpression*
Stage 0: in situ					
Lobular carcinoma in situ	No treatment or consider prophylaxis with tamoxifen[6]	—	—	—	—
Ductal carcinoma in situ	Breast-conserving surgery (consider mastectomy if extensive or multifocal) and radiation therapy	—	—	—	—
Stages I and II: early-stage invasive	Breast-conserving surgery[7] and radiation therapy[8]	SLN biopsy[9-11] or ALN dissection*	Chemotherapy†	Chemotherapy and endocrine therapy[12]	Chemotherapy and trastuzumab (Herceptin)[13,14]
Stage III: locally advanced					
Noninflammatory	Induction chemotherapy,[15] followed by breast-conserving surgery‡[16-19] and radiation therapy	ALN dissection or SLN biopsy[20]	Induction chemotherapy[15,21]	Induction chemotherapy and post-operative endocrine therapy	Induction chemotherapy and postoperative trastuzumab
Inflammatory	Induction chemotherapy, followed by mastectomy and radiation therapy	ALN dissection			
Stage IV: metastatic					
Initial or recurrent	Address patient's treatment goals; radiation therapy or bisphosphonates for bone pain	—	Chemotherapy	Endocrine therapy with or without chemotherapy	Trastuzumab with or without chemotherapy
Recurrent					
Local after breast-conserving surgery	Mastectomy	ALN dissection§	Chemotherapy‖	Chemotherapy and endocrine therapy¶	Chemotherapy and trastuzumab
Local after mastectomy	Wide excision	ALN dissection**			
Local inoperable	Induction chemotherapy	ALN dissection			

ALN = axillary lymph node; SLN = sentinel lymph node.

**—SLN biopsy if clinically negative nodes; otherwise, ALN dissection is recommended.*

†—Except lowest risk (i.e., tumor ≤ 1 cm, node negative).

‡—Mastectomy may be considered if tumor does not sufficiently respond to induction chemotherapy.

§—If nodes are clinically negative and SLN biopsy is done initially, SLN biopsy can be repeated; if nodes are clinically positive, ALN dissection is needed.

‖—Local recurrence is often associated with distant metastases; therefore, prophylactic chemotherapy theoretically may be of benefit and is currently being studied.[22]

¶—Benefit of adjuvant therapy is uncertain and currently being studied; until results are available, chemotherapy is generally recommended.

***—May not need to explore axilla if ALN dissection is done initially and there are clinically negative nodes with recurrence.*

Information from references 6 through 22.

Die unterschiedlichen biologischen Subtypen des Mammakarzinoms besitzen unterschiedliche Metastasierungspotentiale. Die Subtypen werden nach der Expression von Steroidrezeptoren (Östrogen- (*estrogen receptor*, ER) und Progesteron- (*progesterone receptor*, PR) Rezeptor) und dem humanen epidermalen Wachstumsfaktorrezeptor-2

(*human epidermal growth factor receptor* 2, HER2), als auch dem Proliferationsindex Ki-67 wie folgt (Tabelle 2) klassifiziert [10]:

Tabelle 2: Molekulare Subtypen des Mammakarzinoms. (Eigene Darstellung nach Guiu *et al.* 2012. [10])

Subtypen	IHC Klassifikation (St Gallen)
Luminal A	ER und/oder PR positiv HER2 negativ Ki-67 <14%
Luminal B	Luminal B (HER2 negativ) • ER und/oder PR positiv • HER2 negativ • Ki-67 ≥ 14% Luminal B (HER2 positiv) • ER und/oder PR positiv • Jedes Ki-67 • HER2 positiv
HER2 positiv	HER2 positiv ER und PR negativ
Basaler-Zell-Typ	Triple negative ER und PR negativ HER2 negativ

Die Definition dieser Subtypen wurde 2011 in die internationale St. Gallen Konsensus Konferenz aufgenommen [10,11]. Jeder Subtyp hat ein anderes Metastasierungspotential und unterschiedliche Ansprechraten hinsichtlich der systemischen und lokalen Therapie [11]. Das Basalkarzinom ist im Vergleich zu den Luminalkarzinomen aggressiver und prognostisch ungünstiger wodurch es die niedrigsten Überlebensraten aufweist [12]. Zu der modernen, multimodalen Therapie gehören die Operation, die Radiotherapie und die medikamentöse Therapie. Die 5-Jahres Überlebensrate beträgt durch die verbesserte Früherkennung und die personalisierte Therapie in Deutschland 87% [9]. Brustkrebs kann als eine systemische Erkrankung betrachtet werden, da eine frühe Disseminierung von Tumorzellen bereits von kleinen malignen Tumoren ausgehen kann, lange bevor der Tumor diagnostiziert wird. Folglich kann der Primärtumor zum Zeitpunkt der Diagnose bereits weiter entfernte Organe mit Tausenden von Krebszellen besiedelt haben [3]. Jedoch können nur bei schätzungsweise 5% der Krebspatienten zum Zeitpunkt der Erstdiagnose klinisch detektierbare Metastasen nachgewiesen werden. Weitere 30-50% der Patienten, bei denen keine Metastasen zum Zeitpunkt der Erstdiagnose mit den klassischen, bildgebenden Verfahren nachgewiesen werden konnten, entwickeln im Verlauf ihrer Krebserkrankung schließlich doch Metastasen [13–15].

Die Ausbreitung des Tumors erfolgt durch die Ablösung aggressiver, maligner Zellen vom Primärtumor oder durch bereits vorhandene sogenannte Mikrometastasen und deren Zirkulation im Blut, die sich dann an weiter entfernten Organen absetzen können [16]. Letzen Endes sind es diese Mikrometastasen, die durch ihr Wachstum die Funktionsfähigkeit lebenswichtiger Organe beeinträchtigen und zu einem Rezidiv mit einem verkürzten Überleben der Patienten führen. Sensitive Techniken sind notwendig, um die minimale residuale Tumorlast frühzeitig zu detektieren, um eine Metastasierung zu verhindern und die Prognose der Krebspatienten zu verbessern [13,16–18].

1.2 Die Metastasierungskaskade

1.2.1 Zirkulierende Tumorzellen

Die Metastasierung ist ein komplexer, mehrstufiger Prozess, bei dem Krebszellen zu bestimmten Zeitpunkten unterschiedliche Eigenschaften erwerben müssen. Zellen im peripheren Blut, die sich vom Primärtumor oder seinen Metastasen gelöst haben, der Immunantwort entgehen und im Blutstrom zirkulieren können, werden als sogenannte zirkulierende Tumorzellen bezeichnet (Abb. 1) [14,16,17]. Sie stellen ein Zwischenstadium im Metastasierungsprozess dar und müssen Schlüsseleigenschaften erwerben um letztendlich metastatische Läsionen zu generieren [1]. Bereits 1869 postulierte T. Ashworth die Hypothese das im Blut zirkulierende Tumorzellen mit der Entstehung von Metastasen im Zusammenhang stehen [19]. Die Hypothese, dass sich Zellen vom Primärtumor ablösen und über Blutgefäße und das lymphatische System in weiter entfernte Organe bewegen können, konnte bereits in vielen Studien bestätigt werden [18,20,21]. Eine der wichtigsten Entdeckungen in den vergangenen 15 Jahren hinsichtlich des Metastasierungsprozesses war die Erkenntnis, dass zirkulierende Tumorzellen bereits im sogenannten *in-situ* Stadium disseminieren können, lange bevor der Primärtumor mittels bildgebender Verfahren detektiert werden kann [22]. Bislang liegen noch keinen konkreten Hinweise vor, dass zirkulierende Tumorzellen, die zu einem späteren Zeitpunkt der Erkrankung detektiert werden aggressiver und vermehrt in der Lage sind neue Metastasen zu bilden, im Vergleich zu jenen Zellen, die zu einem frühen Zeitpunkt der Erkrankung detektiert werden [22].

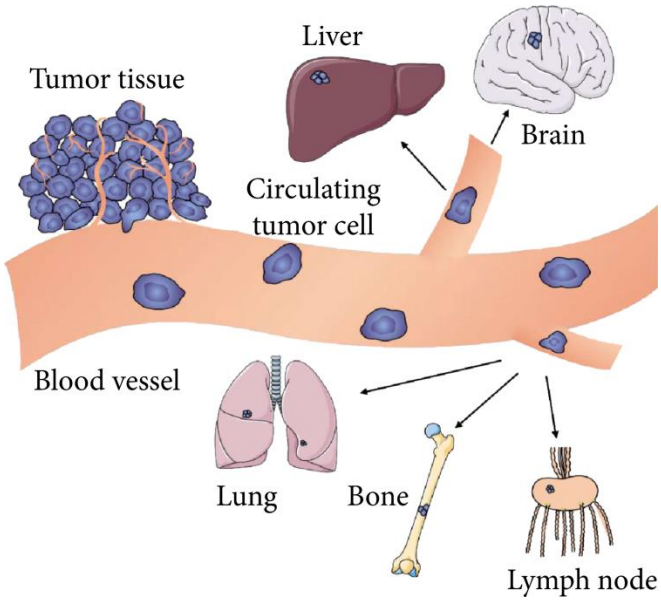

Abb.-Nr. 1: Schematische Darstellung des Metastasierungsprozesses. Tumorzellen können sich vom Primärtumor oder bereits bestehenden Metastasen ablösen, in die Blutzirkulation gelangen und an weiter entfernten Organen neue Läsionen bilden. Tumorzellen können durch die direkte Intravasation tumorassoziierter Blutgefäße in den Blutkreislauf gelangen oder indirekt durch das Lymphsystem. (Eigene Darstellung nach Yang *et al.* 2021. [23])

Durch die Entwicklung sensitiver Methoden konnte eine detaillierte Analyse dieser im Blut zirkulierender Tumorzellen erfolgen. Es wird angenommen, dass aus dem Tumorgewebe 1 x 10^6 zirkulierende Tumorzellen/g Gewebe pro Tag in den Kreislauf gelangen können [24–28]. Die meisten dieser Zellen sterben in der Zirkulation binnen kürzester Zeit, bedingt durch die Kombination aus mehreren Faktoren, wie oxidativer und physikalischer Stress, Anoikis, und dem Fehlen von Wachstumsfaktoren, als auch Zytokinen. Diejenigen Zellen, die im Blutkreis überleben konnten dringen entweder aktiv in das umliegende Gewebe ein oder bleiben in einem Kapillarbett stecken, wo sie anfangen können zu proliferieren und einen neuen Tumor ausbilden [1]. Analog zu der heterogenen Tumorbiologie weisen auch die zirkulierenden Tumorzellen ein sehr heterogenes Metastasierungspotential auf. Versuchsmodelle konnten zeigen, dass schätzungsweise nur 0,02% dieser Zellen in der Lage sind, im sekundären Gewebe neue Läsionen zu bilden [1,24–27,29]. Nachdem die Zellen ihr Ziel erreicht haben fangen sie entweder an zu proliferieren oder verbleiben in einem ruhenden G0/G1 Stadium, wobei sie aber ihre Fähigkeit zur Proliferation nicht verlieren. Bislang ist noch nicht bekannt welches Signal dafür verantwortlich ist, dass die Zellen manchmal erst nach Jahrzehnten erneut anfangen zu proliferieren und neue Läsionen bilden, die meist tödlich enden [2,22]. Die so genannten „ruhenden“ Zellen bleiben klinisch gesehen, bis zu dem Zeitpunkt an dem sie anfangen zu proliferieren, nicht detektierbar. Insbesondere bei Brust- und Prostatakarzinompatienten können diese ruhenden Zellen zu späten Rezidiven führen, die erst Jahre nach der Erstdiagnose und einer

abgeschlossenen Therapie auftreten können [1]. Neue Studien konnten zeigen, dass sich jede Läsion eines Patienten unabhängig von den anderen mit unterschiedlichen *de-novo* Mutationen entwickeln kann. Die Tumorresektion, als auch die einzelnen Biopsien jeder Läsion sind nicht in der Lage das gesamte heterogene Mutationsprofil der gesamten Zellpopulation darzustellen. Mit Hilfe der *Liquid Biopsy* und der darin enthaltenen zirkulierenden Tumorzellen hingegen kann die gesamte Population jeder einzelnen Läsion widergespiegelt und zu jedem Zeitpunkt der Erkrankung auf neue Eigenschaften hin untersucht werden. Zirkulierende Tumorzellen stellen somit eine dynamische Zellpopulation dar. Neue Zellen mit unterschiedlichen Eigenschaften können kontinuierlich aus multiplen Quellen in die Blutzirkulation gelangen. Veränderungen in den Eigenschaften der zirkulierenden Tumorzellen, die durch bestimmte Therapien induziert werden, können ebenfalls in Echtzeit erkannt werden [1,22,27]. Dies ermöglicht eine schnelle und unkomplizierte Anpassung der Therapie auf die fortschreitende Erkrankung. Verbesserte Technologien zum Nachweis zirkulierender Tumorzellen konnten neue Erkenntnisse hinsichtlich der metastatischen Ausbreitung der Krebserkrankung liefern, wodurch neue potentielle *targets* identifiziert werden konnten [1].

Die quantitative Bestimmung der zirkulierenden Tumorzellen bei Patienten mit soliden Tumoren kann wie eine *Liquid Biopsy* zur wiederholten, nicht invasiven Nachuntersuchung verwendet werden. Bei dem Begriff *Liquid Biopsy* handelt es sich um die Entnahme biologischer Flüssigkeiten (am häufigsten Blut- und Speichelproben, Urin, Pleura- und Cerebrospinalflüssigkeit), die unter anderem auch verschiedene Bestandteile von Tumorzellen enthalten kann, die in den Blutkreislauf freigesetzt wurden. Dieses Material kann verschiedene Elemente umfassen, wie z.B. extrazelluläre Vesikel, zirkulierende Tumor DNA (*circulating tumor DNA,* ctDNA) und zirkulierende Tumorzellen (*circulating tumor cells,* CTCs) [30]. Zirkulierende Tumorzellen haben im Vergleich zu den anderen Bestandteilen der *Liquid Biopsy* den Vorteil, dass sie von lebenden, zur Proliferation fähigen Regionen abstammen. Zudem enthalten sie das volle Repertoire an Information, das für die Therapieoptimierung notwendig ist: RNA, DNA, Proteine, etc. Aus diesem Grund sind nur zirkulierende Tumorzellen in der Lage neue Metastasen an weiter entfernten Regionen zu bilden, wodurch sie eine zentrale Rolle beim Fortschreiten der Erkrankung spielen [30].

Große klinischen Studien zu Brust- und Prostatakrebs konnten zeigen, dass zirkulierende Tumorzellen als ein Marker für das Ansprechen auf eine aktuelle Therapie verwendet

werden können und in der frühen und metastatischen Erkrankung mit ungünstigen, klinischen Ergebnissen korrelieren (wie das Progressionsfreie- und Gesamtüberleben) [4,31–35]. Die hohe Heterogenität der Brustkrebszellen im Primärtumor, als auch in den disseminierten Tumorzellen ist von prognostischer Bedeutung und kann für die Entwicklung neuer therapeutischer Strategien nützlich sein [36]. In den letzten Jahren konnten kritische Signalwege, die bei der Entwicklung von Tumoren involviert sind identifiziert werden, wodurch neue *targets* entdeckt und zielgerichtete Therapien entwickelt wurden [37]. Die longitudinale Überwachung der zirkulierenden Tumorzellen während einer Therapie könnte dabei behilflich sein auftretende Medikamentenresistenz frühzeitig zu erkennen und einen Therapiewechsel schnell einzuleiten [38,39]. Dies ermöglicht die Identifizierung von Patienten, die von einer individuellen, zielgerichteten Therapie profitieren [40,41]. Die personalisierte Therapie sollte eine hohe Spezifität für Moleküle haben, die eine entscheidende Rolle im Zellwachstum, Überleben, Invasion, Metastasierung, Progression und Angiogenese spielen [37]. Die weitere Charakterisierung dieser Zellen könnte für ein besseres Verständnis der Tumorbiologie und des Metastasierungsprozesses herangezogen werden [40,41]. Der Fokus einer zielgerichteten Krebstherapie liegt auf der individuell abgestimmten Therapie, die weniger toxisch aber wirksamer für den einzelnen Patienten ist [38].

1.2.2 Nachweis zirkulierender Tumorzellen mit der maintrac® Methode

Aufgrund ihrer niedrigen Konzentration im peripheren Blut stellt der Nachweis der zirkulierenden Tumorzellen eine enorme technische Herausforderung dar (eine Zelle unter 10^3 - 10^7 normaler Blutzellen) und bedarf einer hohen Sensitivität und Spezifität für die Nachweismethode [42][18]. Es wurden bereits viele Detektionsverfahren für den Nachweis und der weiteren Charakterisierung zirkulierender Tumorzellen entwickelt, die in zwei Hauptkategorien aufgeteilt werden können: immunologisch- und molekularbiologisch basierte Detektionsverfahren. Aufgrund ihrer Seltenheit werden bei den meisten Verfahren vor der eigentlichen Detektion der Zellen ein oder mehrere Anreicherungsschritte vorgenommen. Nachteil dabei ist zum einen der enorme Zellverlust während jedes Anreicherungsschrittes und zum anderen die offenbar massive Zerstörung der Zellen, wodurch eine Unterscheidung in lebende und tote Zellen nicht mehr möglich ist. Die genaue Quantifizierung und weitere Charakterisierung lebender zirkulierender

Tumorzellen ist aber essentiell, damit sie als klinische Biomarker in der klinischen Routine verwendet werden können [20,43].

Die Bezeichnung der zirkulierenden Tumorzellen als CTCs (*circulating tumor cells*) beinhaltet auch die zirkulierenden hematopoetischen Tumorzellen. Die Untersuchungen in dieser Studie beziehen sich jedoch ausschließlich auf die epithelialen Tumore, weshalb die Terminologie CETCs (*circulating epithelial tumor cells*) im Vergleich zu CTCs plausibler erscheint. Mit der maintrac® Methode werden die tumorverdächtigen epithelialen Zellen im Blut auf der Grundlage der Expression des epithelialen Zelladhäsionsmoleküls (*epithelial cell adhesion molecule*, EpCAM) der Tumorzellen nachgewiesen. Die Expression von EpCAM ist eng gekoppelt mit benignen oder malignen Tumoren, die von epithelialen Geweben abgeleitet sind. Sie korreliert mit der Tumorentität, dem Stadium der Krankheit und der Antitumorimmunität [44]. Zudem wurde die EpCAM-Expression mit einer verstärkten Proliferation in Verbindung gebracht [45]. Beim primären Mammakarzinom ist die EpCAM-Expression mit einem schlechten progressionsfreien- und Gesamtüberleben assoziiert [45]. Die maintrac® Methode ermöglicht den sensitiven Nachweis der CETCs ohne Anreicherungsschritte und die quantitative Bestimmung lebender Zellen aus dem peripheren Blut von Krebspatienten (Abb. 2). Sie ist schnell, zuverlässig und reproduzierbar [29]. Der Nachweis erfolgt mit einem spezifischen Antikörper gegen EpCAM der mit einem Fluorochrom markiert ist und einem Fluoreszenz Scanning Mikroskop (Olympus). Die anschließende Analyse ermöglicht eine morphologische Beurteilung jeder einzelnen Zelle. Diese Methode ermöglicht es die CETCs relativ verlustfrei in den unterschiedlichen Stadien der Krankheit zu detektieren und somit das Ansprechen auf eine Therapie abzulesen [4,46]. Für die Unterscheidung zwischen lebenden und toten Zellen wird eine Kernfärbung mit Propidiumiodid (*propidium iodide*, PI) durchgeführt bei der nur die toten Zellen aufgrund der Membranpermeabilität eine intranukleäre Färbung mit einem DNA interkalierenden Farbstoff aufweisen. Mit dieser Methode wird eine begleitende Überwachung der CETCs während des Krankheitsverlaufes ermöglicht und für den Patienten eine optimierte, rechtzeitige, angepasste und personalisierte Therapie bereitgestellt [42].

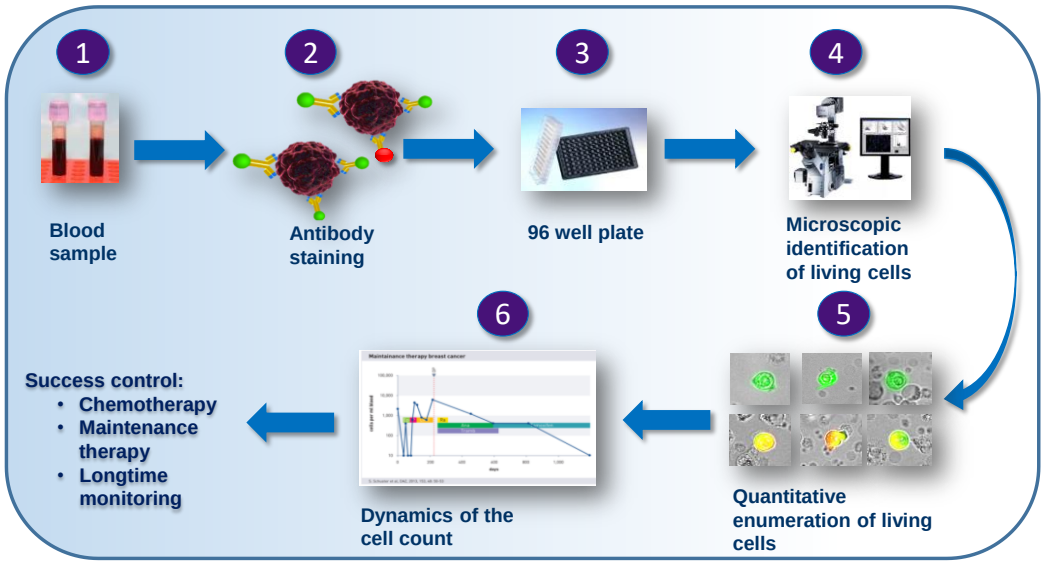

Abb.-Nr. 2: Schematische Darstellung der maintrac® Methodik. Für den Nachweis zirkulierender Tumorzellen wird als Ausgangsmaterial EDTA-Blut (für die anti-Koagulation) benötigt. In einem ersten Schritt werden die Erythrozyten lysiert, die Proben zentrifugiert und anschließend mit einem spezifischen anti-EpCAM Antikörper markiert. Die Proben werden anschließend in 96 *well* Platten überführt und an einem Fluoreszenz Scanning Mikroskop gemessen. Mit Hilfe des automatisierten Analyseprogramms können anschließend die Tumorzellen identifiziert, quantifiziert und zwischen lebenden und toten Zellen unterschieden werden. Durch wiederholte Zellzahlbestimmungen während des Krankheitsverlaufs kann mit dieser Methode jede Behandlungsart kontrolliert und entstehende Resistenzen frühzeitig erkannt werden.

1.2.3 Zirkulierende Krebsstammzellen

Die hohe intratumorale Heterogenität und die molekulare Komplexizität vieler Tumore sind die Ursache für eine primäre oder erworbene Resistenz gegen zytotoxische und biologische Wirkstoffe. Während ein Großteil der Krebszellen durch die verabreichte Medikation zerstört wird, können andere Tumorzellen überleben und zum Wiederauftreten und dem Progress der Krankheit beitragen [47]. Immer mehr Daten, die aus klinischen und experimentellen Studien hervorgehen, deuten darauf hin, dass die Ursachen für die Resistenzen und Rezidive genetische Mutationen, die Interaktion der Krebszellen mit der Mikroumgebung und das Vorhandensein der sogenannten Krebsstammzellen (*cancer stem cells*, CSCs) sind [47,48].

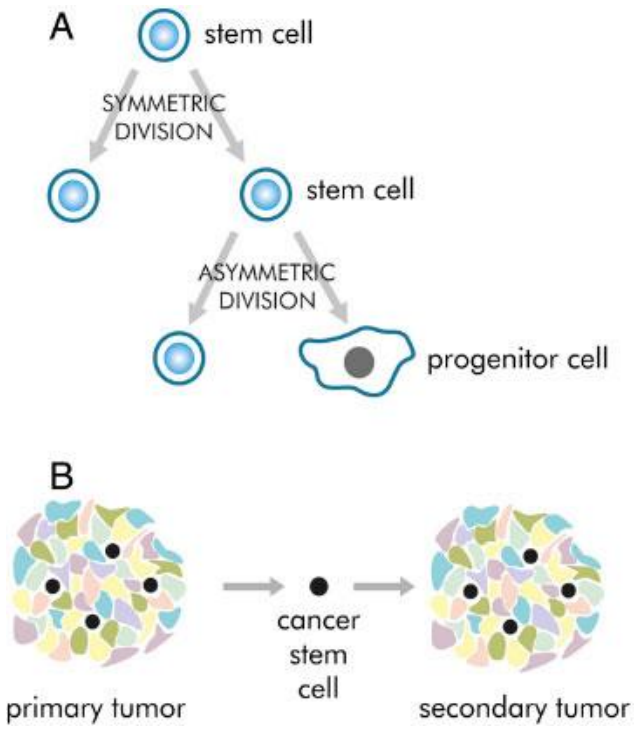

Abb.-Nr. 3: Krebsstammzellmodell. a) Krebsstammzellen besitzen eine Selbsterneuerungskapazität, wodurch die Stammzelleigenschaften während der Zellteilung beibehalten und an die Tochterzellen weitergegeben werden. Bei einer symmetrischen Zellteilung entstehen zwei Tochterzellen, die ihre Stammzelleigenschaften behalten. Bei der asymmetrischen Zellteilung entstehen eine Tochterzelle mit Stammzelleigenschaften und eine Tochterzelle, die ihre Stammzelleigenschaft verliert. b) Die heterogene Zellpopulation wird an den Sekundärtumor weitergegeben. (Eigene Darstellung nach Fulawka *et al.* 2014. [49])

Krebsstammzellen sind in der Lage sich selbst zu erneuern, einen Tumor nach einer abgeschlossenen Behandlung neu zu besiedeln und ein metastatisches Wachstum zu initiieren (Abb. 3). Des Weiteren weisen sie eine Radio- und Chemoresistenz auf, was möglicherweise die Erklärung dafür ist, dass die derzeitigen Behandlungsmethoden oft nicht ausreichen, um die Patienten zu heilen [48]. Die Krebsstammzellhypothese wurde 1800 erstmals von Rudolf Virchow in *Cellular Pathologie* postuliert, jedoch konnten erst 1994 erstmals Krebsstammzellen von Patienten mit einer akuten myeloiden Leukämie (*acute myeloid leukemia*, AML) von Dick und seinen Kollegen identifiziert und isoliert werden. Unter Verwendung immundefizienter Mausmodelle (*nonobese diabetic/severe combied immunodeficient mice*, NOD/SCID) konnten sie demonstrieren, dass die implantierten Zellen (CD34+/CD38-) sich primär im Knochenmark der Mäuse absiedelten, dort proliferierten und hämatopoetische Malignitäten initiierten konnten [50,51]. Von da an wurden Krebsstammzellen in den letzten Jahren in einer Vielzahl anderer, solider Tumore nachgewiesen [47]. CSCs wurden mit Hilfe unterschiedlicher Methoden, wie der Expression CSC-spezifischer Zelloberflächenmarker oder intrazellulärer Moleküle, aber auch der Aldehyd-Dehydrogenase 1 (ALDH1)-Aktivität identifiziert und isoliert [52,53]. Zu den gängigsten Markern des Mammakarzinoms gehören CD133, CD24 und CD44.

Jedoch reichen diese Markerkombinationen für deren Nachweis alleine nicht aus und müssen durch funktionelle Methoden, wie beispielsweise deren Fähigkeit in Suspension in einer 3D Konformität heranzuwachsen (Tumorsphären), belegt werden [47]. Solide Tumore sind während ihres Wachstums von einer Vielzahl nicht-maligner Zellen und einer extrazellulären Matrix umgeben, die zusammen die Mikroumgebung des Tumors bilden. Die Mikroumgebung des Tumors verschafft den Tumorzellen suboptimale Wachstumsbedingungen, wie Hypoxie und Nährstoffmangel. Zudem sind die Tumorzellen den Signalen der umgebenden Zellen ausgesetzt und werden durch direkte Zell-Zellkontakte beeinflusst. Konventionellen 2D Kultursystemen fehlen all diese Umgebungsfaktoren, weshalb die originalgetreue Nachbildung dieser Mikroumgebung *in-vitro* eine große Herausforderung darstellt. Biologische Merkmale der Tumorzellen können durch die erforderlichen zellulären Anpassungen, um *in-vitro* überleben zu können, verloren gehen. Aus diesem Grund sind 3D Kultursysteme notwendig, um die *in-vivo* Wachstumsbedingungen so gut wie möglich zu simulieren und die Aspekte der Tumorbiologie zu verstehen. Mit Hilfe der Spheroid Kultursysteme werden die seltenen CSCs identifiziert und angereichert, wodurch deren Eigenschaften *in-vitro* untersucht werden können [54].

Einige experimentelle Ergebnisse haben gezeigt, dass eine aggressivere Subpopulation der zirkulierenden Tumorzellen ebenfalls Merkmale von Krebsstammzellen aufweist, so dass sie als zirkulierende Krebsstammzellen (*circulating cancer stem cells*, cCSC) bezeichnet werden können. Man geht davon aus, dass insbesondere die cCSCs für die metastatische Ausbreitung aus dem Primärtumor bzw. den Metastasen verantwortlich sind [55,56]. Deren Identifizierung und weitere Charakterisierung stellt eine große Herausforderung dar. Doch hat das Vorhandensein der cCSCs im Blutkreislauf der Patienten klinische Auswirkungen auf die Behandlungsstrategie. Die nachgewiesene Chemo- und Radioresistenz macht sie somit zum wichtigsten *target* für jede Krebstherapie.

Nach wie vor gehört die Xenotransplantation von Krebsstammzellen in immundefiziente Mäuse, trotz ihrer ethischen und finanziellen Bedenken als Goldstandard in der Krebsforschung. Seit dem späten 19. Jahrhundert wurden Hühnereier als eines der ersten Tiermodelle für die Anzucht von Tumortransplantaten verwendet. Die Weiterentwicklung der Methode führte zur Identifizierung der Chorioallantoismembran (*chorio-allantoic-membrane*, CAM) als leicht zugängliche, gut vaskularisierte anatomische Struktur, die sich für vielseitige Experimente eignet. Im Gegensatz zu den Mausmodellen hat sich das CAM

Modell als ein kostengünstiges, reproduzierbares und zuverlässiges Modell für die *in-vivo* Tumorforschung etabliert. Es ermöglicht die Untersuchung der Tumorbiologie [57]. Das CAM-Modell erfordert keine künstliche Induktion einer Immundefizienz, da dem Hühnerei bis zum 14. Tag der Entwicklung ein spezifisches Immunsystem fehlt. Die Xenotransplantate können auf diese Weise auf der nicht-innervierten CAM ohne Schmerzen oder Beeinträchtigung des Embryos wachsen [58]. Im Gegensatz dazu kann es in Mausmodellen durch das Vorhandensein eines Immunsystems zur Abstoßung von Xenotransplantaten kommen, weshalb eine Zucht von Mäusen mit einer schweren Immundefizienz eine Grundvoraussetzung für die Xentransplantation ist. Zusätzlich dazu führen die Implantation des Xenotransplantats, die Überwachung des Tumorwachstums sowie alle therapeutischen Eingriffe zu Stress, Schmerzen und körperlichen Beeinträchtigungen des Wirtstieres. Die CAM ist eine stark vaskularisierte extraembryonale Membran, die dadurch optimale Nährstoffbedingungen für das Wachstum menschlicher Tumorzellen bietet. Innerhalb weniger Tage nach der topischen Applikation von Tumorzellen auf die CAM kann es zu einer Tumorbildung kommen. Bei aggressiven Tumoren können sich metastasierende Zellen über die hämatogene Ausbreitung in Organen des Embryos ansiedeln. In der Humanmedizin wird dieses Modell häufig in der präklinischen Tumorforschung verwendet, um das Wachstum vieler Tumorarten zu untersuchen [57]. Darüber hinaus wird dieses Modell für die Untersuchung der Wirksamkeit unterschiedlicher Krebsmedikamente [59] und der Radiotherapie verwendet [60]. Ein neuer wichtiger Fortschritt des CAM Modells besteht darin, dass Tumorproben von Patienten auf die CAM transplantiert werden können. Dadurch kann binnen kürzester Zeit ein *patient-derived* Tumor auf der CAM entstehen, an dem individualisierte Behandlungen getestet werden können [61]. Mit der *Liquid Biopsy* können Tumorzellen für die Generierung eines *patient-derived* Tumors auf der CAM zu jedem Zeitpunkt des Krankheitsverlaufs und unabhängig von dem Vorhandensein des Primärtumors bereitgestellt werden, um deren Tumorigenität und das Metastasierungspotential zu untersuchen.

1.3 Wie zirkulierende Tumorzellen der Immunantwort entkommen und in der Blutbahn überleben können

1.3.1 Die Rolle des PD-L1 (B7-H1)

Die Immunantwort ist ein komplexes Phänomen, das auf einem Gleichgewicht zwischen aktivierenden und inhibierenden Signalwegen beruht und somit die Aktivität der T-Zellen reguliert. Dieses Gleichgewicht kann bei bestimmten pathologischen Zuständen, wie beispielsweise einer Krebserkrankung, gestört sein, wodurch die Hemmung des Immunsystems das Fortschreiten des Tumors begünstigt [62].

Zirkulierende Tumorzellen müssen eine Reihe physiologischer und immunologischer Hürden überwinden, um disseminieren zu können. Sie müssen sich in einem ersten Schritt durch ihre lokale Mikroumgebung navigieren, das Endothel durchqueren und von ihrem Ursprung in die Blutzirkulation eindringen (Intravasation) [22]. Um sich dort der Immunüberwachung erfolgreich zu entziehen, nutzen zirkulierende Tumorzellen sowie die Tumorzellen im Tumorgewebe eine Vielzahl unterschiedlicher Strategien. Eine davon ist die Expression des programmierten Zelltodes-Liganden 1 (*progremmed cell death ligand* 1, PD-L1, CD247, B7-H1) auf der Zelloberfläche der Tumorzellen [63]. PD-L1 ist ein 40 kDa großes Transmembranprotein, das von aktivierten Immunzelltypen wie natürliche Killerzellen, Makrophagen, myeloide dendritische Zellen, B-Zellen und vaskuläre Endothelzellen sowie von zahlreichen Epithelzellen, einschließlich der Krebszellen, exprimiert wird. Die physiologische Rolle von PD-L1 besteht in der Bindung an den programmierten Zelltodes-Rezeptor 1 (*programmed cell death 1 receptor*, PD-1), der auf der Oberfläche aktivierter zytotoxischer T-Zellen exprimiert wird [63,64]. Physiologisch stellt die PD-1/PD-L1-Interaktion einen wichtigen Kontrollpunkt (*checkpoint*) dar, um eine übermäßige adoptive Immunantwort auf Antigene und eine Autoimmunität zu unterbinden (Abb. 4). Durch diese Bindung werden die IL-2-Produktion und die T-Zellaktivierung inhibiert. Diesen Mechanismus nutzen die Krebszellen aus, um sich erfolgreich vor dem Angriff des Immunsystems zu schützen. Der zweite Ligand für PD-1 ist PD-L2 (auch bekannt als B7-DC und CD273). Seine Rolle bei der Modulation der Immunantwort ist weniger klar und es sind nur wenige Informationen verfügbar. Die Affinität von PD-L2 an PD-1 zu binden ist zudem 2-6-fach niedriger im Vergleich zu PD-L1 [65]. Die Expression von PD-L1 wurde bei einer Vielzahl unterschiedlicher Tumorentitäten evaluiert, das Mammakarzinom mit einbezogen [64]. Es wurden mehrere monoklonale Antikörper entwickelt, die sich gegen PD-1 und PD-L1 richten, um die hemmende Immunantwort zu unterbinden und die zelluläre Zytotoxizität der T-Zellen zu fördern, wodurch die

Tumorzellen eliminiert werden sollen [66]. Viele dieser zielgerichteten Medikamente befinden sich bereits im klinischen Einsatz, um die Immunität gegen die Krebszellen wiederherzustellen. Zahlreiche neue *checkpoint*-Inhibitoren werden derzeit in klinischen Studien getestet [62].

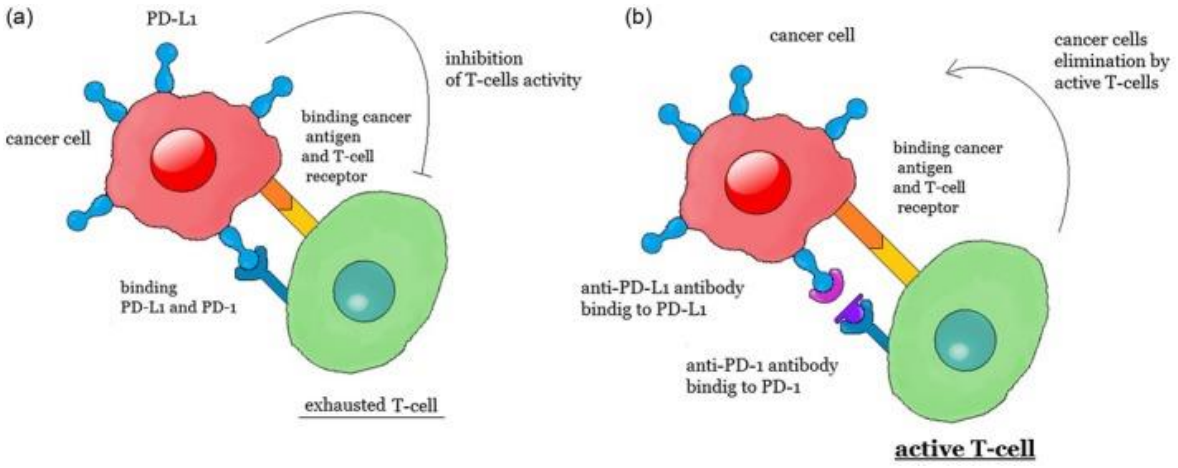

Abb.-Nr. 4: Interaktion von PD-L1 und PD-1. a) Inhibierung der T-Zell-Aktivität durch die Bindung des Liganden PD-L1 (auf den Tumorzellen) an den Rezeptor PD-1 (auf den T-Zellen). b) Wiederherstellung der T-Zell-Aktivität durch anti-PD-L1 oder anti-PD-1 Antikörper. Die aktivierten T-Zellen können nun die Krebszellen erkennen und eliminieren. (Eigene Darstellung nach Pawlowska *et al.* 2019. [67])

Die Immuntherapie hat die Krebstherapie revolutioniert und in Kombination mit den konventionellen Therapien wie Operation, Radiotherapie und Chemotherapie konnte die Überlebensrate der Patienten verbessert werden. Um eine erfolgreiche Strategie für die Immuntherapie zu entwickeln müssen diagnostische, prädiktive, prognostische und therapeutische Marker identifiziert werden. Es konnte bereits gezeigt werden, dass die PD-L1-Expression in den Tumorzellen des Primärtumors mit einem therapeutischen Ansprechen korreliert [68]. Die Informationen die durch eine einzelne Biopsie erhalten werden liefern aber nur limitierte Einblicke in die Eigenschaften eines Tumors und spiegeln nicht die Heterogenität des ganzen Tumors wieder. Die hohe intra-Tumorheterogenität bezüglich des PD-L1 Status zwischen dem Primärtumor und den Metastasen, mit einer höheren PD-L1 Expression in den Metastasen, deutet darauf hin, dass eine einzige Kernbiopsie möglicherweise nicht ausreicht, um die PD-L1 Expression zu bestimmen [62]. Zudem fehlt es den herkömmlichen klinischen Methoden, die für die Planung der Immuntherapie verwendet werden an Sensitivität und Spezifität. Aus diesem Grund ist der Primärtumor kein geeignetes Surrogat für die Bestimmung der PD-L1 Expression insbesondere bei metastasierten Patienten [69]. Die Metastasen stellen aggressive Subklone dar, die in der Lage sind vom Primärtumor zu disseminieren und der

Immunantwort in der Blutbahn zu entgehen. Des Weiteren kann die konventionelle Tumorbiopsie nicht beliebig oft wiederholt werden, um beispielsweise den PD-L1 Status im Tumorgewebe während des Krankheitsverlaufs zu überwachen. Daher könnte der Nachweis von PD-L1 auf zirkulierenden Tumorzellen als ein neuer Biomarker für eine bessere Auswahl von Patienten für die Behandlung mit PD-1/PD-L1-Antikörpern herangezogen werden [70,71].

1.3.2 Die Rolle von B7-H3

B7-H3 ist ein Typ I Transmembranprotein und analog zu B7-H1 ein wichtiges Mitglied der *immune-checkpoint* B7-Ligandenfamilie (Abb. 5). Neueste Studien haben gezeigt, dass die Hochregulation von B7 Molekülen in der Mikroumgebung des Tumors mit der Immunabwehr des Tumors assoziiert ist [66]. B7-H3 ist ein Oberflächenantigen, gegen das eine gezielte Therapie ins Auge gefasst werden kann. Im Unterschied zu B7-H1 konnte sein Rezeptor jedoch bislang noch nicht identifiziert werden. B7-H3 wird von Immunzellen, insbesondere von Antigen-präsentierenden Zellen und Makrophagen exprimiert [72,73]. Es wird angenommen, dass B7-H3 an der Hemmung von T-Zellen beteiligt ist. Andererseits wurde festgestellt, dass B7-H3 auch stimulierende immunologische Funktionen aufweist [74,75]. Das B7-H3-Protein konnte bereits in einer Vielzahl unterschiedlicher Zelllinien [74,76] und Tumorentitäten [72,77–81] nachgewiesen werden. Im Vergleich zu anderen *immune-checkpoints* beeinflusst B7-H3 nicht nur die angeborene und adaptive Immunität, sondern auch die Aggressivität der Krebszellen über verschiedene, nicht-immunologische Signalwege [66]. Neben der Immunevasion spielt B7-H3 eine Rolle bei der Krebsprogression, wie der Invasion und Migration, der Angiogenese und Genregulation [75]. Zahlreiche Studien haben gezeigt, dass die Expression von B7-H3 mit einem fortgeschrittenen Tumorstadium und einem höheren Tumorgrad in Zusammenhang steht [73]. Zudem konnten experimentelle Modelle zeigen, dass die Hochregulation von B7-H3 mit einer Resistenz hinsichtlich zytotoxischer Medikamente in Verbindung gebracht werden kann. Im Gegensatz dazu verbessert die Suppression von B7-H3 die Sensitivität von Zelllinienzellen hinsichtlich zytotoxischer Medikamente [66]. Somit konnte die B7-H3 Expression sowohl mit einer schlechteren Prognose, als auch einem schlechteren klinischen *outcome* in Verbindung gebracht werden [72]. Die Immuntherapie, bei der *immune-checkpoints*, wie CTLA-4, PD-1 oder PD-L1 blockiert werden, hat bei vielen Patienten mit unterschiedlichen Tumorentitäten einen klinischen

Nutzen gezeigt. Da B7-H3 eine ähnliche Rolle bei der Unterdrückung, bzw. der Umgehung des Immunsystems spielt und in einer Vielzahl unterschiedlicher Tumorentitäten aber selten in normalen Zellen überexprimiert wird, ist es ein interessantes *target* für neue immuntherapeutische Konzepte zur Behandlung von Krebs geworden [72,77,82,83]. Derzeit befindet sich eine Vielzahl von anti-B7-H3-Ansätzen in prä-klinischen und klinischen Studien. Aufgrund des wachsenden Interesses an der Immuntherapie kann die Analyse der B7-H3-Expression im primären Tumorgewebe und auf den zirkulierenden Tumorzellen von entscheidender Bedeutung sein. Durch die große Homologie zwischen B7-H3 und B7-H1 ist die Blockade dieser beiden Moleküle äußerst wahrscheinlich [72,74]. Es werden weitere Untersuchungen notwendig sein, um den genauen Mechanismus der beiden regulatorischen Funktionen von B7-H3 zu ergründen und neue Medikamente zu konzipieren [66].

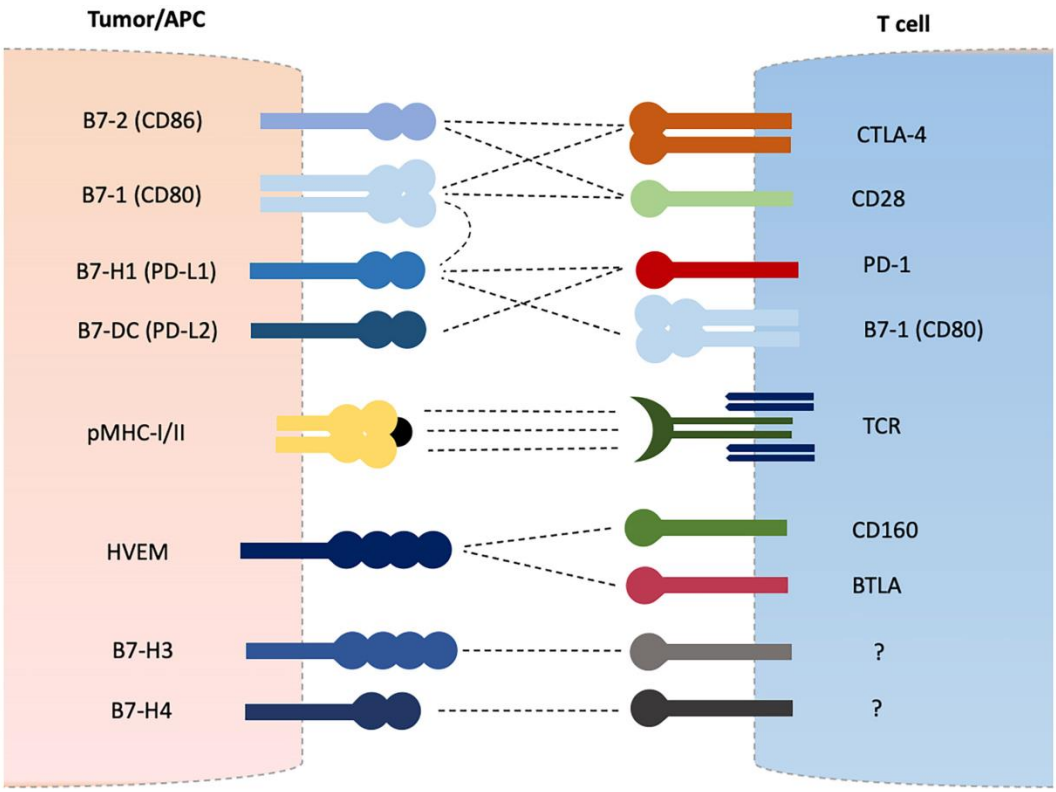

Abb.-Nr. 5: Ko-inhibitorische *checkpoint* Moleküle der B7-Familie. Eine Vielzahl an ko-inhibitorischen Molekülen der B7-Familie wurden zusammen mit ihren dazugehörigen Rezeptoren entdeckt und werden auf ihre Funktion hinsichtlich der Immunevasion untersucht. Bislang ist der Rezeptor für B7-H3 und B7-H4 nicht bekannt. (Eigene Darstellung nach Khan *et al.* 2021. [84])

1.3.3 Die Rolle der Thrombozyten

Eine Voraussetzung für die Entstehung von Fernmetastasen ist das Überleben von Tumorzellen in der Blutbahn. Dafür nutzen sie, wie bereits beschrieben, unter anderem

Oberflächenantigene, wodurch sie in der Lage sind sich vor dem Immunsystem zu tarnen und es gleichzeitig zu inhibieren. Damit die zirkulierenden Tumorzellen im Blutkreislauf überleben können werden sie aber auch durch die Hilfe anderer Zellen geschützt [85,86]. Thrombozyten tragen auf vielfältige Weise zur Persistenz und der hämatogenen Ausbreitung zirkulierender Tumorzellen bei (Abb. 6). Sie haben einen pro-metastatischen Effekt, der sowohl physikalischer, als auch molekularer Natur ist. Sobald die zirkulierenden Tumorzellen in die Blutbahn gelangen heften sich die Thrombozyten sehr schnell an ihre Oberfläche an und schützen sie somit vor starken Scherkräften, aber auch vor der immunologischen Eliminierung beispielsweise durch die natürlichen Killerzellen (NK) [22,85].

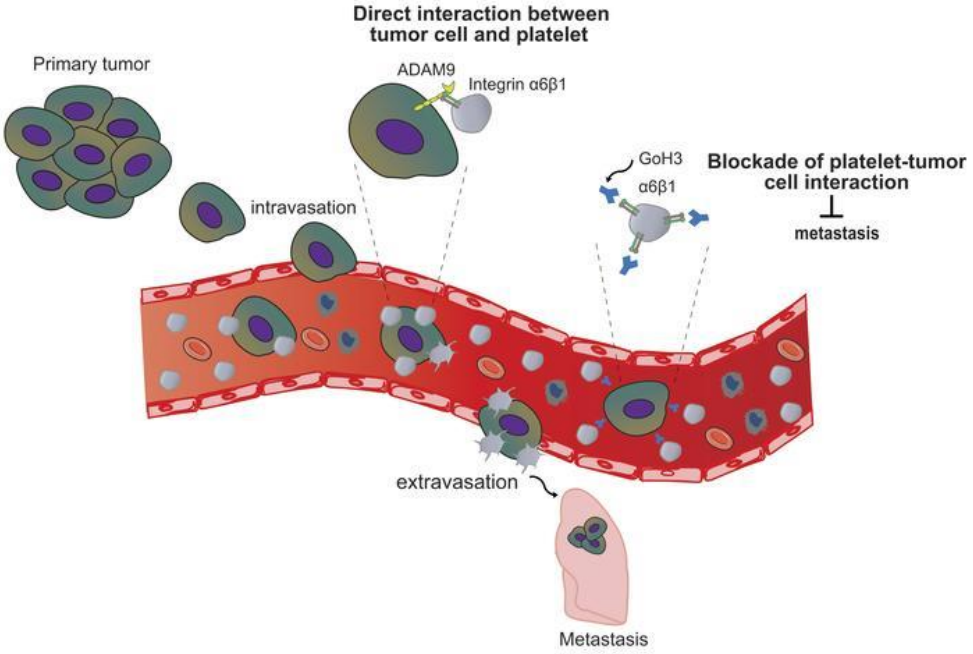

Abb.-Nr. 6: Tumorzell-Thrombozyten-Interaktion. Nach dem Eintritt der Tumorzellen in den Blutkreislauf kommt es zu einer stabilen Interaktion zwischen den zirkulierenden Tumorzellen und den Thrombozyten, die als Tumorzell-induzierte Thrombozytenaggregation bezeichnet wird. Dadurch schützen die Thrombozyten die Tumorzellen vor Scherstress und vor dem Angriff des Immunsystems. Zudem begünstigt die Thrombozyten-Tumorzell-Interaktion die Extravasation der Tumorzellen in das umliegende Gewebe. (Eigene Darstellung nach Mammadova-Bach *et al.* 2016. [86])

Neben ihrer lebenswichtigen Rolle bei der Aufrechterhaltung der Blutbarriere und der Hämostase exprimieren die Thrombozyten Zelloberflächenmoleküle und sezernieren eine Fülle von Proteinen, die Entzündungen und den Progress von Krebserkrankungen fördern. Die Thrombozyten begünstigen zudem die Ausbreitung von Metastasen, indem sie die Tumorangiogenese durch die Interaktion mit dem Endothel und ihren Angiogenesemodulatoren kontrollieren. Die Tumorzellen wiederum induzieren eine Thrombozytenaktivierung die ebenfalls die Angiogenese fördert, indem Proteasen und

Phospholipide freigesetzt werden [87]. Sie bilden sogenannte Tumorzell-induzierte Thrombozytenaggregate. Diese Aggregate haben eine höhere Wahrscheinlichkeit in dünnen Blutgefäßen hängen zu bleiben, wodurch die Tumorzellen mehr Zeit für das Durchdringen (Extravasation) in das benachbarte Gewebe haben. Die Interaktion der Tumorzellen mit den Thrombozyten erfolgt über Integrine, wodurch das *crosslinking* und die Koagulation gefördert werden. Es ist bekannt, dass Thrombozyten gutartige, abgelöste Epithelzellen binden, die in den Blutkreislauf gelangen (z. B. bei Wunden) [85]. Während normale Epithelzellen nicht in der Lage sind sich zu vermehren und nach Tagen bzw. Wochen durch das Immunsystem eliminiert werden [25], können zirkulierende Tumorzellen für einen deutlich längeren Zeitraum im Blutkreislauf bestehen [88], so lange sie mit Blutplättchen beschichtet sind [89]. Die Oberflächenbeschichtung der Tumorzellen mit Thrombozyten dient dabei als Schutzschild gegen Immunangriffe [90]. Dies ist möglicherweise aber auch der Grund dafür, dass Antikörper nicht an die Oberflächenantigene der Tumorzellen gelangen und folglich die zirkulierenden Tumorzellen in frisch entnommenen Blutproben nicht nachgewiesen werden können. Zudem könnte diese Beobachtung auch eine Erklärung dafür sein, weshalb eine zielgerichtete anti-EpCAM Antikörpertherapie in Phase III Studien bei der Behandlung von Epitheltumoren begrenzt wirksam war [89,91,92].

1.4 Einfluss unterschiedlicher adjuvanter Therapien auf die zirkulierenden epithelialen Tumorzellen und zirkulierenden Krebsstammzellen

1.4.1 Einfluss der adjuvanten Radiotherapie

Die Behandlung des Mammakarzinoms hat sich in den letzten Jahren durch neue Operationstechniken, neue systemische Therapieoptionen und durch Fortschritte im Bereich der Radiotherapie verbessert [93]. Die Radiotherapie in Kombination mit einer brusterhaltenden Operation/Mastektomie, einer Chemotherapie oder einer anderen zielgerichteten Therapie ist ein wichtiger Bestandteil der Krebstherapie, da sie die Lokalrezidive und die Mortalitätsrate bei Mammakarzinompatientinnen reduziert [94]. Große randomisierte kontrollierte Studien konnten zeigen, dass Patientinnen in einem frühen Stadium unabhängig davon, ob sie mit einer brusterhaltenden Therapie oder Mastektomie behandelt wurden, ein gleiches Gesamtüberleben aufwiesen. Folglich ist die

brusterhaltende Therapie die bevorzugte Behandlungsoption für diese Patientinnen geworden. Zu der brusterhaltenden Therapie gehören die lokale Entfernung des Tumors (mit freien Rändern) und eine anschließende adjuvante Radiotherapie. Studien konnten zeigen, dass in 41% der Patientinnen residuale Tumorzellen in mehr als 2 cm Entfernung vom ursprünglichen Tumor gefunden werden können. Das Hauptziel der Radiotherapie besteht darin, Tumorrestzellen die nach einer Operation zurückgeblieben sind zu eliminieren, den lokalen Tumor unter Kontrolle zu halten und die Schädigung des gesunden Gewebes zu minimieren [95,96]. Die Radiotherapie hat eine Reihe von Antitumoreffekten, die die Proliferation, die Morphologie und den Zelltod beeinflusst und folglich in einer Verkleinerung des Tumors resultiert. Auf molekularer Ebene führt die Radiotherapie zu irreversiblen DNA Brüchen, die in einer zellulären Seneszenz und Apoptose resultieren. Klinische Beobachtungen konnten zudem zeigen, dass die Radiotherapie auch stimulierende immunologische Effekte aufweist, die unter anderem zu einer Tumorremission außerhalb des Bestrahlungsfeldes bei sekundären Tumoren führt. Diese Beobachtung wurde in klinischen Studien bestätigt und wird als „abskopaler Effekt“ bezeichnet. Die größten Erfolge des abskopalen Effekts wurden bei Patientenkohorten beobachtet, die eine Kombination aus Radiotherapie und Immuntherapie erhalten haben. Hierbei verstärkt die Radiotherapie die antitumorale Wirksamkeit unterschiedlicher Immuntherapien. Präklinische Ergebnisse konnten bereits zeigen, dass die Radiotherapie zu einer Hochregulation von PD-L1 in dendritischen Zellen und auch in den Tumorzellen, im Vergleich zu nicht bestrahlten Regionen führt. Die Hochregulation von PD-L1 ermöglicht die Gabe einer Immuntherapie, wodurch das volle zytotoxische Potential der eigenen Immunität gegen den Tumor zugänglich und gleichzeitig die Wirksamkeit einer der beiden Therapieoptionen erhöht wird [97]. Die Kombination aus Radiotherapie und Immuntherapie könnte eine neue Behandlungsstrategie sein, insbesondere bei den Patienten, die auf eine Immuntherapie alleine nicht ansprechen [98].

Die Indikation für eine adjuvante Radiotherapie richtet sich primär nach klinisch-pathologischen Faktoren, wie Alter der Patientinnen, Tumorgröße, Lymphknotenstatus und Hormon- bzw. HER2 Status [94]. Seit der Einführung der Radiotherapie treten bei nur 2,5% der Frauen Lokalrezidive auf, jedoch erleiden zwischen 20% und 30% der Frauen, unabhängig von der durchgeführten Behandlung ein Fernrezidiv, welches oftmals tödlich endet [99–101]. Die Operation soll bei Patientinnen insbesondere in einem frühen Stadium der Erkrankung die nachweisbare makroskopische Erkrankung vollständig entfernen. Es können jedoch in der erhaltenen Brust oder Brustwand mikroskopische Tumorherde

verbleiben. Die derzeitigen Verfahren zur Tumoreinstufung und die hochauflösenden bildgebenden Verfahren sind nicht sensitiv genug, um Mikrometastasen oder die frühe Dissemination von Tumorzellen zu detektieren. Zudem verringert die hohe intra- und intertumorale Heterogenität, aber insbesondere Veränderungen in den Tumorzelleigenschaften, die durch die Behandlung selbst hervorgerufen werden, deren Effektivität. Des Weiteren bestehen trotz standardisierter und einheitlicher Behandlungsprotokolle interindividuelle Unterschiede in der Radiosensitivität [96]. Langzeitanalysen zirkulierender Tumorzellen könnten ein vielversprechendes Werkzeug sein, um die komplexen Prozesse die mit einem Therapieerfolg oder einem Rezidiv einhergehen besser zu verstehen und die Radiotherapie besser zu individualisieren [32,96]. Biomarker aus dem Primärtumor können dabei helfen die Prognose der Patienten besser einzuschätzen, jedoch liefert die Analyse der disseminierten Tumorzellen im Blut Echtzeitinformationen über das Tumorgeschehen zu jedem Zeitpunkt der Erkrankung [32]. Folglich kann die Analyse der zirkulierenden Tumorzellen während der Radiotherapie nützlich sein, um den Erfolg oder Misserfolg einer Radiotherapie frühzeitig zu erkennen und zeitnah über Therapieoptionen bei entstehenden Radioresistenzen zu entscheiden.

1.4.2 Einfluss der endokrinen Therapie auf den Anteil Androgen- Östrogenrezeptor positiver CETCs

Es ist schon lange bekannt, dass das Wachstum von Brustkrebs maßgeblich hormonabhängig ist. Folglich spielen der Östrogen- und der Progesteronrezeptor eine entscheidende Rolle bei der Brustkrebsentstehung, bei der Regulierung der Zellproliferation und –differenzierung. Endokrine Therapien wie Tamoxifen, Aromataseinhibitoren und anti-HER2-Therapien werden bei Patientinnen, deren Primärtumor positiv für die entsprechenden Rezeptoren ist, erfolgreich bei der Behandlung eingesetzt. Im Gegensatz dazu fehlen für Patientinnen, deren Primärtumor negativ für diese Rezeptoren ist, effektive Therapieansätze [102–105]. In den letzten Jahren hat der Androgenrezeptor (*androgen receptor*, AR) auch bei der Behandlung des Mammakarzinoms an Bedeutung gewonnen und könnte als ein neuer therapeutischer Marker für die Behandlung des Mammakarzinoms herangezogen werden. Der AR ist beim Prostatakarzinom bereits ein wichtiges therapeutisches *target* und die selektiven AR Inhibitoren zeigen beim Prostatakarzinom gute Erfolge [106]. Der AR wird abhängig vom

Subtyp bei etwa 80% der Brustkrebsarten exprimiert und das häufig in einem höheren Level als der ER [107–109]. Daten deuten darauf hin, dass der AR der am häufigsten nachgewiesene Steroidrezeptor in Brustkrebszellen ist und eine zentrale Rolle in der Pathologie und Progression des Mammakarzinoms spielt [102,105]. Dies eröffnet die Möglichkeit AR Inhibitoren, die für das Prostatakarzinom zugelassen sind auch für bestimmte Subtypen des Mammakarzinoms als ein therapeutisches Instrument einzusetzen [105,106]. Der AR ist ein Mitglied der Steroidhormonrezeptorfamilie und ist wie ER und PR ein nukleärer Transkriptionsfaktor, die gemeinsame Strukturen aufweisen. Die Bindung von Steroidhormonen und Androgenen aktiviert den AR [102,110]. Studien konnten zeigen, dass über 70% der ER-positiven Brusttumore, etwa 60% der HER2-positiven und 30% bis 45% der *triple* negativen Tumore den AR exprimieren. AR hat beim Mammakarzinom eine multifunktionale Rolle und es ist bislang noch nicht vollständig geklärt ob er als ein Tumorsuppressor oder als Onkogen agiert. Vergleichbar zu ER und PR ist der AR ein wichtiger prognostischer Marker und korreliert mit dem rezidivfreien und dem Gesamtüberleben [104,110–112]. Zusätzlich dazu ist er ein unabhängiger prognostischer Marker, der mit günstigen klinisch-pathologischen Merkmalen im Zusammenhang steht und ein zuverlässiger Prädiktor für das Ansprechen auf eine Chemotherapie ist [113,114]. Der AR wird mit einem gut differenzierten Stadium und einem indolenten Brustkrebs in Verbindung gebracht [111,115]. Im Widerspruch dazu stehen Studien, die wiederum zeigen konnten, dass der AR das Wachstum ER positiver und *triple* negativer Tumore durch unterschiedliche Mechanismen fördert [116]. Darüber hinaus steht die AR Expression mit einer Resistenz für anti-Östrogene Therapien in Zusammenhang [113]. Die Resistenz gegen eine etablierte endokrine Therapie ist ein gut dokumentiertes Phänomen, welches *de novo* bei 30% bis 50% aller ER+ Tumore und letztlich bei allen metastasierenden ER+ Tumoren auftritt. Der Grund dafür könnte die Adaption der Tumorzellen von einer Östrogenabhängigkeit zur Androgenabhängigkeit sein [111]. Neueste Studien deuten darauf hin, dass nicht das Expressionslevel des AR allein den Nutzen einer adjuvanten endokrinen Therapie mit Tamoxifen vorhersagt, sondern dass sein Expressionslevel im Verhältnis zum Expressionslevel des ER im Primärtumor von Bedeutung ist, da ein *cross talk* zwischen diesen beiden Rezeptoren besteht [110,112]. Das suboptimale Ansprechen auf eine ER-gerichtete endokrine Therapie kann daher auf das AR:ER-Verhältnis zurückzuführen sein. Ein hohes AR:ER-Protein-Verhältnis ist Studien zufolge ein Indikator für das Therapieversagen einer Hormontherapie und korreliert mit einem kürzeren rezidivfreien Überleben bei Patientinnen, die mit Tamoxifen behandelt

werden [105,111]. Folglich spielen sowohl der ER als auch der AR eine entscheidende Rolle bei der Unterscheidung von Tumoren, die rezidivieren können oder nicht [106]. Trotz dieser neuen Daten ist die Rolle des AR bei Brustkrebs jedoch noch immer nicht vollständig geklärt, und die Biologie des AR bei Brustkrebs ist nach wie vor unvollständig verstanden [113]. Bislang sind keine Biomarker vorhanden, mit denen sich Veränderungen in der AR-Expression unter einer zielgerichteten AR-Therapie im Laufe der Zeit verfolgen lassen, insbesondere wenn der Primärtumor bereits entfernt wurde. Zirkulierende Tumorzellen, die als *Liquid Biopsy* verwendet werden könnten zu einem tumorspezifischen Biomarker werden, mit dem sich das Ansprechen auf eine Therapie kontrollieren und die Tumorentwicklung überwachen lässt [106]. Kürzlich konnte gezeigt werden, dass ungefähr 31% der zirkulierenden Tumorzellen beim Mammakarzinom den Androgen Rezeptor exprimieren. Diese Ergebnisse zeigen, dass der AR in zirkulierenden Tumorzellen eine prognostische und prädiktive Bedeutung für die Identifizierung von Patienten hat, die von einer AR-gerichteten Therapie profitieren könnten [105].

2 Zielsetzung

Die genaue Bestimmung der Langzeitprognose und die frühzeitige Erkennung von Rezidiven sind Schlüsselelemente des diagnostischen und therapeutischen Prozesses. Das Wichtigste aus klinischer Sicht ist die Identifizierung der Patienten mit einer potenziell schlechteren Prognose und die frühzeitige Erkennung residualer Tumorherde, damit eine geeignete Therapie möglichst schnell eingeleitet werden kann. In 70-80% der Fälle kann es auch nach Jahren der Erstdiagnose zu einem Rezidiv der Erkrankung kommen. Im Gegensatz zu der metastasierten Situation, in der der Krebspatient nur noch palliativ behandelt werden kann, ist das Ziel in der frühen Tumorerkrankung eine wenig belastende, möglichst effektive, personalisierte Krebstherapie. Für die Therapieentscheidung werden prädiktive Marker im Primärtumor untersucht, jedoch steht dieser nur zu Beginn der Erkrankung zur Verfügung und bestehende Metastasen sind für eine Biopsie nur schwer zugänglich. Die *Liquid Biopsy* liefert Informationen über die sogenannte minimale residuale Tumorerkrankung und spiegelt die ganze Komplexizität der heterogenen Krebserkrankung wider, da die Zellen im Blut von unterschiedlichen Tumorläsionen im Körper abgegeben werden können. Für die Detektion einer minimalen Resterkrankung sind neue sensitive Verfahren notwendig, um die disseminierten Tumorzellen im peripheren Blut von Krebspatienten nachzuweisen und die verschiedenen Entwicklungsstufen der Metastasierungskaskade besser verstehen zu können. Ziel der vorliegenden Arbeit war die Entwicklung einer neuartigen *in-vitro* Methode für die Identifizierung der zirkulierenden Krebsstammzellen aus dem peripheren Blut von Krebspatienten, ihre phäno- und genotypische Charakterisierung und die Untersuchung ihrer klinischen Relevanz in präklinischen Studien. Zudem sollte mit Hilfe des CAM Models die Tumorigenität der kultivierten Tumorsphären *in-vivo* untersucht werden, um ihr Tumor-initiierendes Potential zu demonstrieren und somit ein *patient-derived* Xenograft Model zu etablieren.

Ein weiteres Ziel der vorliegenden Arbeit war die Untersuchung der unterschiedlichen Mechanismen, die die zirkulierenden Tumorzellen im peripheren Blut der Krebspatienten nutzen, um die metastatische Ausbreitung des Tumors zu ermöglichen und sich vor dem Immunsystem zu schützen. Dafür sollten im Rahmen dieser Arbeit neuartige Oberflächenmarker wie PD-L1 und B7-H3 auf den CETCs untersucht werden, die die Zellen im peripheren Blut vor einem immunologischen Angriff schützen und gleichzeitig bei der Stratifizierung von Patienten behilflich sein könnten, die von einer Immuntherapie profitieren würden. Gleichzeitig sollte die Fähigkeit der CETCs Interaktionen mit anderen

Komponenten des Blutes einzugehen, geprüft werden, was einen weiteren Mechanismus für die Immunevasion darstellen könnte. Die Einführung der *Liquid Biopsy* in die Behandlungsstandards könnte in einer personalisierten Krebstherapie resultieren.

Zwar handelt es sich bei der *Liquid Biopsy* um eine Momentaufnahme, jedoch kann sie aufgrund ihrer einfachen Zugänglichkeit beliebig oft wiederholt werden. In präklinischen Studien sollte mit der maintrac® Methode die klinische Relevanz der CETCs, als auch der cCSCs während unterschiedlicher Therapieoptionen, wie der Immuntherapie, der Radiotherapie und der Hormontherapie untersucht werden. Zudem sollte die Anzahl der Zellen mit klinisch-pathologischen Merkmalen korreliert werden, um die Aggressivität des Primärtumors zu bestimmen. Im Rahmen dieser präklinischen Untersuchungen sollte zudem die prognostische Bedeutung der Androgen- und Östrogenrezeptorexpression in den CETCs hinsichtlich ihrer Rolle bei der Entwicklung einer Resistenz gegen die Tamoxifen-Erhaltungstherapie geprüft werden.

Insgesamt sollen die Erkenntnisse aus den präklinischen Studien die klinische Relevanz der Langzeitüberwachung zirkulierender Tumorzellen verdeutlichen, mit besonderem Fokus auf die sehr seltene Subpopulation der zirkulierenden Krebsstammzellen. Die weitere Charakterisierung der CETCs und der cCSCs hinsichtlich therapierelevanter Marker in verschiedenen therapeutischen Situationen könnten dazu beitragen, die Methode für den routinemäßigen Einsatz zu etablieren. Somit könnte die residuale Tumorlast zuverlässig erkannt und die Wirksamkeit systemischer Therapien schnell bestimmt werden.

3 Synopsis

3.1 Übersicht der Teilprojekte

Die vorliegende kumulative Dissertation basiert auf sechs Publikationen und einem Manuskript. Sie ist in drei Themenbereiche gegliedert ist, die primär den Nutzen der *Liquid Biopsy* für eine personalisierte und zielgerichtete Krebstherapie veranschaulichen sollen. Im Fokus standen die Identifizierung der zirkulierenden epithelialen Tumorzellen (*circulating epithelial tumor cells*, CETCs) und der zirkulierenden Krebsstammzellen (*circulating cancer stem cells*, cCSCs) aus dem peripheren Blut von Mammakarzinompatientinnen und ihre prognostische und prädiktive Bedeutung als Biomarker für unterschiedliche Therapiearten. Zusätzlich dazu sollte mit den publizierten Daten die Fähigkeit der zirkulierenden Tumorzellen in der Blutbahn zu überleben, der Immunantwort zu entgehen, und somit der Metastasierungsprozess untersucht werden.

Im ersten Teil der vorliegenden Arbeit, der die Publikationen I und II beinhaltet, wird die erstmalige Identifizierung, Charakterisierung und klinische Relevanz der aggressivsten Subpopulation zirkulierender Tumorzellen, der sogenannten zirkulierenden Krebsstammzellen aus dem peripheren Blut von Mammakarzinompatientinnen beschrieben, die die Ursache für den Therapiemisserfolg und die Metastasierung sind. Publikation II knüpft an die Ergebnisse aus der Publikation I an und unterstreicht durch *in-vivo* Experimente die Hypothese des Vorhandenseins zirkulierender Krebsstammzellen und deren Tumorigenität im peripheren Blut von Krebspatienten.

Der Metastasierungsprozess beinhaltet komplexe Prozesse, die im Wachstum sekundärer Läsionen in entfernten Organen resultieren. Ein entscheidender Schritt hierbei ist die Disseminierung von Tumorzellen aus dem Primärtumor und deren Überleben im peripheren Blut. Im zweiten Teil dieser Arbeit, der die Publikationen III, IV und Manuskript I beinhaltet, werden die unterschiedlichen Anpassungsmöglichkeiten zirkulierender Tumorzellen im peripheren Blut veranschaulicht, mit denen es ihnen gelingt der Immunantwort zu entgehen, in der Blutbahn zu überleben und letztendlich neue Metastasen zu generieren.

Zirkulierende Tumorzellen stellen wertvolle Biomarker für gezielte molekulare Therapien bei Krebspatienten dar. Trotz ihrer extrem niedrigen Konzentration im peripheren Blut können sie wichtige Informationen zur Diagnose und Prognose liefern. Die *Liquid Biopsy*, die beliebig oft wiederholt werden kann, spiegelt die Komplexizität der heterogenen

Krebserkrankung wider, da die Zellen von unterschiedlichen Tumorläsionen im Körper ins Blut abgegeben werden können. Das *Monitoring* zirkulierender Tumorzellen während unterschiedlicher Arten der Krebstherapie spiegelt direkt das Ansprechen der Patientinnen auf die jeweilige Therapie wider und ist von prognostischer Bedeutung. Dieser Ansatzpunkt wurde im dritten Teil der vorliegenden Arbeit, der Publikation III, V und VI beinhaltet, verfolgt, in der die Bedeutung der longitudinalen Überwachung der zirkulierenden Tumorzellen und der zirkulierenden Krebsstammzellen bei der Immuntherapie, der Radiotherapie und der endokrinen Therapie beschrieben wird.

Alle im Rahmen dieser Arbeit publizierten Untersuchungen bezüglich der zirkulierenden Tumorzellen und zirkulierenden Krebsstammzellen wurden im Labor Dr. Pachmann durchgeführt. Die Untersuchung der Tumorigenität der zirkulierenden Krebsstammzellen aus dem peripheren Blut von Krebspatienten in Publikation II erfolgte in Kooperation mit dem Lehrstuhl Organische Chemie I der Universität Bayreuth. Die Patientenkohorte für die Untersuchung des Einflusses der Radiotherapie auf die CETCs und cCSCs in Publikation V stammte aus dem Universitätsklinikum Jena.

3.2 Die Anzahl der aus dem peripherem Blut kultivierten Tumorsphären ist ein Prädiktor für das Vorhandensein von Metastasen bei Mammakarzinompatientinnen

Die erste im Rahmen dieser Dissertation veröffentlichte Publikation behandelt den Nachweis zirkulierender Krebsstammzellen aus dem peripheren Blut von Mammakarzinompatientinnen und deren prognostische Bedeutung.

Tumormetastasen sind die Hauptursache für die Krebsmorbidität und Sterblichkeit. Es wird angenommen, dass eine Unterpopulation von Tumorzellen mit stammzellähnlichen Eigenschaften für die Tumorinvasion, Metastasierung, Heterogenität und Therapieresistenz verantwortlich ist. Diese Population wird als Krebsstammzellen (*cancer stem cells*, CSCs) bezeichnet. Sie weisen eine erhöhte Zellmotilität und Invasivität auf und exprimieren Gene, die die Metastasierung fördern [117,118]. Sie besitzen Eigenschaften, die denen normaler adulter Stammzellen ähneln. Sie zeichnen sich durch eine erhöhte Therapieresistenz aus, weshalb sie für das Wiederauftreten des Tumors (Rezidive) und die Metastasierung verantwortlich sind. Jene CSCs die den Selektionsdruck einer Therapie überlebt haben, können diese Resistenzmechanismen an die Tochterzellen weitergeben, wodurch das Auftreten einer Resistenz gefördert und gleichzeitig die Entstehung eines aggressiveren Tumors ermöglicht wird [118]. In der vorliegenden Studie wurde ein neuer funktioneller 3D-Zellkulturansatz entwickelt, um Zellen mit proliferativer Aktivität und Krebsstammzelleigenschaften *in-vitro* aus dem peripheren Blut von insgesamt 49 nicht-metastasierten und 23 metastasierten Mammakarzinompatientinnen zu identifizieren und charakterisieren. Bei diesem Ansatz wurden die Leukozyten zusammen mit den zirkulierenden epithelialen Tumorzellen (*circulating epithelial tumor cells*, CETCs) für bis zu 21 Tage in einer Suspensionskultur kultiviert. Dadurch konnte eine Subpopulation von CETCs mit proliferativer Aktivität identifiziert werden, die zu sogenannten Tumorsphären heranwächst (Abb. 7).

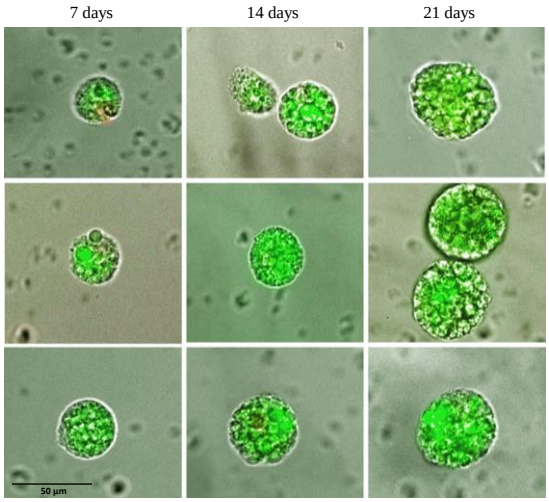

Abb.-Nr. 7: Typisches Wachstum von cCSCs zu Tumorsphären während der Zellkultur. Nach 3-5 Tagen der Zellkultur können die ersten Anzeichen für die Entstehung von Tumorsphären beobachtet werden. Die Sphären wachsen während der Zellkultur kontinuierlich und erreichen nach 21 Tagen einen Durchmesser zwischen 30 µm - 60 µm.

Die Art des Wachstums, als auch die phäno- und genotypische Charakterisierung der Tumorsphären legten nahe, dass es sich bei der identifizierten Subpopulation der CETCs um Zellen mit Krebsstammzell-Eigenschaften handelte, die als Vorläuferzellen von Metastasen gelten und für Rezidive und Therapieresistenzen verantwortlich sind. Nur ein geringer Anteil der CETCs war in der Lage *in-vitro* zu Tumorsphären heranzuwachsen. Bei gesunden Probanden konnten zudem keine Tumorsphären gezüchtet werden, was die Entstehung der Sphären als Folge der Aggregation von Leukozyten ausschließt. Für die Identifizierung von Krebsstammzellen im Tumorgewebe werden unterschiedliche Markerkombinationen verwendet. Um die generierten Tumorsphären in der vorliegenden Studie als Krebsstammzellen identifizieren zu können, wurde die am häufigsten verwendete Markerkombination für das Mammakarzinom verwendet: CD44, CD24 und ALDH1 Aktivität [53][118]. Die Immunfluoreszenzfärbungen zeigten sowohl eine $CD44^{+}/CD24^{-/low}$-Expression, als auch eine hohe ALDH1-Aktivität. Auch die Expression von Pluripotenz-assoziierten Faktoren spielt eine entscheidende Rolle bei der Entwicklung und dem bösartigen Fortschreiten verschiedener Krebsarten [119]. Für die relative Genexpressionsanalysen wurden mit Hilfe eines semi-automatisierten Mikroskops und einer Glaskapillare einzelne CETCs und Tumorsphären der gleichen Patienten aus einer Zellsuspension isoliert und im Anschluss eine RT-PCR durchgeführt. Die kultivierten Tumorsphären zeigten im Vergleich zu den CETCs eine Überexpression von Transkriptionsfaktoren wie *Oct4*, *Sox2* und *Nanog*, die für die Aufrechterhaltung der Pluripotenz und Selbsterneuerungsfähigkeit von Krebsstammzellen erforderlich sind und eine wichtige Rolle bei der unkontrollierten Vermehrung der Tumorzellen spielen [120].

Die vorliegende Studie zeigte, dass die Anzahl der Tumorsphären *in-vitro* mit der Aggressivität des Primärtumors korrelierte, wie z.B. dem Lymphknotenstatus und der Metastasierung. Patientinnen im Stadium I hatten signifikant weniger Tumorsphären im Vergleich zu Patientinnen mit distanten Metastasen (Stadium IV). Unter Verwendung des statistischen Verfahrens der Grenzwertoptimierungskurve (*Receiver-Operating Characteristics*, ROC) konnte gezeigt werden, dass die Anzahl an Tumorsphären bei einem *cut-off* von ≥ 30 Tumorsphären/100µL Blut als ein unabhängiger Prädiktor für das Vorhandensein einer metastatischen Erkrankung verwendet werden kann. Die Fläche unter der ROC Kurve (*area under the ROC curve*, AUC) war für die Anzahl der Tumorsphären höher, als für die Anzahl der CETCs und betrug 0,71 (p = 0,005) (Abb. 8a). Bei einem *cut-off* von 30 Tumorsphären/100µL Blut lag die Sensitivität bei 70% und die Spezifität bei 65% (Abb. 8b). Demzufolge könnten bei einer Anzahl von mehr als 30 Tumorsphären/100µL Blut bei Mammakarzinompatientinnen weitere diagnostische Bildgebungen empfehlenswert sein, um möglicherweise bereits vorhandene Fernmetastasen frühzeitig zu entdecken.

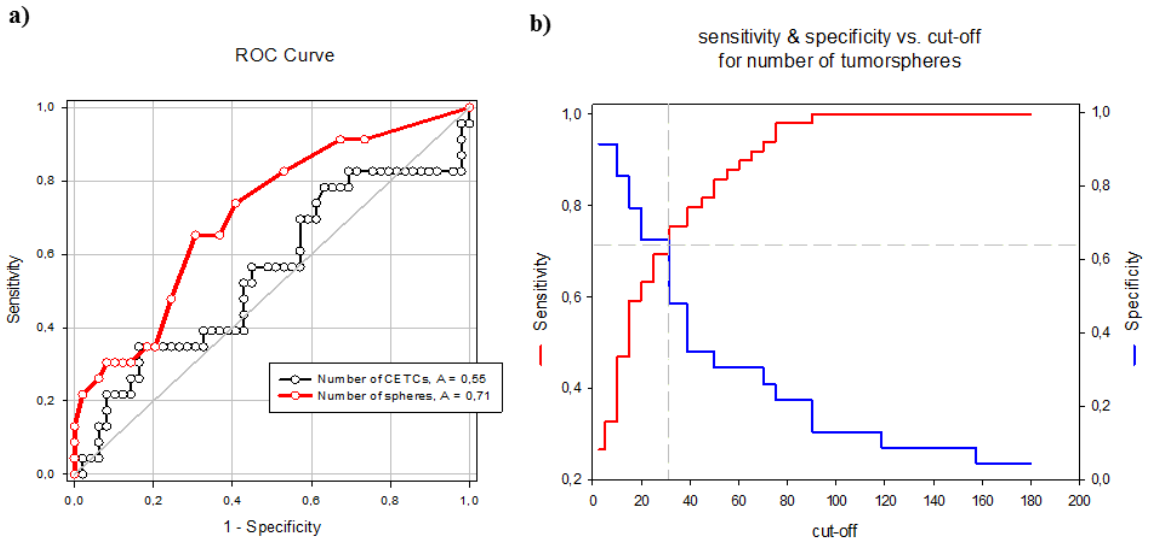

Abb.-Nr. 8: a) ROC Kurve für die Anzahl an CETCs und Tumorsphären zur Unterscheidung von metastasierten und nicht-metastasierten Mammakarzinompatientinnen. Die ROC Kurve ist ein Diagramm, in dem die Richtig-Positiv-Rate gegen die Falsch-Positiv-Rate aufgetragen wird. Die AUC zeigt die Klassifizierungsfähigkeit des Klassifikators. Je besser die Klassifikationsfähigkeit des Klassifikators ist, desto höher ist der AUC-Wert und beträgt max. 1. Die AUC für die Klassifizierung zwischen einer metastatischen und nicht-metastatischen Erkrankung lag bei einem *cut-off* von ≥ 30 Tumorsphären/100µL Blut bei A = 0,71, p < 0,01. **b) Sensitivität und Spezifität für ausgewählte *cut-off* Punkte hinsichtlich der Anzahl an Tumorsphären.** Der Punkt, an dem sich beide Kurven kreuzen zeigt den *cut-off* mit der höchsten Sensitivität und Spezifität für die Klassifizierung zwischen einer metastatischen und nicht-metastatischen Erkrankung.

Zudem war die Anzahl der Tumorsphären bei Patientinnen mit HER2-positivem Primärtumor signifikant höher als bei HER2-negativen Tumoren. Diese Ergebnisse sind vergleichbar mit den Ergebnissen von Korkaya *et al*., die ebenfalls zeigen konnten, dass eine HER2-Überexpression die Stammzellpopulation im Brustkrebs erhöht und die Fähigkeit zur Tumorsphärenbildung in Gewebekulturen steigert [121]. In der adjuvanten Situation, in der keine erkennbare residuale Erkrankung mehr vorhanden ist, stellt die Überwachung der CETCs ein wertvolles Werkzeug zur routinemäßigen Überwachung der Therapieeffizienz dar [4]. In der vorliegenden Arbeit konnte gezeigt werden, dass als ein Zeichen einer erfolgreichen Therapie auch die Anzahl der Tumorsphären gleichzeitig mit der Anzahl an CETCs während der Chemotherapie abfallen kann. Im Gegensatz dazu könnte eine zunehmende Anzahl der CETCs und Tumorsphären weitere diagnostische Schritte rechtfertigen, um rechtzeitig eine Therapie zu ändern oder einzuleiten. Die Überwachung der Therapie mit Hilfe der CETCs und der Tumorsphären *in-vitro* könnten bei der individuellen Anpassung der Therapie hilfreich sein.

Es ist vielfach beschrieben worden, dass es durch die epithelial-mesenchymale Transition (*epithelial-mesenchymal-transition*, EMT) zu phänotypischen Veränderung von Tumorzellen kommen kann, was wiederum ein entscheidender Schritt bei der Tumorinvasion und Metastasierung ist. Während dieses Prozesses wird die Expression epithelialer Marker wie EpCAM und Cytokeratin herunterreguliert [122]. Bislang konnte nicht festgestellt werden, aus welchen Vorläuferzellen die Tumorsphären in der vorliegenden Arbeit entstehen, da bei zwei Patientinnen ohne EpCAM-positive zirkulierende Tumorzellen, dennoch eine EpCAM-positive Tumorsphärenbildung beobachtet werden konnte. Aus diesem Grund ermöglicht der in dieser Studie publizierte Ansatz die Identifizierung der wichtigsten Subpopulation der CETCs mit proliferativer Kapazität, ohne auf Oberflächenmarker angewiesen zu sein und somit ohne Selektion und Verlust von Zellpopulationen. Zudem konnte gezeigt werden, dass die Anzahl der Tumorsphären als ein Prädiktor für das Vorhandensein von Metastasen verwendet werden kann.

Weitere Details in: M. Pizon, D. Schott, U. Pachmann, K. Pachmann

The number of tumorspheres cultured from peripheral blood is a predictor for presence of metastasis in patients with breast cancer.

Oncotarget. **2016**;7(30):48143-48154

[Publikation I]

3.3 Das Chorioallantoismembran (CAM) Modell für *patient-derived Xenografts* aus zirkulierenden Krebsstammzellen (cCSCs) von Mammakarzinompatientinnen

Es existiert eine außerordentlich große Vielfalt an Tumorentitäten und Subtypen, die jede Krebserkrankung individuell und einzigartig machen. Tumore bestehen aus heterogenen Zellpopulationen, die unterschiedliche Eigenschaften aufweisen. Unter diesen Subpopulationen befinden sich jene Tumorzellen mit Stammzelleigenschaften, die als Krebsstammzellen bezeichnet werden und mit einem besonders aggressiven Phänotyp korrelieren [123]. Interessanterweise konnten einige experimentelle Ergebnisse zeigen, dass eine kleine Subpopulation zirkulierender Tumorzellen ebenfalls Eigenschaften von Krebsstammzellen aufweist, die zu einer Tumorinitiierung fähig ist [124,125]. Sie werden als zirkulierende Krebsstammzellen bezeichnet (*circulating cancer stem cells*, cCSCs). Funktionelle Tests, die es den Krebsstammzellen ermöglichen *in-vitro* zu sogenannten Tumorsphären heranzuwachsen sind gut beschriebene Kulturmethoden, um die Krebsstammzellen aus unterschiedlichen Gewebeproben zu identifizieren [126–129]. Die Ergebnisse der zweiten Publikation knüpften an die Ergebnisse der ersten Publikation an. Erneut konnten durch die *in-vitro* Zellkultur das Wachstum der cCSCs zu Tumorsphären aus dem peripheren Blut von insgesamt 75 Mammakarzinompatientinnen in unterschiedlichen Stadien beobachtet werden (Abb. 9).

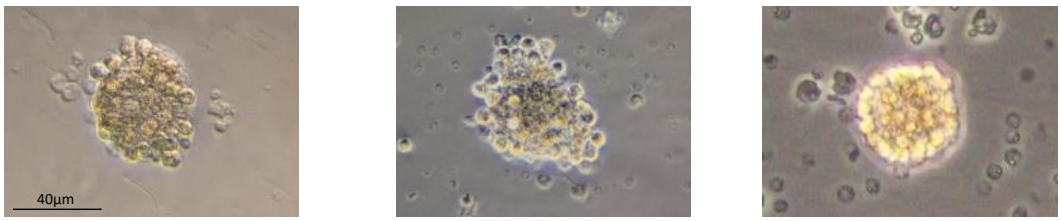

Abb.-Nr. 9: Typische Tumorsphären unter dem Lichtmikroskop. Mikroskopische Aufnahmen exemplarischer Tumorsphären nach 3-wöchiger Zellkultur. Die Tumorsphären haben einen Durchmesser von ungefähr 60 µm, wobei die einzelnen Zellen in einer Tumorsphäre gut erkennbar sind.

Die Anzahl der *in-vitro* gewachsenen Tumorsphären korrelierte erneut mit der Anwesenheit von Metastasen und einem positiven HER2 Status. Zudem wiesen Patientinnen mit multiplen Metastasen die höchste Anzahl an Tumorsphären auf. Diese Beobachtungen deuten darauf hin, dass die Anzahl der *in-vitro* gewachsenen Tumorsphären die Aggressivität des Tumors widerspiegeln und zusätzliche Informationen über die Progression der Erkrankung liefern.

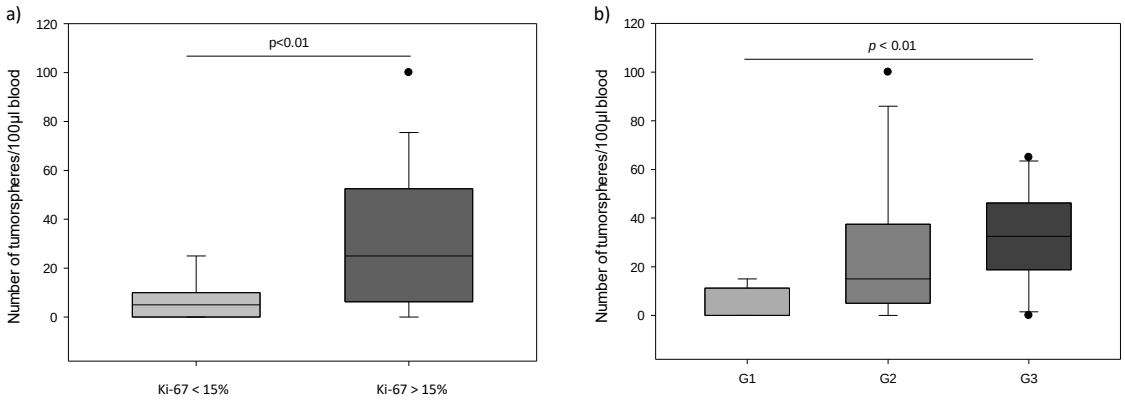

Abb.-Nr. 10: a) Anzahl an Tumorsphären bei Patientinnen mit einem primären Ki-67 < 15% und Ki-67 > 15% Index. Bei Patientinnen mit einem Ki-67-Index > 15% war die Anzahl der Tumorsphären statistisch signifikant höher als bei Patientinnen mit einem Ki-67-Index < 15% (Median 34 vs. 8 Tumorsphären/100µL Blut, p < 0,01). **b) Anzahl an Tumorsphären in Bezug auf den histologischen Grad des Primärtumors.** Patientinnen mit G3-Primärtumoren hatten signifikant mehr Tumorsphären, als Patientinnen mit G1 Tumoren (Median 32,5 vs. 0, p < 0,01) (G1-gut differenzierter Tumor; G2-mäßig differenzierter Tumor; G3-schlecht differenzierter Tumor).

Zudem konnte ein Zusammenhang zwischen der Tumorsphärenbildungsrate und einem hohen Ki-67-Index bzw. dem histologischen Differenzierungsgrades des Primärtumors beobachtet werden. Ki-67 wird in proliferierenden Zellen exprimiert und dient bei Mammakarzinompatientinnen als ein prädiktiver und prognostischer Marker. Der klinisch am weitesten verbreitete *cut-off* für die Einteilung der Patientinnen mit einem geringen und hohen Rezidivrisiko liegt bei 15% Ki-67-positiver Zellen [130]. Die Ergebnisse der vorliegenden Arbeit zeigten, dass bei Patientinnen mit einem Ki-67-Index > 15% im Primärtumor mehr Tumorsphären *in-vitro* gewachsen sind, als bei Patientinnen mit einem Ki-67-Index < 15% (Abb. 10a). Der histologische Grad des Primärtumors (Drüsenbildung, Kerngehalt, Mitoserate) ist beim Mammakarzinom ein weiterer, wichtiger prognostischer Marker für das Gesamtüberleben [131,132]. Er gibt an, wie sehr die invasiven Karzinome normalen Brustepithelzellen ähneln, bzw. basiert auf dem Grad der Differenzierung des Tumorgewebes [133]. Patientinnen mit Grad 1 Tumoren haben signifikant bessere Überlebensraten, als Patientinnen mit Grad 2,3 Tumoren [134]. Bislang sind in der Literatur kaum Daten beschrieben, die einen Zusammenhang zwischen der Anzahl an Krebsstammzellen und dem histologischen Grad beschreiben. In der vorliegenden Studie konnte gezeigt werden, dass Patientinnen mit einem schlecht differenzierten Primärtumor (G3) signifikant mehr Tumorsphären *in-vitro* gebildet haben, als Patientinnen mit einem gut differenzierten Tumor (G1) (Abb. 10b). Neben der klinischen Bedeutung der Anzahl an Tumorsphären und deren weiteren Charakterisierung, konnte in dieser Studie erstmals die Tumorigenität der *in-vitro* gewachsenen Tumorsphären aus dem peripheren Blut der Patientinnen *in-vivo* mit Hilfe des Chorioallantoismembran (*chorioallantoic-membrane*,

CAM)-Models gezeigt werden. In den letzten Jahren hat sich das CAM-Modell für viele wissenschaftliche Zwecke gut etabliert. Es stellt eine gute Alternative zu den konventionellen Tierversuchen dar. Zellmaterial wird auf die stark vaskularisierte extraembryonale Membran des sich entwickelnden Embryos von befruchteten Hühnereiern implantiert [135]. Bei Tumorexperimenten gelten jedoch nach wie vor immundefiziente Mausmodelle mit subkutan implantierten Xenotransplantaten als "Goldstandard" für die Bewertung der Tumorigenität und Invasivität der Tumorzellen. Da jedoch sowohl in der CAM, als auch im Hühnerembryo bis zum 14. Tag keine Nozizeption vorhanden ist, ist dieses Modell minimal invasiv und ethisch vorteilhafter als die gut etablierten Mausmodelle. Die Versuche dieser Studie konnten erstmals zeigen, dass nach der Applikation der *in-vitro* gewachsenen Tumorsphären aus dem peripheren Blut von Mammakarzinompatientinnen diese in der Lage waren, Tumore auf der CAM zu generieren (Abb. 11a). Dabei korrelierte die Anzahl an *in-vitro* gewachsenen Tumorsphären mit der Anwachsrate auf der CAM. Darüber hinaus legten die Ergebnisse der Studie nahe, dass die Anwachsrate mit dem Ki-67-Index und dem histologischen Grad des Primärtumors zusammenhing. Tumore konnten nur bei den Patientinnen auf der CAM induziert werden, deren Primärtumor einen hohen Ki-67-Index hatte. Die histopathologische Analyse war der wichtigste Endpunkt dieser Studie. Mit Hilfe der Präparate konnte bestätigt werden, dass die auf der CAM induzierten Tumore das ursprüngliche morphologische Profil des Primärtumors der Patientinnen aufwiesen (Abb. 11b).

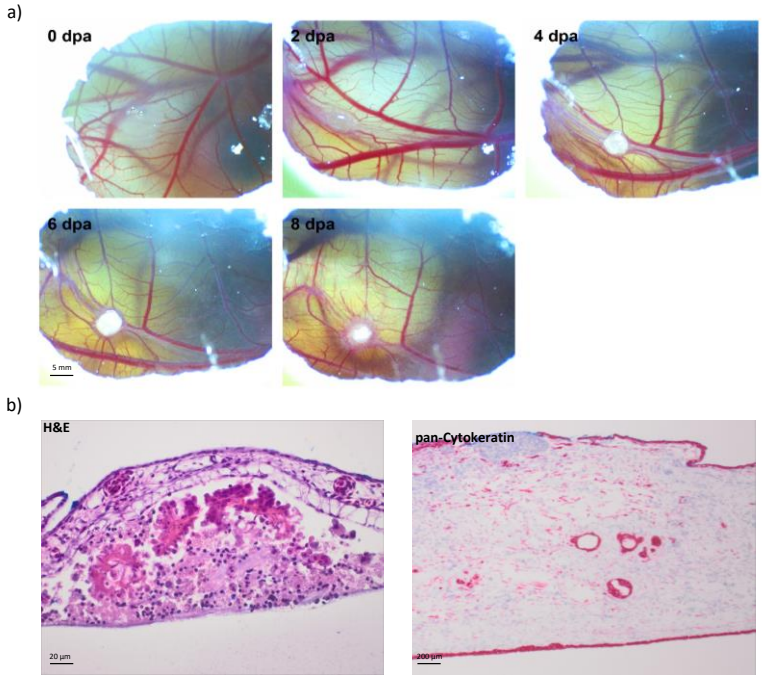

Abb.-Nr. 11: a) Entstehung eines Mikrotumors auf der CAM aus *in-vitro* gewachsenen Tumorsphären einer Mammakarzinompatientin. Die topisch applizierten Tumorsphären einer Patientin konnten innerhalb von 8 Tagen auf der CAM einen Mikrotumor generieren, der in die CAM eindringen konnte und stark vaskularisiert wurde. **b) H&E- und Pan-Zytokeratin-Färbung eines aus Tumorsphären entstandenen CAM-Xenotransplantats.** Die CAM Tumore enthielten neoplastisches Gewebe mit einer gut organisierten homogenen Struktur, die sich deutlich vom umgebenden Stroma und dem Membranrand unterschied. Zudem ähnelte die Morphologie der des Primärtumors. Die Zytokeratinfärbung bestätigte die Anwesenheit von Tumorzellen in der CAM.

In Anbetracht der hohen Anwachsrate der *patient-derived Xenografts* auf der CAM in einem kurzen Zeitraum von 8 Tagen stellt dieses Modell eine vielversprechende *in-vitro* Plattform dar, um zukünftig Medikamente an Tumoren individueller Patienten zu testen. Dieses Modell hat den großen Vorteil, dass das für den CAM-Test verwendete biologische Material durch die *Liquid Biopsy* leicht zugänglich und wiederholt verfügbar ist. Das Ziel der personalisierten Medizin ist es, Krebspatienten eine maßgeschneiderte Therapie zur Verfügung zu stellen. Aus diesem Grund sind individualisierte Tumormodelle notwendig, um experimentelle und neuartige Strategien sowohl für diagnostische als auch therapeutische Zwecke zu bewerten.

Weitere Details in: M. Pizon, D. Schott, U. Pachmann, R. Schobert, M. T. Pizon, M. Wozniak, R. Bobinski, K. Pachmann

Chick Chorioallantoic Membrane (CAM) Assays as a model of patient-derived xenografts from circulating cancer stem cells (cCSCs) from breast cancer patients.

Cancers. 2022; 14(6):1476

[Publikation II]

3.4 Sensitiver Nachweis PD-L1 positiver CETCs als ein potentieller Biomarker für die Selektion von Patienten für die Behandlung mit PD-1/PD-L1 Inhibitoren

Sobald die Tumorzellen die Blutbahn erreichen sind sie einer Reihe physiologischer und immunologischer Hürden ausgesetzt, die deren Überleben im peripheren Blut erschweren. Um sich vor der immunologischen Eliminierung erfolgreich zu schützen, nutzen die CETCs eine Reihe unterschiedlicher Mechanismen. Dazu gehört die Expression der programmierten Zelltodes-Liganden 1 und 2 (*programmed cell death ligand-1/2*, PD-L1/2, auch B7-H1 genannt). Es ist bekannt, dass Tumorzellen durch die Expression von PD-L1 auf ihrer Zelloberfläche Ausweichstrategien entwickeln, um der Immunantwort zu entkommen und die Immunreaktion zu hemmen [63]. Zudem kann durch die Unterdrückung des Immunsystems der Progress der Erkrankung und somit die Metastasierung vorangetrieben werden. Viele Studien konnten zeigen, dass PD-L1 bei einer Reihe unterschiedlicher Krebsarten häufig hochreguliert wird [64]. Die Krebsforschung konzentriert sich aktuell stark auf Immuntherapien, bzw. die Entwicklung von *checkpoint*-Inhibitoren mit denen in den letzten Jahren ein Durchbruch in der Tumortherapie erzielt werden konnte. Zu den am umfangreichsten untersuchten *checkpoints* gehört der programmierte Zelltodes Rezeptor 1 (*programmed cell death receptor 1*, PD-1) mit seinen Liganden PD-L1/2 [136,137]. Leider lässt sich ein Therapieerfolg für den einzelnen Patienten bisher noch nicht ausreichend vorhersagen.

Im Rahmen der dritten Publikation sollte mit dem Nachweis von PD-L1 und PD-L2 auf den CETCs mit einer erweiterten maintrac® Methode ein Mechanismus der Immunevasion zirkulierender Tumorzellen untersucht werden. Zudem sollte geprüft werden, ob der Nachweis von PD-L1 und PD-L2 auf den CETCs als ein Biomarker für die Stratifizierung von Patienten, die mit PD-1/PD-L1 Inhibitoren behandelt werden könnten, herangezogen werden kann. Zudem sollte die klinische Relevanz PD-L1 positiver CETCs während einer Immuntherapie untersucht werden. Insgesamt wurden in die Studie 128 Mamma-, 72 Prostata-, 18 kolorektale- und 11 Lungenkarzinompatienten in unterschiedlichen Stadien einbezogen. Bei 94,6% der Mammakarzinompatientinnen konnten PD-L1 positive CETCs nachgewiesen werden. Mazel *et al*. konnten mit der *CellSearch*-Methode bei 11/16 (68,8%) metastasierten Mammakarzinompatientinnen PD-L1 positive zirkulierende Tumorzellen identifizieren. Der Anteil PD-L1-positiver zirkulierenden Tumorzellen variierte bei den individuellen Patienten zwischen 0,2% und 100% [138], was mit den

publizierten Daten in dieser Studie übereinstimmt. Sowohl die PD-L1, als auch die PD-L2 Expression auf der Oberfläche der CETCs zeigte eine hohe Heterogenität (Abb. 12).

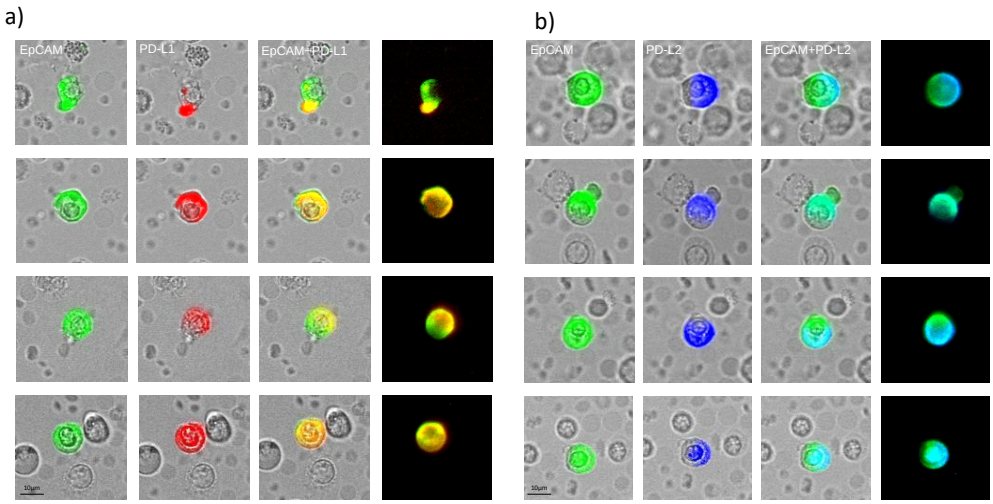

Abb.-Nr. 12: Darstellung der Zweitantikörperfärbung der CETCs mit spezifischen Antikörpern gegen a) EpCAM und PD-L1 und b) EpCAM und PD-L2. EpCAM war mit FITC, PD-L1 mit Phycoerythrin und PD-L2 mit Alexa Fluor® 350 konjugiert. Die Expressionsintensität von EpCAM, PD-L1 und PD-L2 variierte von Zelle zu Zelle einer Patientin.

Die Rolle von PD-L2 bei der Umgehung des Immunsystems ist noch nicht vollständig geklärt. Beim Vergleich beider PD-1-Liganden auf den CETCs konnte festgestellt werden, dass die Häufigkeit PD-L2 positiver CETCs im Vergleich zu der Häufigkeit PD-L1 positiver Zellen deutlich geringer war. Daher könnte PD-L2 bei der Immuntherapie nur eine untergeordnete Rolle spielen [65,139]. Der Nachweis von Kopienzahlvariationen, der im Gegensatz zu den Schwierigkeiten bei Antikörper abhängigen Ansätzen unabhängig von den meisten Fixierungsverfahren ist, kann eine alternative Methode zur Immunhistochemie sein. In einem weiteren Schritt wurde der Grad der Übereinstimmung zwischen der PD-L1 Expression mit Hilfe der Immunfluoreszenz und der PD-L1 Kopienzahl unter Verwendung der Fluoreszenz *in-situ* Hybridisierung (*fluorescence in-situ hybridization*, FISH) in den CETCs bei insgesamt 13 Mammakarzinompatientinnen untersucht. In allen untersuchten Fällen wurden PD-L1-amplifizierte CETCs detektiert. Der Prozentsatz amplifizierter CETCs reichte von 62% bis 96% mit einem Medianwert von 75% und war bei den untersuchten Patientinnen signifikant mit der PD-L1-Expression verbunden.

Der Anteil PD-L1 positiver Zellen korrelierte mit keinen klinisch-pathologischen Parametern außer mit dem Vorhandensein von Fernmetastasen und der Radiotherapie bei

Mammakarzinompatientinnen. Entzündungen, die während der Radiotherapie auftreten, können die PD-L1 Expression in Tumorzellen stimulieren, um sich besser vor einem aktivierten Immunsystem zu schützen. Diese Beobachtung konnte in dieser Studie bestätigt werden, da Patientinnen mit einer vorangegangenen Radiotherapie signifikant mehr PD-L1 positive CETCs hatten als Patientinnen, die bislang keine Radiotherapie erhalten haben (Abb. 13a). Eine kombinierte Radiotherapie mit PD-1 und PD-L1 Inhibitoren hingegen könnte die Effektivität der Radiotherapie wiederherstellen und den Tumor unter lokaler Kontrolle halten, indem die zytotoxische T-Zellantwort reaktiviert wird [140].

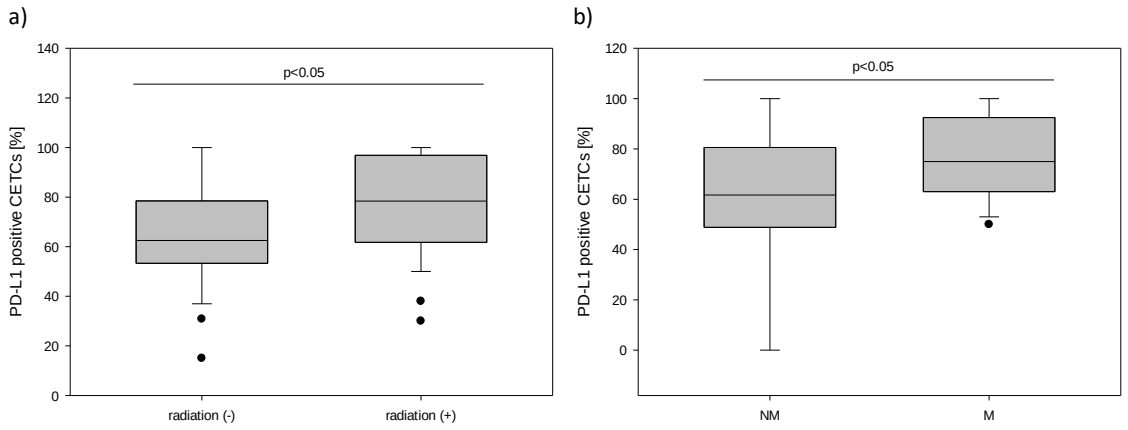

Abb.-Nr. 13: a) Anteil PD-L1 positiver CETCs bei Patientinnen mit und ohne Radiotherapie. Patientinnen nach einer Radiotherapie hatten einen höheren Anteil PD-L1 positiver CETCs verglichen mit Patientinnen, die keine Radiotherapie erhalten haben (Median 77,4% vs. 62,5%, $p < 0,05$), unabhängig vom Bestrahlungsregime. **b) Anteil PD-L1 positiver CETCs bei nicht metastasierten und metastasierten Patientinnen.** Patientinnen mit bereits vorhandenen Metastasen hatten einen signifikant höheren Anteil PD-L1 positiver CETCs, als nicht metastasierte Patientinnen (Median 75% vs. 61%, $p < 0,05$).

Metastasierte Patientinnen wiesen einen signifikant höheren Anteil PD-L1 positiver CETCs im Vergleich zu nicht metastasierten Patientinnen auf (Abb. 13 b). Tumorzellen, die invasiv sind und sich den zytotoxischen T-Zellen wirksam entziehen können, haben einen größeren selektiven Vorteil und tragen wahrscheinlich stärker zum Fortschreiten der Krebserkrankung bei. PD-L1-positive CETCs konnten auch bei Patienten ohne Metastasen nachgewiesen werden. Dies kann Behandlungsentscheidungen bereits in der adjuvanten Situation ermöglichen.

Die hohe Prävalenz PD-L1 positiver CETCs veranschaulichte, dass eine PD-1/PD-L1 Blockade bereits in der adjuvanten Situation von großer Bedeutung sein könnte, um die Immunantwort, die sich gegen die Tumorzellen richtet, zu verstärken. Die Ergebnisse veranschaulichten, dass zirkulierende Tumorzellen für die metastatische Ausbreitung des Tumors Mechanismen nutzen, um sich vor dem Angriff des Immunsystems zu tarnen und diese gleichzeig zu inhibieren. Des Weiteren kann der Nachweis von PD-L1 auf CETCs

nützlich für die Patientenstratifizierung und Therapieüberwachung sein. Serienmäßige Tests durch die Verwendung der *Liquid Biopsy* während der Behandlung könnten die Erkennung einer frühen Resistenz ermöglichen, insbesondere in Therapiesituationen bei der durch die operative Entfernung des Tumors keine weiteren Kontrollmöglichkeiten zum Therapieerfolg bestehen.

Weitere Details in: D. Schott, M. Pizon, U. Pachmann, K. Pachmann
Sensitive detection of PD-L1 expression on circulating epithelial tumor cells (CETCs) could be a potential biomarker to select patients for treatment with PD-1/PD-L1 inhibitors in early and metastatic solid tumors
Oncotarget. **2017**; 8(42): 72755-72772
[Publikation III]

3.5 B7-H3 auf zirkulierenden epithelialen Tumorzellen korreliert mit dem Proliferationsmarker Ki-67 und kann mit der Aggressivität von Tumoren bei Brustkrebspatientinnen in Verbindung gebracht werden

Immuntherapien, wie PD-1/PD-L1 Inhibitoren zielen darauf ab, das Immunsystem bei der Bekämpfung von Krebs zu unterstützen. Periphere Membranproteine, zu denen die B7-Familie gehört, sind nachweislich an der T-Zellreaktion beteiligt. Neueste Studien haben belegt, dass die Hochregulation von B7-Molekülen in der Mikroumgebung des Tumors mit der Immunevasion von Tumorzellen zusammenhängt [66]. Das B7-H3 Protein (CD276) ist wie PD-L1 (B7-H1) ein *immune checkpoint*-Molekül, welches sowohl kostimulatorische, als auch koinhibitorische Funktionen besitzt und somit eine doppelte Rolle im Immunsystem spielt [141]. Sein Vorhandsein wird mit einer schlechteren Prognose, einer verkürzten Überlebenszeit und Rezidiven in Verbindung gebracht [142]. Zahlreiche experimentelle Daten deuten darauf hin, dass B7-H3 die Aggressivität der Krebszellen und somit den Progress des Tumors über immunabhängige und immununabhängige Wege regulieren kann. Durch die Kombination von PD-1/PD-L1 und B7-H3 Inhibitoren könnte möglicherweise die Effektivität der Immuntherapie gesteigert werden, indem das Immunsystem reaktiviert und eine zielgerichtete Eliminierung der Tumorzellen ermöglicht wird. Die vierte Publikation im Rahmen dieser Dissertation schließt an die vorangegangene Publikation an, in der ein weiterer Mechanismus der Immunevasion zirkulierender Tumorzellen untersucht werden sollte. Zudem sollte die B7-H3 Expression mit klinisch-pathologischen Parametern korreliert und folglich die Aggressivität des Primärtumors bestimmt werden.

Ki-67 (auch bekannt als MKI67) ist ein zellulärer Marker, der eng mit dem Zellzyklus gekoppelt ist [143]. In der Literatur wurde ein enger Zusammenhang zwischen dem Anteil Ki-67-positiver Zellen und der Tumorgröße, der Aggressivität des Tumors und dem Grad der Angiogenese beschrieben. Mammakarzinompatientinnen mit einem Ki-67-Index von mehr als 15% haben ein kürzeres rezidivfreies- und Gesamtüberleben [11]. Der Nachweis von B7-H3 und Ki-67 in den CETCs erfolgte in dieser Studie analog zum PD-L1-Nachweis mit der maintrac® Methode. Insgesamt wurden 50 Mammakarzinompatientinnen in unterschiedlichen Stadien einbezogen. B7-H3 konnte mittels Immunfluoreszenz bei 82% der Mammakarzinompatientinnen nachgewiesen werden. Eine vergleichbar hohe B7-H3 Expression wurde auch im Primärtumor gefunden

[74,77]. Auch der Proliferationsmarker Ki-67 konnte in einem hohen Prozentsatz bei den Mammakarzinompatientinnen detektiert werden (90%). In der Literatur wurden sehr unterschiedliche Daten hinsichtlich Ki-67-positiver zirkulierender Tumorzellen publiziert. Spiliotaki *et al.* konnten bei nur 27,5% Ki-67-positive zirkulierende Tumorzellen detektieren [144], wohingegen Kallergi *et al.* Ki-67-positive zirkulierende Tumorzellen bei 51,7% bzw. 44% der Patientinnen mit frühem bzw. metastasiertem Brustkrebs beobachten konnten [145].

Des Weiteren zeigten die Ergebnisse dieser Studie, dass der Anteil sowohl B7-H3, als auch Ki-67-positiver CETCs mit einem aggressiveren Subtyp des Mammakarzinoms korrelierte. Beide Biomarker waren signifikant häufiger bei ER/PR negativen Subtypen exprimiert im Vergleich zu ER/PR positiven Subtypen. Zudem konnte eine hohe Korrelation zwischen dem Prozentsatz B7-H3- und Ki-67-positiver CETCs beobachtet werden. Kolokalisationsfärbungen, die bei 20 Patientinnen durchgeführt wurden, ergaben in 90% eine starke Überlappung der EpCAM, B7-H3 und Ki-67 Fluoreszenzsignale (Abb. 14). Demnach wurden beide Biomarker zu einem hohen Prozentsatz von ein und derselben Zelle exprimiert, was die Aggressivität dieser Zellen verdeutlicht. Es handelt sich dabei um Tumorzellen, die nicht nur einen hohen Proliferationsindex aufweisen, sondern gleichzeitig in der Lage sind, der Immunantwort zu entkommen.

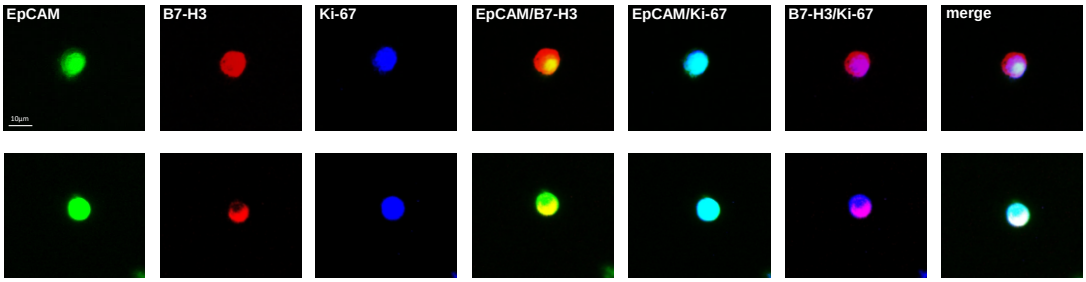

Abb.-Nr. 14: Kolokalisationsfärbungen der CETCs mit spezifischen Antikörpern gegen EpCAM, B7-H3 und Ki-67. EpCAM war mit FITC, B7-H3 mit Phycoerythrin und Ki-67 mit Pacific Blue konjugiert. Die Färbungen zeigen eine starke Überlappung zwischen den verschiedenen fluoreszierenden Markierungen.

Zusätzlich dazu konnte ein Zusammenhang zwischen Ki-67 bzw. B7-H3 positiven CETCs und einem positiven Lymphknotenstatus der Patientinnen herausgefunden werden. Es wurde bereits ein Zusammenhang zwischen einem hohen Ki-67-Index im Tumorgewebe und einem positiven Lymphknotenstatus beim Mammakarzinom beschrieben [146]. In anderen Studien wurde ebenfalls eine Korrelation zwischen einem hohen B7-H3 Status im Gewebe und einem positiven Lymphknotenstatus gefunden [83]. Folglich spielen beide Biomarker eine entscheidende Rolle bei der Tumorprogression und Invasivität.

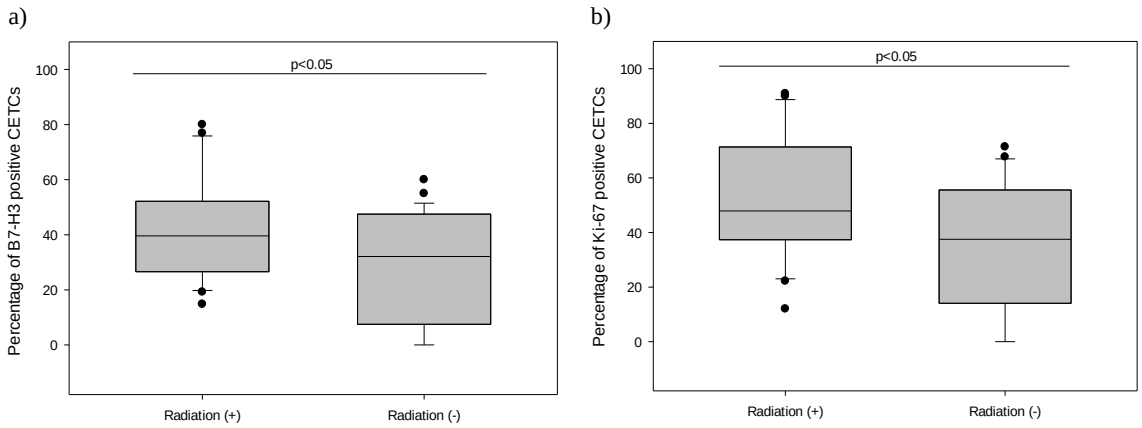

Abb.-Nr. 15: a) Radiotherapie und der Anteil B7-H3 positiver CETCs. Patientinnen nach einer Radiotherapie hatten mehr B7-H3 positive CETCs verglichen mit Patientinnen die keine Radiotherapie erhalten haben (Median 40% vs. 23%, $p < 0{,}05$). **b) Radiotherapie und der Anteil Ki-67 positiver CETCs.** Patientinnen die eine Radiotherapie erhalten haben, hatten signifikant mehr Ki-67 positive CETCs im Vergleich zu Patientinnen ohne Radiotherapie (Median 48% vs. 38%, $p < 0{,}05$).

Nach der Operation sind adjuvante Therapien wie Chemo- und Strahlentherapie für Mammakarzinompatientinnen unverzichtbar, doch leider kommt es bei vielen Patientinnen zu einem Lokalrezidiv oder zur Metastasierung. In der vorliegenden Studie konnte, wie bereits für PD-L1, gezeigt werden, dass die Radiotherapie den Anteil B7-H3-positiver und Ki-67-positiver CETCs erhöhen kann (Abb. 15 a, b). Somit konnten bei Patientinnen nach einer Radiotherapie aggressivere Zellen beobachtet werden, als bei Patientinnen ohne eine Radiotherapie. Dies könnte auf entzündliche Prozesse zurückzuführen sein, die während der Strahlentherapie auftreten können. Folglich könnte die bei Mammakarzinompatientinnen beobachtete Resistenz gegen eine Radiotherapie auf die Hochregulation nicht nur von PD-L1, sondern ebenfalls auf die Hochregulation von B7-H3 in den CETCs mit einem hohen Proliferationsindex zurückzuführen sein. Kombinationstherapien mit PD-1/PD-L1 oder B7-H3 Inhibitoren könnten in Zukunft die Radioresistenz überwinden. B7-H3 sowie PD-L1 stellen vielversprechende therapierelevante Biomarker für Immuntherapie dar, die eine personalisierte Patientenstratifizierung ermöglichen könnten.

Weitere Details in: M. Pizon, D.Schott, U. Pachmann, K. Pachmann

B7-H3 (CD276) on circulating epithelial tumor cells (CETCs) correlates with proliferation marker Ki-67 and may be associated with aggressiveness of tumor in breast cancer patients

Int J Oncol. **2018**; 53(5): 2289-2299

[Publikation IV]

3.6 Nachweis von Tumorzell-Thrombozytenaggregaten im peripheren Blut von Mammakarzinompatientinnen

Thrombozyten spielen bei der Hämostase und der Thrombose eine Schlüsselrolle; es gibt jedoch immer mehr Hinweise darauf, dass sie auch bei der Entstehung von Krebs und der Metastasierung eine wichtige Rolle spielen [147]. Es wird ein Zusammenhang zwischen der Tumorentstehung und Metastasierung durch die Wechselwirkung von Thrombozyten mit Tumorzellen angenommen. Eine Gerinnungsaktivierung und die Malignom-assoziierte Thrombose sind die häufigsten klinischen Manifestation bei Krebspatienten, die mit einer schlechten Prognose und verkürzten Überlebensraten einhergehen [148,149]. Thrombozyten besitzen viele Adhäsionsmoleküle, die es ihnen ermöglichen, Aggregate mit Tumorzellen zu bilden. Die sogenannten Tumorzell-induzierten Thrombozytenaggregate schützen die Tumorzellen in der Blutbahn vor den Scherkräften des Blutes und begünstigen gleichzeitig deren Tarnung vor einem aktivierten Immunsystem [149]. Thrombozyten, die die zirkulierenden Tumorzellen ummanteln, verhindern jedoch gleichzeitig den Zugang von Antikörpern zur Oberfläche der Tumorzellen und sind wahrscheinlich der Grund dafür, dass diese Zellen in frisch abgenommenen und fixierten Blutproben nicht nachgewiesen werden können. Somit stellt die Thrombozyten-Tumorzellinteraktion einen weiteren Mechanismus der Immunevasion zirkulierender Tumorzellen im peripheren Blut dar.

Das Manuskript im Rahmen dieser Dissertation sollte die Ergebnisse der dritten und vierten Publikation ergänzen und einen weiteren Mechanismus der Immunevasion zirkulierender Tumorzellen beschreiben. Bei der experimentell durchgeführten Studie handelt es sich um ein *proof-of-principal* für den Nachweis von Tumorzell-Thrombozytenaggregaten im peripheren Blut von Mammakarzinompatientinnen. Thrombozyten sind aufgrund ihrer geringen Größe und des Fehlens eines Zellkerns im Durchlicht schwer zu erkennen. Allerdings exprimieren sie (in ihrem ruhenden und aktivierten Stadium) den CD36 Rezeptor, einen Protease-resistenten Oberflächen-Glykoprotein-IV-Scavenger-Rezeptor, der zur Thrombozytenadhäsion und kollagenabhängigen Thrombenstabilisierung beiträgt [150]. Unter Verwendung der maintrac® Methode konnte eine parallele Färbung der Patientenproben mit einem anti-EpCAM-FITC und einem anti-CD36-PE Antikörper erfolgen, wodurch der gleichzeitige Nachweis der CETCs und der Thrombozyten in der Patientenprobe ermöglicht wurde. Insgesamt wurden 36 Patientinnen in unterschiedlichen Stadien in die Studie einbezogen.

Bei keiner Patientenprobe konnten am Tag 0 der Blutentnahme CETCs mittels Immunfluoreszenz nachgewiesen werden, wohingegen bei 94% der Patientinnen an Tag 1 mehr als 50 CETCs/mL Blut detektiert werden konnten. Typischerweise wurden in den frisch entnommen Patientenproben (Tag 0) Thrombozytenaggregate beobachtet, die sich nicht mit dem anti-EpCAM Antikörper angefärbt haben (Abb. 16a).

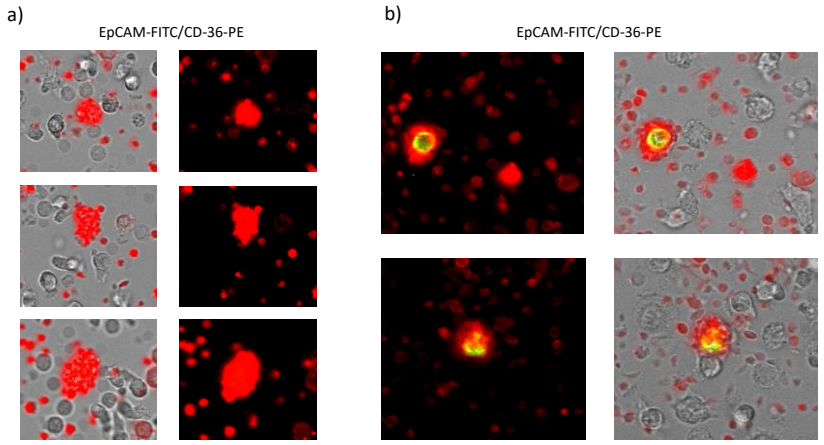

Abb.-Nr. 16: a) Immunfluoreszenzaufnahmen der Zellsuspensionen nach der Erythrozytenlyse in frisch entnommen Patientenproben (Tag 0). Bei allen Patientinnen konnte an Tag 0 nach der Blutentnahme keine EpCAM-Färbung beobachtet werden. Es konnten Thrombozytenaggregate beobachtet werden, die sich nicht mit dem anti-EpCAM Antikörper angefärbt haben. **b) Immunfluoreszenzaufnahmen der Zellsuspensionen an Tag 1 (24 Stunden nach der Blutentnahme).** Die Zellpellets wurden auf die gleiche Weise wie zum Zeitpunkt 0 gefärbt. EpCAM positive Zellen (grün) konnten nach 24 Stunden detektiert werden. Die Dichte der Thrombozytenaggregate war geringer, wodurch ein Nachweis EpCAM-positiver Zellen möglich war.

Nachdem die Blutproben 24 Stunden lang bei Raumtemperatur aufbewahrt wurden, konnten noch zahlreiche Thrombozytenaggregate detektiert werden, allerdings wurden die darunter liegenden Zellen für den anti-EpCAM-Antikörper zugänglich (Abb. 16b). Die Dichte der aggregierten Thrombozyten war geringer, was ein Zeichen dafür sein könnte, dass sich die Thrombozyten 24 Stunden nach der Blutentnahme von den CETCs abgelöst haben und die Antigene für den anti-EpCAM-Antikörper zugänglich wurden. Nach weiteren 24 Stunden (Tag 2) wurde die EpCAM-Färbung intensiver und es konnten nur noch vereinzelt Thrombozyten an den Tumorzellen detektiert werden (Abb. 17).

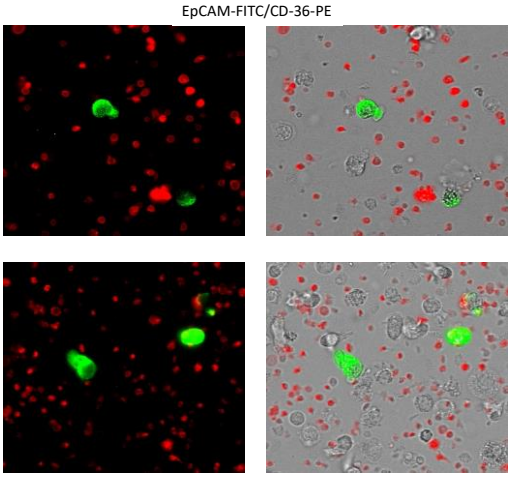

Abb.-Nr. 17: Immunfluoreszenzaufnahmen der Zellsuspensionen mit einem anti-EpCAM-FITC und einem anti-CD36-PE Antikörper 48 Stunden nach der Blutentnahme. Es konnten nahezu keine Thrombozytenaggregate nachgewiesen werden.

Die vorliegende Studie konnte zeigen, dass die Tumorzellen im Blut mit Thrombozyten Aggregate bilden, die als Schutzschild für die Scherkräfte im Blut und vor der Eliminierung durch das Immunsystem dienen. Dadurch konnten die Hinweise aus der Literatur, die auf eine enge Wechselwirkung zwischen den Tumorzellen und den Thrombozyten hindeuten, unterstrichen werden. Ein gezielter Eingriff in die Wechselwirkung zwischen den Thrombozyten und den Tumorzellen könnte eine neue und vielversprechende Antitumorstrategie darstellen, um die Metastasierung zu verhindern. Neue Studien konnten zeigen, dass die Wirkung unterschiedlicher Thromobozytenaggregationshemmer von der Inhibierung der Thrombozytenfunktion abhängen könnte [151]. Gleichzeitig erschweren die Tumorzell-induzierten Thrombozytenaggregate den Nachweis zirkulierender Tumorzellen unmittelbar nach der Blutentnahme. Dies könnte eine Erklärung für die beschriebenen Diskrepanzen in der Anzahl detektierter zirkulierender Tumorzellen zwischen den unterschiedlichen Arbeitsgruppen sein [152]. Die Verwendung von unterschiedlichen Blutröhrchen bei der Blutentnahme, die z.T. Fixative enthalten, verhindern das Ablösen der Thrombozyten von den Tumorzellen in der Blutprobe, wodurch die Maskierung der Oberflächenantigene dauerhaft erhalten bleibt und die Tumorzellen dadurch zu keinem Zeitpunkt der Analyse detektiert werden können.

Weitere Details in: D.Schott, M. Pizon, E.Schill, K. Pachmann

Evidence of platelet-covered circulating tumor cells in peripheral blood of breast cancer patients - under revision with Current Oncology

[Manuskript I]

3.7 Einfluss der adjuvanten Radiotherapie auf zirkulierende epitheliale Tumorzellen und zirkulierende Krebsstammzellen bei primär nicht-metastasiertem Mammakarzinom

Die *Liquid Biopsy*, die für die Patienten einen minimal invasiven Eingriff darstellt kann beliebig oft während des Krankheitsverlaufs wiederholt werden und liefert wichtige prognostische und prädiktive Informationen für eine personalisierte Krebstherapie. Die Effektivität einer aktuellen Therapie kann somit in Echtzeit abgebildet werden. Die fünfte Publikation sollte die Ergebnisse der dritten Publikation ergänzen und die klinische Relevanz der Identifizierung und Charakterisierung zirkulierender Tumorzellen und zirkulierender Krebsstammzellen während der adjuvanten Radiotherapie untersuchen.

Große randomisierte Studien konnten bei Mammakarzinompatientinnen im Frühstadium belegen, dass die brusterhaltende Operation in Kombination mit einer post-operativen Radiotherapie Gesamtüberlebensraten erreicht, die mit einer radikalen Mastektomie vergleichbar sind. Die post-operative Radiotherapie ist nach wie vor ein fester Bestandteil der brusterhaltenden Therapie, um die mikroskopische Resterkrankung zu kontrollieren. Sie führt zu einer signifikanten Reduktion von Lokalrezidiven im Vergleich zur brusterhaltenden Operation alleine [153,154]. Im Gegensatz zur Chemotherapie hängt die Indikation zur Radiotherapie meist von klinischen und pathologischen Merkmalen ab. Jedoch sollte auch die Radiotherapie selektiv eingesetzt werden aufgrund möglicher kurz- und langfristiger Morbidität [155]. Bislang ist noch kein vielversprechender Biomarker vorhanden, mit dem die Effektivität einer Radiotherapie kontrolliert bzw. entstehende Radioresistenzen frühzeitig erkannt werden können. Die Überwachung der Anzahl der zirkulierenden Tumorzellen und insbesondere der zirkulierenden Krebsstammzellen ist der konventionellen bildgebenden Methode zur Bewertung des Ansprechens auf unterschiedliche Therapien überlegen, wurde aber bislang für die Überwachung der Radiotherapie noch nicht eingesetzt.

Die vorliegende Studie wurde als translationale Forschungsstudie konzipiert, um die Durchführbarkeit zur Identifizierung und Charakterisierung zirkulierender Tumorzellen und zirkulierender Krebsstammzellen während der Radiotherapie mit der maintrac® Methode zu untersuchen. Dafür wurden den Patientinnen Blutproben zu drei fest definierten Zeitpunkten während der Radiotherapie entnommen: erster Tag der Radiotherapie, in der Hälfte der Radiotherapie, letzter Tag der Radiotherapie. Insgesamt

wurden in die Studie 52 Patientinnen aufgenommen, bei denen ein primär nicht metastasiertes Mammakarzinom diagnostiziert worden war, die ihre brusterhaltende Therapie am Universitätsklinikum Jena abgeschlossen hatten und für eine kurative adjuvante Radiotherapie vorgesehen waren. Da jede Therapie einen Einfluss auf die Anzahl der CETCs hat, wurde in der vorliegenden Studie ein möglicher Zusammenhang zwischen der Anzahl an CETCs und dem Hormonrezeptor- bzw. Lymphknotenstatus vor der Radiotherapie untersucht. Es konnte gezeigt werden, dass die Anzahl an CETCs vor der Radiotherapie mit einem aggressiveren Subtyp des Mammakarzinoms korrelierte. So hatten *triple negative* (ER-/PR-/HER2-) und HER2 positive Patientinnen signifikant mehr CETCs im Vergleich zu Luminal A, B Subtypen. Positive axilläre Lymphknoten sind ein Prädiktor für ein erhöhtes Risiko eines lokalen und distanten Rezidivs und stehen in direktem Zusammenhang mit dem Gesamtüberleben. Patientinnen mit einem positiven Lymphknotenstatus die bislang noch keine Chemotherapie erhalten haben, hatten vor der Radiotherapie statistisch signifikant mehr CETCs verglichen mit Patientinnen ohne befallene Lymphknoten.

Bei 90% der Patientinnen wurde in dieser Studie ein brusterhaltendes Verfahren (*breast conserving surgery*, BCS) durchgeführt. Vom Beginn der Radiotherapie bis zum Ende der Radiotherapie wurde bei 40% der Patientinnen ein Anstieg in den CETC-Zahlen und bei 44% der Patientinnen ein Abfall in den CETC-Zahlen beobachtet. Hinsichtlich der klinisch-pathologischen Merkmale gab es keinen Unterschied zwischen den Gruppen mit steigenden und sinkenden Zellzahlen. Der einzige Unterschied zwischen beiden Gruppen bestand darin, dass in der Patientengruppe mit abnehmenden Zellzahlen während der Radiotherapie nahezu alle Patientinnen (80%) mit einer neoadjuvanten Chemotherapie behandelt worden waren. Das Ziel einer neoadjuvanten Chemotherapie ist die Verkleinerung des Tumorgewebes für eine BCS. Durch die neoadjuvante Chemotherapie kann *in-vivo* das Ansprechen auf eine Therapie beurteilt werden, wodurch wichtige prognostische Informationen bereitgestellt werden. Patientinnen die durch die neoadjuvante Chemotherapie eine pathologische komplette Remission (*pathologic complete response*, pCR) erreichen, haben bessere Überlebensraten, verglichen mit Patientinnen, die trotz der neoadjuvanten Chemotherapie keine pCR erreichen. Die Verkleinerung des Tumors und die Zerstörung des Tumorgewebes führen jedoch gleichzeitig zur Mobilisierung und Freisetzung von Tumorzellen in die Blutbahn [156]. Dies könnte eine Erklärung dafür sein, dass Patientinnen nach einer neoadjuvanten Chemotherapie statistisch signifikant mehr CETCs vor Beginn der Radiotherapie hatten,

verglichen mit Patientinnen nach einer adjuvanten Chemotherapie oder verglichen mit Patientinnen, die bislang keine Chemotherapie erhalten hatten (Abb. 18a). Die abfallenden Zellzahlen während der adjuvanten Radiotherapie bei Patientinnen nach einer neoadjuvanten Chemotherapie spiegeln die Effektivität der Radiotherapie wider und verdeutlichen zudem ihren Nutzen (Abb. 18b).

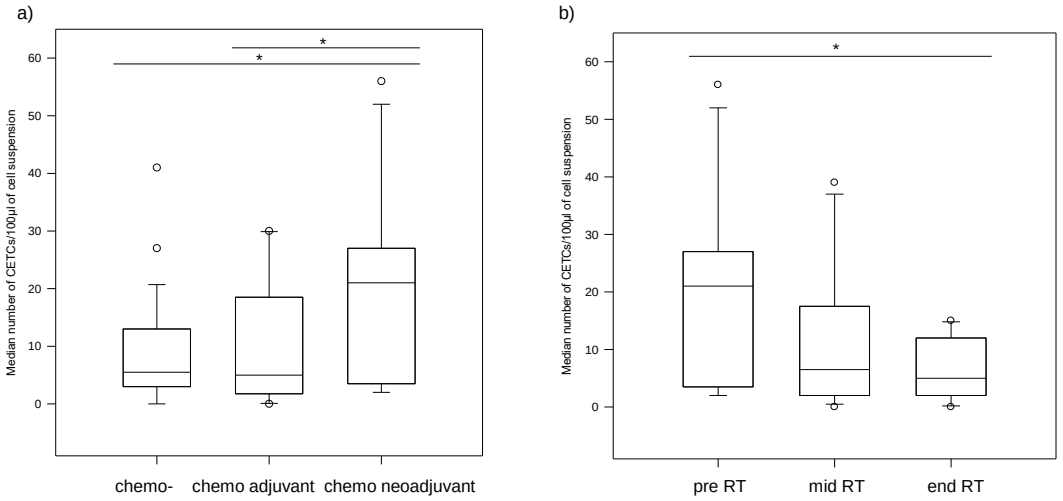

Abb.-Nr. 18: a) Anzahl an CETCs vor Beginn der Radiotherapie im Vergleich zur Chemotherapie. Patientinnen nach einer neoadjuvanten Chemotherapie hatten die höchste Anzahl an CETCs im Vergleich zu Patientinnen nach einer adjuvanten Chemotherapie (Median 21 vs. 4 CETCs/100µL Zellsuspension, $p < 0,05$), bzw. verglichen zu Patientinnen, die bislang keine Chemotherapie erhalten haben (Median 21 vs. 6 CETCs/100µL Zellsuspension, $p < 0,05$) **b) Anzahl an CETCs während der Radiotherapie bei Patientinnen nach einer neoadjuvanten Chemotherapie.** Bei Patientinnen, die nach einer neoadjuvanten Chemotherapie die höchsten Zellzahlen vor Beginn der Radiotherapie hatten, konnte ein kontinuierlicher, statistisch signifikanter Abfall der CETC Zahlen während der Radiotherapie beobachtet werden (Median 21 vs. 5 CETCs/100µL Zellsuspension, $p < 0,05$).

Parallel zu den CETCs wurden auch die zirkulierenden Krebsstammzellen bei den Patientinnen unter Verwendung des funktionellen 3D-Zellkulturansatzes untersucht, bei dem die zirkulierenden Krebsstammzellen *in-vitro* zu den sogenannten Tumorsphären heranwachsen können. Bei fast der Hälfte der Patientinnen, unabhängig vom molekularen Subtyp, konnten zu mindestens einem Messzeitpunkt während der Radiotherapie die Entstehung von Tumorsphären *in-vitro* beobachtet werden, die nach der Charakterisierung mittels Immunfluoreszenz die typischen Stammzellmarker exprimierten. Vergleichbar zu der Anzahl an CETCs, war die Anzahl an *in-vitro* gewachsenen Tumorsphären nach einer neoadjuvanten Chemotherapie höher, als bei Patientinnen, die eine adjuvante Chemotherapie erhalten hatten. Die höchste Anzahl an Tumorsphären konnte bei Patientinnen beobachtet werden, die bislang keine Chemotherapie erhalten hatten (Abb. 19a). Bei Patientinnen nach einer neoadjuvanten Chemotherapie konnte die Entstehung von Tumorsphären *in-vitro* häufiger beobachtet werden (61%), als bei Patientinnen, die eine adjuvante (42%) oder keine Chemotherapie (48%) erhalten hatten.

In Hinblick auf die Anzahl der Tumorsphären konnten erneut zwei Patientengruppen mit steigenden und abfallenden Zellzahlen während der Radiotherapie festgestellt werden. Die Patientengruppe mit ansteigenden Zahlen an Tumorsphären *in-vitro* beinhaltete Patientinnen, mit einem ungünstigen Hormonrezeptorstatus und einem hohen Ki-67 Index. Außerdem hatte über die Hälfte der Patientinnen in dieser Gruppe bislang noch keine Chemotherapie erhalten. In 63% der Patientinnen nach einer neoadjuvanten Chemotherapie und in 69% der Patientinnen ohne eine Chemotherapie konnte ein Anstieg in der Anzahl an Tumorsphären *in-vitro* während der Radiotherapie festgestellt werden. Neben den CETCs können folglich auch Zellen mit Krebsstammzelleigenschaften während einer neoadjuvanten Chemotherapie freigesetzt werden. Ein antidromischer Verlauf in der Anzahl an CETCs und in der Anzahl an Tumorsphären *in-vitro* während der Radiotherapie konnte häufig in der Patientengruppe, die zuvor eine neoadjuvante Chemotherapie erhalten hatte beobachtet werden. Während die Anzahl an CETCs bei Patientinnen nach einer neoadjuvanten Chemotherapie abnahm, stieg gleichzeitig die Anzahl an Tumorsphären *in-vitro*. Dies veranschaulichte, dass die Krebsstammzellen, wie bereits in einer Vielzahl von Studien beschrieben, eine hohe Radioresistenz aufweisen.

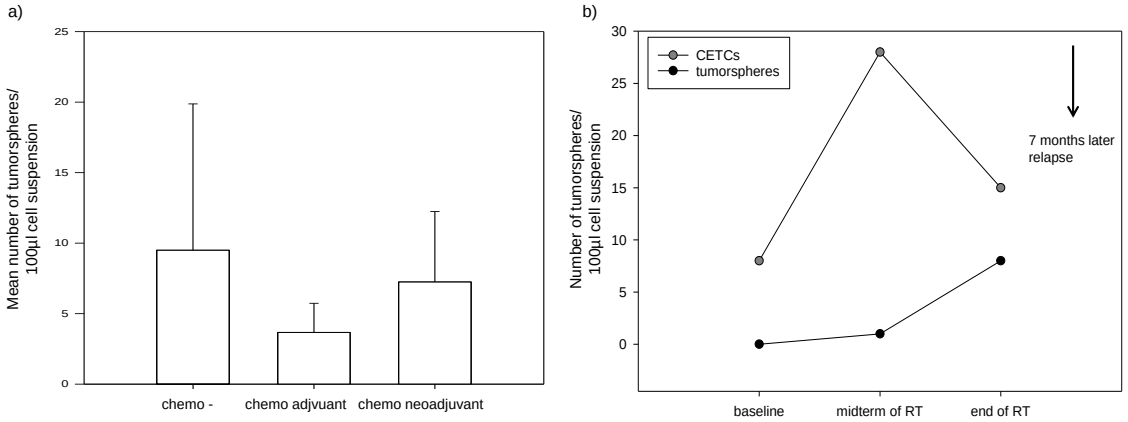

Abb.-Nr. 19: a) Anzahl an Tumorsphären vor Beginn der Radiotherapie im Vergleich zur Chemotherapie. Patientinnen ohne Chemotherapie hatten die höchste Anzahl an Tumorsphären im Vergleich zu Patientinnen nach einer adjuvanten oder neoadjuvanten Chemotherapie. **b) Verlauf in der Anzahl an CETCs und in der Anzahl an *in-vitro* gewachsener Tumorsphären während der Radiotherapie bei einer Mammakarzinompatientin.** Die Patientin wurde mit einem Stadium II Tumor und einem *triple* negativem Hormonrezeptorstatus diagnostiziert. Die Patientin erhielt sowohl eine neoadjuvante, als auch eine adjuvante Chemotherapie. Während der Radiotherapie konnte ein Anstieg in der Anzahl an CETCs, als auch ein Anstieg in der Anzahl an *in-vitro* gewachsener Tumorsphären beobachtet werden. 7 Monate nach Ende der Radiotherapie erlitt die Patientin ein Rezidiv.

In der kurzen Nachbeobachtungszeit von 7 Monaten konnte bei einer *triple* negativen Patientin bereits ein Rezidiv beobachtet werden. Die Patientin wurde bei der Erstdiagnose mit einem Stadium II (T2N1M0) Tumor diagnostiziert und mit einer neoadjuvanten Chemotherapie behandelt. Da sie keine pCR erreicht hatte, wurde sie zusätzlich mit einer

adjuvanten Chemotherapie behandelt. Während der Radiotherapie konnte ein Anstieg sowohl in der Anzahl der CETCs, als auch in der Anzahl der *in-vitro* gewachsenen Tumorsphären beobachtet werden. 7 Monate nachdem die Radiotherapie erfolgreich abgeschlossen wurde, erlitt die Patientin ein Rezidiv (Abb. 19b). Sollte sich diese Beobachtung bei einer größeren Anzahl von Patientinnen bestätigen, könnte die Überwachung der Anzahl an CETCs und insbesondere der Anzahl an kultivierten Tumorsphären mit Hilfe der *Liquid Biopsy* während der adjuvanten Radiotherapie die Aktivität der residualen Tumorlast nach einer brusterhaltenden Operation direkt widerspiegeln. Dies könnte dazu beitragen Patientinnen mit einem erhöhten Risiko für ein Lokalrezidiv frühzeitig zu identifizieren und möglicherweise zeitnah zusätzliche Therapieansätze einzuleiten.

Weitere Details in: D.Schott, M. Pizon, E.Schill, K. Pachmann, R. Schobert, A.Wittig, M. Mäurer

Influence of adjuvant radiotherapy on circulating epithelial tumor cells and circulating cancer stem cells in primary non-metastatic breast cancer

Transl Oncol. **2021**; 14(3):101009

[Publikation V]

3.8 Einfluss der endokrinen Therapie auf das Verhältnis von Androgenrezeptor (AR) zu Östrogenrezeptor (ER) positiven zirkulierenden epithelialen Tumorzellen (CETCs) beim Mammakarzinom

In den letzten Jahren gewinnt der Androgenrezeptor (*androgen receptor*, AR) beim Mammakarzinom immer mehr an Bedeutung. Er spielt eine entscheidende Rolle bei der Pathologie und dem Fortschreiten des Mammakarzinoms. Interessanterweise wird der Androgenrezeptor in mehr als 70% der Mammakarzinome exprimiert, jedoch scheint seine Funktion in den verschiedenen Subtypen des Mammakarzinoms unterschiedlich zu sein [105]. Somit könnte der AR ein potenzielles therapeutisches *target* bei ER positiven, aber auch bei ER negativen Mammakarzinomen sein. Der AR rückt aufgrund seines Zusammenspiels mit dem ER als ein interessanter prognostischer und therapierelevanter Marker in den Fokus der Forschung. Es konnte bereits gezeigt werden, dass nicht das Expressionslevel des AR alleine mit dem rezidivfreien Überleben korreliert, sondern dass dem AR:ER-Verhältnis eine viel größere Bedeutung zukommt. Patientinnen mit einem hohen AR:ER Verhältnis (*ratio* $\geq$ 2) hatten ein kürzeres rezidivfreies Überleben, als Patientinnen mit einem niedrigeren AR:ER Verhältnis wenn sie mit anti-Östrogenen oder einer Chemotherapie behandelt wurden [157]. Der Grund dafür könnte die Adaption der Tumorzellen von einer Östrogenabhängigkeit zur Androgenabhängigkeit sein [111]. Folglich kann der AR als ein prädiktiver Marker für die Hormontherapie herangezogen werden. Anti-AR Therapien könnten zudem dabei helfen, das hohe AR:ER-Verhältnis bei Tamoxifen resistenten Patientinnen zu reduzieren und somit eine erneute Tamoxifensensibilität erzielen. Aus diesem Grund könnte die kontinuierliche Überwachung des ER- und des AR-Status in den CETCs mit Hilfe der *Liquid Biopsy* dabei helfen, Resistenzen während einer endokrinen Therapie frühzeitig zu entdecken. Im Rahmen der sechsten Publikation dieser Dissertation, die an die dritte und fünfte Publikation anschließt, sollte die klinische Relevanz der Langzeitanalyse zirkulierender Tumorzellen während der Hormontherapie untersucht werden. Dafür wurde das Verhältnis von AR zu ER in den CETCs mit einer erweiterten maintrac® Methode bei insgesamt 66 Mammakarzinompatientinnen in unterschiedlichen Stadien untersucht. Im Gegensatz zu den Daten von Fuji *et al*., die nur bei 18% der metastasierten Mammakarzinompatientinnen AR-positive zirkulierende Tumorzellen fanden [113], konnten in der vorliegenden Studie AR-positive CETCs mittels Immunfluoreszenz und spezifischer Antikörper bei allen

untersuchten Patientinnen detektiert werden (Abb. 20a). ER-positive CETCs konnten bei 94% der Patientinnen nachgewiesen werden (Abb. 20b).

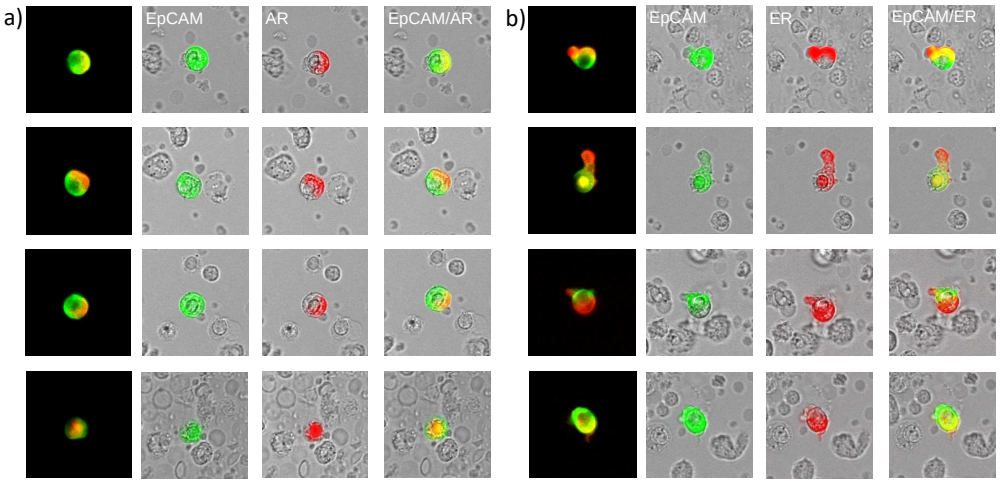

Abb.-Nr. 20: a) Darstellung der Zweitantikörperfärbung der CETCs mit spezifischen Antikörpern gegen a) EpCAM und AR und b) EpCAM und ER. EpCAM war mit FITC, AR und ER mit Phycoerythrin konjugiert. Die Expressionsintensität von EpCAM, AR und ER variierten von Zelle zu Zelle bei einer Patientin.

Der Prozentsatz AR-positiver CETCs lag median bei 90% und variierte von 25% bis 100%. Es ist bekannt, dass *triple* negative Patientinnen häufiger einen axillären Lymphknotenbefall und ein kürzeres Gesamtüberleben aufweisen. Aufgrund des Fehlens eines hormonellen Rezeptors als *target* sind die Chemo- und Strahlentherapie die einzige Option für die Behandlung von *triple* negativen Mammakarzinompatientinnen [158]. Es konnte in der vorliegenden Studie ein Zusammenhang zwischen einem aggressiveren Subtyp des Mammakarzinoms und dem prozentualen Anteil AR-positiver CETCs beobachtet werden. Patientinnen mit einem *triple* negativen Mammakarzinom hatten statistisch signifikant mehr AR-positive CETCs im Vergleich zu Hormonrezeptor-positiven Patientinnen. Eine AR-gerichtete Therapie könnte eine neue Option für die Behandlung von *triple* negativen Mammakarzinompatientinnen sein.

Interessanterweise war in der gesamten Studienpopulation der Anteil AR-positiver CETCs signifikant höher als der Anteil ER-positiver CETCs (Abb. 21a). So lag der mediane Anteil AR-positiver CETCs bei 90%, wohingegen der Anteil ER-positiver CETCs bei 50% lag. Auch beim Primärtumor konnte in vielen Studien eine höhere AR Expression im Vergleich zur ER und PR Expression beschrieben werden [159]. Primär scheint der AR für ein schlechtes Ansprechen auf eine ER-gerichtete endokrine Therapie, insbesondere eine Tamoxifen Therapie, verantwortlich zu sein. Dies wird durch die vorliegenden Ergebnisse bestätigt, die zeigen konnten, dass Patientinnen nach einer Tamoxifen-Therapie ein höheres AR:ER-Verhältnis aufwiesen als Patientinnen ohne eine endokrine Therapie

(Abb. 21b). Die Behandlung mit Aromataseinhibitoren hatte keinen Einfluss auf das AR:ER-Verhältnis.

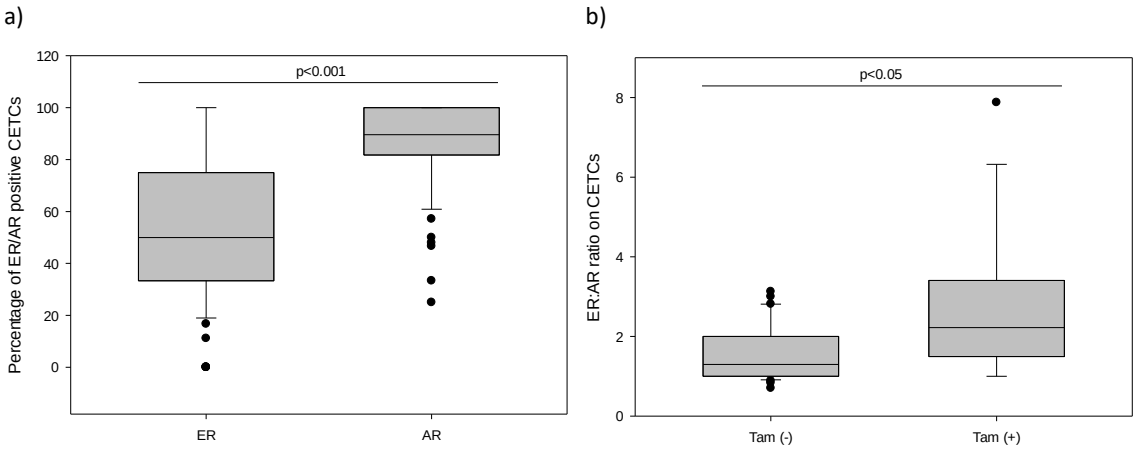

Abb.-Nr. 21: a) Prozentualer Anteil ER- und AR-positiver CETCs bei allen untersuchten Mammakarzinompatientinnen. Der prozentuale Anteil AR-positiver CETCs war statistisch signifikant höher als der Anteil ER positiver CETCs (Median 90% vs. 50%, $p < 0{,}001$). **b) Boxplot-Analyse des ER:AR-Verhältnisses auf den CETCs bei Patientinnen mit und ohne Tamoxifen-Therapie.** Bei Patientinnen die eine Tamoxifen Therapie erhalten haben, konnte ein statistisch signifikant höheres ER:AR-Verhältnis auf den CETCs beobachtet werden im Vergleich zu Patientinnen ohne eine Tamoxifen Therapie (medianes *ratio* 2,2 vs. 1,3, $p < 0{,}05$).

In-vitro Studien haben gezeigt, dass die Sensitivität für Tamoxifen bei Tamoxifen-resistenten Zelllinienzellen durch die Inhibierung des AR-Signalweges wiederhergestellt werden konnte. Diese Ergebnisse deuten darauf hin, dass die hohe AR-Expression bei ER-positiven Tumoren einen Mechanismus der Tamoxifenresistenz darstellt [157]. Zusammengenommen deuten diese Daten darauf hin, dass die Tumore mit einem AR:ER Verhältnis von ≥ 2 von einem ER-abhängigen zu einem AR-abhängigen Wachstum wechseln, wobei die ER-Expression kontinuierlich abnimmt [160]. Folglich könnte die Blockade des AR als ein therapeutisches *target* für die Behandlung Tamoxifen-resistenter Mammakarzinome dienen. Die Ergebnisse der vorliegenden Studie deuten darauf hin, dass der Nachweis und die Überwachung des AR und des ER Status, insbesondere während einer Tamoxifen Therapie, als ein Biomarker für die Identifizierung von Patientinnen verwendet werden kann, die eine Tamoxifen Resistenz entwickeln könnten.

Weitere Details in: M. Pizon, D. Lux, U. Pachmann, K. Pachmann, D. Schott

Influence of endocrine therapy on the ratio of androgen receptor (AR) to estrogen receptor (ER) positive circulating epithelial tumor cells (CETCs) in breast cancer

J Transl Med. **2018**; 16(1):356

[Publikation VI]

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5 Publikationen

5.1 Darstellung des Eigenanteils

Die in dieser Arbeit enthaltenen Publikationen wurden in Zusammenarbeit und Mitwirkung anderer Mitarbeiter der Arbeitsgemeinschaft des Labors Dr. Pachmann und der Simfo GmbH, sowie mit anderen Arbeitsgruppen anderer Institutionen erarbeitet. Hierzu zählt die Klinik für Strahlentherapie und Radioonkologie am Universitätsklinikum Jena, der Lehrstuhl für Organische Chemie I der Universität Bayreuth und die zahlreichen nationalen, als auch internationalen niedergelassenen Onkologen und Gynäkologen, die ihre Patientenproben in das Labor Dr. Pachmann zur Untersuchung geschickt und ihre Patienten hinsichtlich der Forschungsvorhaben aufgeklärt haben, sodass das Restblut der Patienten für wissenschaftliche Zwecke genutzt werden durfte.

Es folgt eine detaillierte Darstellung der Beiträge der Co-Autoren zu den jeweiligen Publikationen und des Manuskripts.

5.1.1 Eigenanteil: Publikation I

Die Ergebnisse zu diesem Thema wurden im Journal ***Oncotarget*** mit dem folgenden Titel veröffentlicht (DOI: 10.18632/oncotarget.10174):

"The number of tumorspheres cultured from peripheral blood is a predictor for presence of metastasis in patients with breast cancer"

von den Autoren

Monika Pizon, Dorothea Schott, Ulrich Pachmann, Katharina Pachmann

Eigenanteil: Vorbereitung, Konzeption, Durchführung und Auswertung der genetischen, immunfluorimetrischen und 3D-Zellkultur *Assays*:
Quantifizierung der CETCs mit der etablierten maintrac® Methode, Etablierung und Validierung des eigenständig entwickelten Zellkulturverfahrens zum Nachweis cCSCs aus dem peripheren Blut von Krebspatienten, weitere immunologische Charakterisierung der kultivierten Tumorsphären, Nachweis der ALDH1-Aktivität, relative Genexpressionsanalysen mittels RT-PCR, Optimierung der Olympus Software zur Messung und Auswertung der Immunfluoreszenz Proben; *Single Cell Picking*;
Statistische Auswertung der Daten mittels SigmaPlot;
Verfassen des Manuskripts inklusive Diskussion und Interpretation der Ergebnisse, sowie Korrektur und Revision des Manuskriptes, graphische Repräsentation und Bearbeitung der Abbildungen.

M. Pizon: Konzeption und Durchführung der biochemischen, genetischen und 3D-Zellkultur *Assays*:
Etablierung und Validierung des eigenständig entwickelten Zellkulturverfahrens zum Nachweis cCSCs aus dem peripheren Blut von Krebspatienten, weitere immunologische Charakterisierung der kultivierten Tumorsphären, Interpretation der Ergebnisse, statistische Auswertung der Daten mittels SigmaPlot, Verfassen des Manuskriptes.

U. Pachmann: Korrektur und Revision des Manuskriptes.

K. Pachmann: Konzeption des neuen 3D-Zellkultur *Assays*, Interpretation der Daten, Diskussion und Korrektur des Manuskriptes.

Geschätzter Eigenanteil: circa 45%

5.1.2 Eigenanteil: Publikation II

Die Ergebnisse zu diesem Thema wurden im Journal ***Cancers*** mit dem folgenden Titel veröffentlicht (DOI.org/10.3390/cancers14061476)

"Chick Chorioallantoic Membrane (CAM) Assays as a model of patient- derived xenografts from circulating cancer stem cells (cCSCs) in breast cancer patients"

von den Autoren

Monika Pizon, Dorothea Schott, Ulrich Pachmann, Rainer Schobert, Marek Pizon, Marta Wozniak, Rafal Bobinski, Katharina Pachmann

Eigenanteil: Konzeption, Durchführung, Validierung und Auswertung der immunfluorimetrischen und 3D- Zellkultur *Assays*:
Quantifizierung der CETCs mit der maintrac® Methode, Kultivierung von Tumorsphären aus Patientenblut, weitere immunologische Charakterisierung der Tumorsphären, ALDH1-Assay, Vorbereitung der Proben für den CAM *Assay*;
Statistische Auswertung der Daten mittels SigmaPlot;
Verfassen des Manuskripts inklusive Diskussion und Interpretation der Ergebnisse, sowie Korrektur und Revision des Manuskriptes, graphische Repräsentation und Bearbeitung der Abbildungen.

M. Pizon: Konzeption, Durchführung, Validierung und Auswertung der immunfluorimetrischen und 3D- Zellkultur *Assays*:
Kultivierung von Tumorsphären aus Patientenblut, weitere immunologische Charakterisierung der Tumorsphären;
statistische Auswertung der Daten mittels SigmaPlot;
Interpretation der Ergebnisse, Verfassen des Manuskripts inklusive Diskussion sowie Korrektur und Revision des Manuskripts.

U. Pachmann: Korrektur und Revision des Manuskripts.

R. Schobert: Vorbereitung und Konzeption der Studie, Verfassen von Studienprotokoll und Ethikantrag, Projektadministration, Beauftragung und Überwachung des durch Mitarbeiter durchgeführten CAM-*Assays*.

Marek Pizon: Korrektur und Revision des Manuskriptes.

M. Wozniak: Auswertung der histologischen Präparate, Korrektur des Manuskriptes.

R. Bobinski: Korrektur und Revision des Manuskriptes.

K. Pachmann: Konzeption der Studie, Interpretation der Daten, Korrektur und Revision des Manuskriptes.

Geschätzter Eigenanteil: circa 40%

5.1.3 Eigenanteil: Publikation III

Die Ergebnisse zu diesem Thema wurden beim Journal ***Oncotarget*** mit dem folgenden Titel veröffentlicht (DOI: 10.18632/oncotarget.20346):

"Sensitive detection of PD-L1 expression on circulating epithelial tumor cells (CETCs) could be a potential biomarker to select patients for treatment with PD-1/PD-L1 inhibitors in early and metastatic solid tumors"

von den Autoren

Dorothea Schott, Monika Pizon, Ulrich Pachmann, Katharina Pachmann

Eigenanteil: Vorbereitung, Konzeption, Durchführung und Dokumentation der experimentell durchgeführten Studie.
Konzeption, Durchführung und Auswertung der immunfluorimetrischen und molekularzytogenischen *Assays*:
Quantifizierung der CETCs mit der maintrac® Methode, Etablierung und Validierung der erweiterten maintrac® Methode zum Nachweis PD-L1/PD-L2 positiver CETCs, FISH für den Nachweis von PD-L1 Amplifikaten in den CETCs, Zellkultur unterschiedlicher Zelllinienzellen zur Kontrolle und Etablierung des *Assays*;
Statistische Auswertung der Daten mittels SigmaPlot;
Optimierung der Olympus Software zur Messung und Auswertung der Immunfluoreszenz Proben;
Verfassen des Manuskriptes inklusive Interpretation der Ergebnisse sowie Korrektur und Revision des Manuskripts, graphische Repräsentation und Bearbeitung der Abbildungen.

M. Pizon: Vorbereitung und Konzeption der experimentell durchgeführten Studie.
Konzeption, Durchführung und Auswertung der immunhistochemischen *Assays:*
Hilfe bei der Etablierung und Validierung der erweiterten maintrac® Methode zum Nachweis PD-L1/PD-L2 positiver CETCs, Zellkultur unterschiedlicher Zelllinienzellen zur Kontrolle und Etablierung des *Assays*;
Interpretation der Ergebnisse, Manuskriptkonzeption, Diskussion und Korrektur des Manuskriptes.

U. Pachmann: Korrektur und Revision des Manuskriptes.

K. Pachmann: Interpretation der Daten, Korrektur und Revision des Manuskriptes.

Geschätzter Eigenanteil: circa 75%

5.1.4 Eigenanteil: Publikation IV

Die Ergebnisse zu diesem Thema wurden im ***International Journal of Oncology*** mit dem folgenden Titel veröffentlicht (DOI: 10.3892/ijo.2018.4551):

"B7-H3 on circulating epithelial tumor cells correlates with the proliferation marker, Ki-67, and may be associated with the aggressiveness of tumors in breast cancer patients"

von den Autoren

Monika Pizon, Dorothea Schott, Ulrich Pachmann, Katharina Pachmann

Eigenanteil: Vorbereitung, Konzeption, Durchführung und Dokumentation der experimentell durchgeführten Studie.
Konzeption, Durchführung und Auswertung der immunhistochemichen *Assays*:
Quantifizierung der CETCs mit der maintrac® Methode, Etablierung und Validierung der erweiterten maintrac® Methode zum Nachweis B7-H3 und Ki-67 positiver CETCs, Zellkultur unterschiedlicher Zelllinienzellen zur Kontrolle und Etablierung des *Assays*.
Statistische Auswertung der Daten mittels SigmaPlot;
Optimierung der Olympus Software zur Messung und Auswertung der Immunfluoreszenz Proben;
Verfassen des Manuskriptes inklusive Interpretation der Ergebnisse, sowie Korrektur und Revision des Manuskripts, graphische Repräsentation und Bearbeitung der Abbildungen.

M. Pizon: Konzeption und Auswertung der immunfluorimetrischen *Assays*: Etablierung und Validierung der erweiterten maintrac® Methode zum Nachweis B7-H3 und Ki-67 positiver CETCs, Zellkultur unterschiedlicher Zelllinienzellen zur Kontrolle und Etablierung des *Assays*.; Statistische Auswertung der Daten mittels SigmaPlot;
Interpretation der Ergebnisse, Verfassen des Manuskripts inklusive Diskussion sowie Korrektur und Revision des Manuskripts.

U. Pachmann: Korrektur und Revision des Manuskriptes

K. Pachmann: Konzeption der experimentell durchgeführten Studie. Interpretation der Daten, Korrektur und Revision des Manuskriptes.

Geschätzter Eigenanteil: circa 60%

5.1.5 Eigenanteil: Manuskript I

Die Ergebnisse zu diesem Thema wurden im Journal ***Current Oncology*** mit dem folgenden Titel zur Publikation eingereicht:

"Evidence of platelet-covered circulating tumor cells in peripheral blood of breast cancer patients"

von den Autoren

Dorothea Schott, Monika Pizon, Erika Schill, Katharina Pachmann

Eigenanteil: Vorbereitung, Konzeption, Durchführung und Auswertung der experimentell durchgeführten Studie.
Konzeption, Durchführung und Auswertung der immunfluorimetrischen *Assays*:
Quantifizierung der CETCs mit der maintrac® Methode, Nachweis und Validierung der Interaktion von CETCs und Thrombozyten mit der erweiterten maintrac® Methode.
Statistische Auswertung der Daten mittels SigmaPlot;
Optimierung der Olympus Software zur Messung und Auswertung der Immunfluoreszenz Proben;
Verfassen des Manuskriptes inklusive Interpretation der Ergebnisse, graphische Repräsentation und Bearbeitung der Abbildungen.

M. Pizon: Konzeption und Auswertung der immunfluorimetrischen *Assays*: Etablierung und Validierung der erweiterten maintrac® Methode zum Nachweis der Interaktion von CETCs und Thrombozyten.
Interpretation der Ergebnisse, Manuskriptkonzeption, Diskussion und Korrektur des Manuskriptes, graphische Repräsentation und Bearbeitung der Abbildungen.

E. Schill: Hilfe bei der Durchführung der CD36 Immunfluoreszenzfärbungen zum Nachweis der Thrombozyten in der Patientenprobe.

K. Pachmann: Konzeption der experimentell durchgeführten Studie. Interpretation der Daten, Verfassen entsprechender Manuskriptpassagen, Diskussion und Korrektur des Manuskriptes, Projektadministration.

Geschätzter Eigenanteil: circa 70%

5.1.6 Eigenanteil: Publikation V

Die Ergebnisse zu diesem Thema wurden im Journal ***Translational Oncology*** mit dem folgenden Titel veröffentlicht (DOI: 10.1016/j.tranon.2021.101009):

"Influence of adjuvant radiotherapy on circulating epithelial tumor cells and circulating cancer stem cells in primary non-metastatic breast cancer."

von den Autoren

Dorothea Schott, Monika Pizon, Ulrich Pachmann, Katharina Pachmann, Rainer Schobert, Andrea Wittig, Matthias Mäurer

Eigenanteil: Vorbereitung, Konzeption, Durchführung und Dokumentation der translationalen Studie; Verfassen von Studienprotokoll und Ethikantrag.
Konzeption, Durchführung und Auswertung der molekularzytogenischen, immunfluorimetrischen und 3D-Zellkultur *Assays*:
Quantifizierung der CETCs mit der maintrac® Methode, Etablierung und Validierung der erweiterten maintrac® Methode zum Nachweis ER und PR positiver CETCs, FISH für den Nachweis von HER2 Amplifikaten in den CETCs. Kultivierung von Tumorsphären aus Patientenblut, weitere immunologische Charakterisierung der Tumorsphären, ALDH1-Assay;
Statistische Auswertung der Daten mittels SigmaPlot;
Optimierung der Olympus Software zur Messung und Auswertung der Immunfluoreszenz Proben;
Verfassen des Manuskriptes inklusive Interpretation der Ergebnisse sowie Korrektur und Revision des Manuskripts, graphische Repräsentation und Bearbeitung der Abbildungen.

M. Pizon: Konzeption und Auswertung der immunfluorimetrischen und 3D-Zellkultur *Assays*:
Hilfe bei der Validierung der erweiterten maintrac® Methode zum Nachweis ER und PR positiver CETCs;
Verfassen entsprechender Manuskriptpassagen, Bearbeitung der Abbildungen, Diskussion und Korrektur des Manuskriptes.

U. Pachmann: Korrektur und Revision des Manuskriptes.

K. Pachmann: Konzeption der translationalen Studie. Interpretation der Daten, Korrektur und Revision des Manuskriptes.

R. Schobert: Korrektur und Revision des Manuskriptes.

A. Wittig: Korrektur und Revision des Manuskriptes.

M. Mäurer: Konzeption der translationalen Studie. Verfassen entsprechender Manuskriptpassagen, Betreuung und Aufklärung der Patienten. Verfassen von Studienprotokoll und Ethikantrag.

Geschätzter Eigenanteil: circa 75%

5.1.7 Eigenanteil: Publikation VI

Die Ergebnisse zu diesem Thema wurden im ***Journal of Translational Medicine*** mit dem folgenden Titel veröffentlicht (DOI: 10.1186/s12967-018-1724-z):

"Influence of endocrine therapy on the ratio of androgen receptor (AR) to estrogen receptor (ER) positive circulating epithelial tumor cells (CETCs) in breast cancer."

von den Autoren

Monika Pizon, Daniel Lux, Ulrich Pachmann, Katharina Pachmann, Dorothea Schott

Eigenanteil: Konzeption und Auswertung der immunfluorimetrischen und molekularzytogenischen *Assays*:
Validierung der erweiterten maintrac® Methode zum Nachweis ER und AR positiver CETCs, Zellkultur unterschiedlicher Zelllinienzellen zur Kontrolle und Etablierung des *Assays;*
Statistische Auswertung der Daten mittels SigmaPlot;
Verfassen des Manuskriptes inklusive Interpretation der Ergebnisse sowie Korrektur und Revision des Manuskripts, graphische Repräsentation und Bearbeitung der Abbildungen.

M. Pizon: Konzeption und Auswertung der immunfluorimetrischen *Assays*:
Validierung der erweiterten maintrac® Methode zum Nachweis ER und AR positiver CETCs;
Interpretation der Ergebnisse, Verfassen des Manuskripts inklusive Diskussion sowie Korrektur und Revision des Manuskripts. Graphische Repräsentation und Bearbeitung der Abbildungen.

D. Lux: Durchführung der immunfluorimetrischen *Assays*:
Validierung der erweiterten maintrac® Methode zum Nachweis ER und AR positiver CETCs;
Statistische Auswertung der Daten mittels SigmaPlot.

U. Pachmann: Konzeption, Korrektur und Revision des Manuskriptes

K. Pachmann: Konzeption der experimentellen Studie. Interpretation der Daten, Korrektur und Revision des Manuskriptes.

Geschätzter Eigenanteil: circa 45%

5.2 Publikation I

The number of tumorspheres cultured from peripheral blood is a predictor for presence of metastasis in patients with breast cancer

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The number of tumorspheres cultured from peripheral blood is a predictor for presence of metastasis in patients with breast cancer

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Keywords: *breast cancer, circulating epithelial tumor cells, circulating cancer stem cells, predictor for presence of metastasis*

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ABSTRACT

Background: Tumor metastases are the major cause of cancer morbidity and mortality. A subpopulation of tumor cells with stem-like properties is assumed to be responsible for tumor invasion, metastasis, heterogeneity and therapeutic resistance. This population is termed cancer stem cells (CSCs). We have developed a simple method for identification and characterization of circulating cancer stem cells among circulating epithelial tumor cells (CETCs).

Methods: CETCs were cultured under conditions favoring growth of tumorspheres from 72 patients with breast cancer, including a subpopulation of 23 patients with metastatic disease. CETCs were determined using the maintrac® method. Gene expression profiles of single CETCs and tumorspheres of the same patients were analyzed using qRT-PCR.

Results: Sphere formation was observed in 79 % of patients. We found that the number of tumorspheres depended on stage of disease. Furthermore, the most important factor for growing of tumorspheres is obtaining chemotherapy. Patients with chemotherapy treatment had lower numbers of tumorspheres compared to patients without chemotherapy. Patients with HER2 positive primary tumor had higher number of tumorspheres. Analysis of surface marker expression profile of tumorspheres showed that cells in the spheres had typical phenotype of cancer stem cells. There was no sphere formation in a control group with 50 healthy donors.

Conclusion: This study demonstrates that a small fraction of CETCs has proliferative activity. Identifying the CETC subset with cancer stem cell properties may provide more clinically useful prognostic information. Chemotherapy is the most important component in cancer therapy because it frequently reduces the number of tumorspheres.

INTRODUCTION

Breast cancer is the most common cancer in women worldwide and development of distant metastases is a main reason for cancer mortality. Distant metastasis formation is due to spread of cancer cells from the primary location through circulation [1, 2]. Presence of circulating tumor cells was first demonstrated in 1869 [3] and their existence in the blood have been associated with metastasis formation. Enumeration, longitudinal monitoring and characterization of circulating tumor cells can contribute to individually tailor therapy, improve design of personalized therapies, monitor treatment efficiency, enhance prognostic accuracy and advance our understanding of the biology of metastatic disease [4, 5, 6]. Recently the hypothesis has been proposed that a more aggressive subpopulation of circulating tumor cells, circulating cancer stem cells (cCSCs), are the source of metastatic spread from the primary tumor [6, 7]. The presence of cancer stem cells (CSCs) in neoplastic tissue has been a long standing hypothesis, and recently, these cells have first been identified in leukemia and subsequently in different solid tumors [8, 9]. CSCs carry typical stem cells properties; they are capable of undergoing extensive proliferation and self-renewal through asymmetric division and differentiation into non-

tumorigenic cancer cells. Furthermore CSCs have been found to have increased resistance to chemotherapeutic agents and radiation [10, 11]. CSCs have been identified and isolated from solid tumor tissue or cancer cell lines by different methods such as CSC-specific cell surface marker expression and aldehyde dehydrogenase (ALDH1) activity and their ability to grow as floating spheres (tumorspheres) [12, 13]. Multiple markers including CD44, CD24 and EpCAM are used to identify breast cancer stem cells. Up-regulation of the expression of CD44 a marker for stem cells of several types of cancer and the major hyaluronan receptor increases tumor growth and has an anti-apoptotic effect [13, 14]. CD24 has been investigated in combination with CD 44 or other surface antigens in various cancers [13, 15]. The overexpression of the Epithelial Cell Adhesion Molecule (EpCAM) enhances the proliferative and invasive capacities of tumors [16]. Aldehyde dehydrogenase 1 (ALDH1) is a detoxifying enzyme responsible for oxidation of retinol to retinoic acid and may have a role in early differentiation of stem cells [13, 17].

Transcription factors such as Oct4, Sox2 and Nanog are required to maintain pluripotency and self-renewal capacity of cancer stem cells and play an important role in the uncontrolled proliferation of cancer cells [18].

A functional approach, tumorsphere formation assay, based on the unique property of stem cells to survive and grow in suspension culture, is successfully applied to enrich stem cells from invasive tumor samples or cancer cell lines. Tumorspheres have been reported to display typical stem cell surface markers like $CD44^{+}$/$CD24^{-}$, overexpress neoangiogenic and cytoprotective factors and genes of pluripotency [19, 20].

The aim of the present study was to develop a new approach based on functional, pheno-and genotypic features of CSCs for detection and characterization of cells with proliferative activity and cancer stem cell properties among circulating epithelial tumor cells.

RESULTS

Patients characteristics are given in Table 1. Among the 72 patients there were 49(68%) patients with non-metastatic and 23 (32%) with metastatic disease. The primary tumors were histologically positive for ER and PR in 44 (71%) patients, and positive for HER-2/neu in 6 (11%) patients.

During culture in non-adhesive, suspension culture the CETCs with proliferative activity grew as spherical tumorspheres. After 3-5 days of culture first signs of sphere formation were observed. Spheres grew gradually over time and after 21 days of culture all spheroids had a diameter between 30 and 60 µm (median 40) (Figure 1). For cultivation of tumorspheres we included patients with cell numbers ranging from 0 to 2860 CETCs/100µl blood. The fraction of tumorspheres varied between 0-10% of the CETCs. We found no statistically significant correlation between the number of CETCs and tumorspheres ($p=0.7$) indicating that the number of spheres is independent from the number of single circulating tumor cells. Interestingly, in two cases of patients with metastatic breast cancer without EpCAM positive CETCs we observed more than 30 tumorspheres/100µl blood. In contrast, no spheres could be grown from blood of 50 normal subjects. To test for self-renewal capacity established tumorspheres were dissociated into single cells, plated and the number of secondary spheres that formed after 14 days counted. Approximately, 15% to 20% of the tumorsphere cells gave rise to secondary spheres indicating that the tumorspheres contain self-renewing stem cells.

Counterstaining for specific stem cell markers is shown in Figure 2a, 2b. Tumorspheres in patients with breast cancer were positive for EpCAM and also for CD 44 and negative or low positive for CD 24. All tumorspheres showed distinct fluorescence for ALDH1 (Figure 2c) typical for breast cancer stem cells.

The expression levels of the putative stem cell markers Oct4, Sox2, Nanog, EpCAM, ALDH1 and CD133 showed overexpression in relation to house-keeping gene RPL13a in tumorspheres whereas the expression level of these stem cell markers was significantly lower in CETCs ($p<0.05$) (Figure 3).

The number of tumorspheres increased with stage of disease and patients in stage IV had the highest number of tumorspheres differing significantly from patients in stage I (median 30 vs 10; $p=0.002$) (Figure 4). Furthermore, we found that patients with positive axillary lymph node status possessed significantly more tumorspheres as compared to patients with negative lymph nodes (median 30 vs 15; $p<0.05$) (Figure 5).

The frequency of spheroids cultured from CETCs was monitored in patients without and with distant metastases in order to test whether the number of tumorspheres correlated with disease progression. The total amount of tumorspheres was higher in patients with metastatic disease as compared to patients without metastases (median 30 vs 15; $p<0.005$) (Figure 6). Patients with HER2 positive primary tumors had the highest numbers of tumorspheres with a median of 50 (Figure 7). Thus, our results indicate that the frequency of cells from the subpopulation of CETCs that can grow into tumorspheres correlates with the aggressiveness of the tumor.

Surprisingly, patients who received no chemotherapy (56.9%) had higher numbers of tumorspheres compared to patients who had received chemotherapy (34.7%) (median 25 vs 10; $p=0.002$) (Figure 8). Moreover, numbers of tumorspheres were shown to vary depending on chemotherapy. Figure 9a, 9b shows two representative serial analyses of CETCs and tumorspheres in two exemplary breast cancer patients. In Figure 9a) the course of decreasing CETCs and tumorspheres during chemotherapy is shown. Figure 9b) shows increasing CETCs and tumorspheres in a patient without cytotoxic treatment.

Table 1: Clinico-pathological characterisitcs in breast cancer patients

	Number of patients (frequency)	
Age (years)		
	<40	2 (2.7%)
	40-49	18 (25%)
	50-59	27 (37.5%)
	60-69	17 (23.6%)
	70+	8 (11.2%)
Stage of disease		
	I	18 (25%)
	II	17 (24%)
	III	14 (19%)
	IV	23 (32%)
Axillary lymph node		
	Positive	31 (43.1%)
	Negative	37 (51.4%)
	unknown	4 (5.5%)
Metastasis		
	Present	23 (31.9%)
	Absent	49 (68.1%)
HER2 status		
	Positive	6 (8%)
	Negative	54 (75%)
	unknown	12 (17%)
ER/PR status		
	Positive	44 (61%)
	Negative	18 (25%)
	unknown	10 (14%)
Chemotherapy		
	Neoadjuvant	3 (4.2%)
	Adjuvant	22 (30.5%)
	without chemotherapy	41 (56.9%)
	unknown	6 (8.4%)

In contrast to the number of CETCs the number of tumorspheres was an independent factor of the presence of metastases (Odds Ratio 1.1; Coefficient 0.119; p=0.008) in patients with breast cancer. The receiver operating characteristic (ROC) curve shows that the number of spheres that can be grown from a patient's blood is a predictor of the presence of distant metastases. The AUC for number of spheres was higher than for the number of CETCs and it is 0.71 (p=0.005) (Figure 10). The cut-off number of spheres predictive of metastases was 30/100µl blood with a sensitivity of 70% and a specificity of 65%.

DISCUSSION

Culturing blood white cells comprising CETCs from patients with breast cancer we were able to identify a

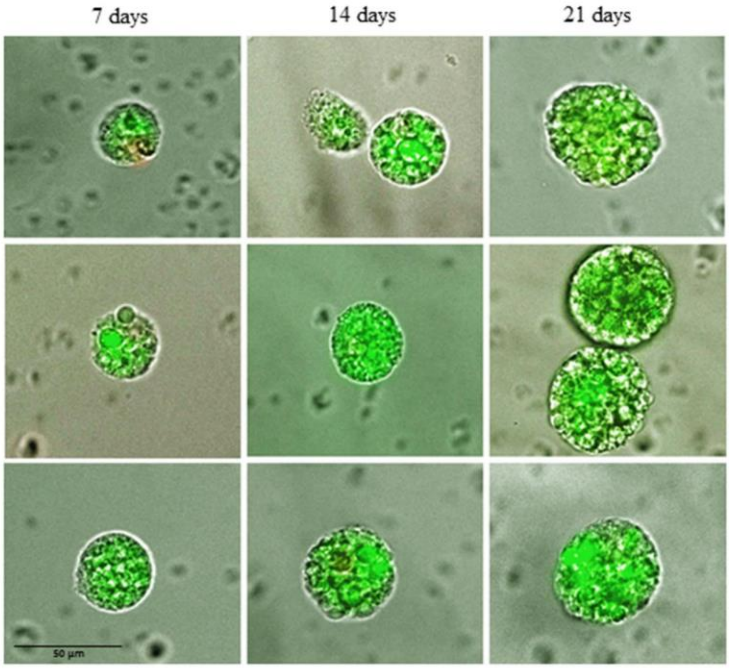

Figure 1: Changes in size of typical tumorspheres after 7, 14 and 21 days of culture.

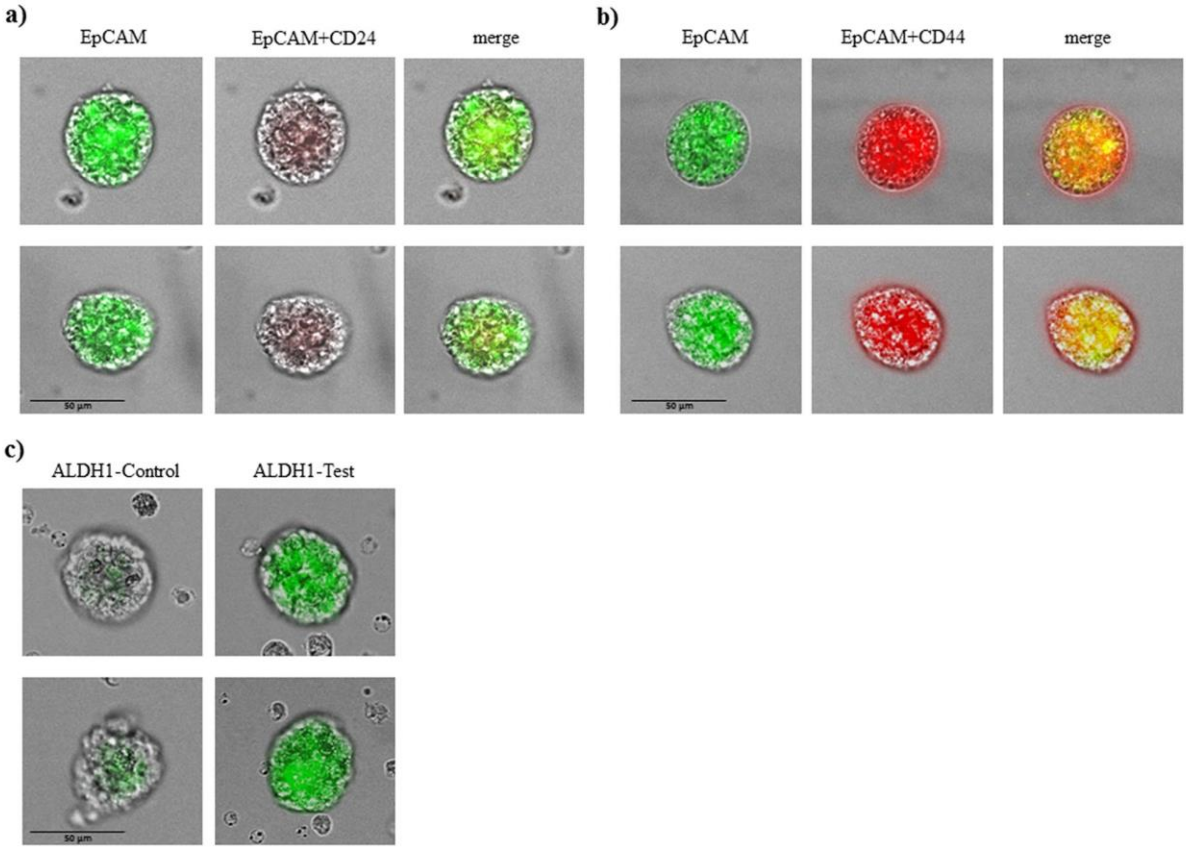

Figure 2: Immunophenotypic analysis of tumorspheres in breast cancer patients. A. tumorspheres are low positive or negative for CD 24 and **B.** positive for CD44 and EpCAM. **C.** tumorspheres had also elevated level of ALDH1 activity.

subpopulation of CETCs with clonal proliferative activity growing into tumorspheres. These tumorspheres can be cultured in special medium supplemented with growth factors during 21 days at 37°C and 5% CO_2 in conventional cell culture flasks. Only a small subpopulation of epithelial tumor cells circulating in patients´ blood had the capacity to form spheres under these conditions and no spheres could be grown from normal subjects. In contrast to Yu et al we were able to successfully culture CETCs from breast cancer patients in early stage as well as in advanced stage

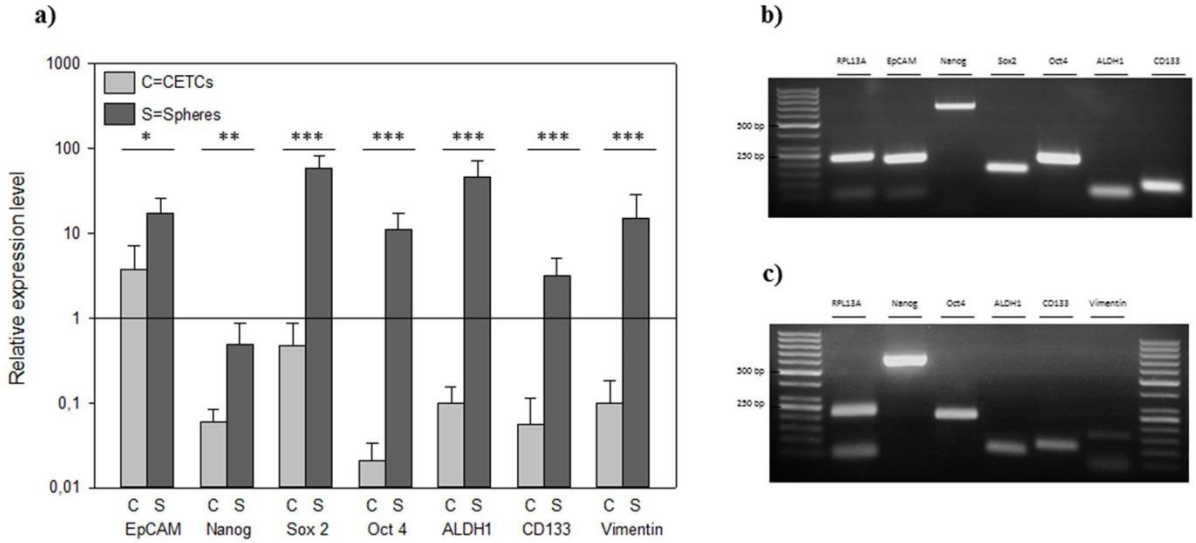

Figure 3: A. Relative expression levels of typical cancer stem cell genes in individual CETCs and tumorspheres from the same patient. Gel picture of the qRT-PCR assay of **B.** single tumorsphere and **C.** single CETC.

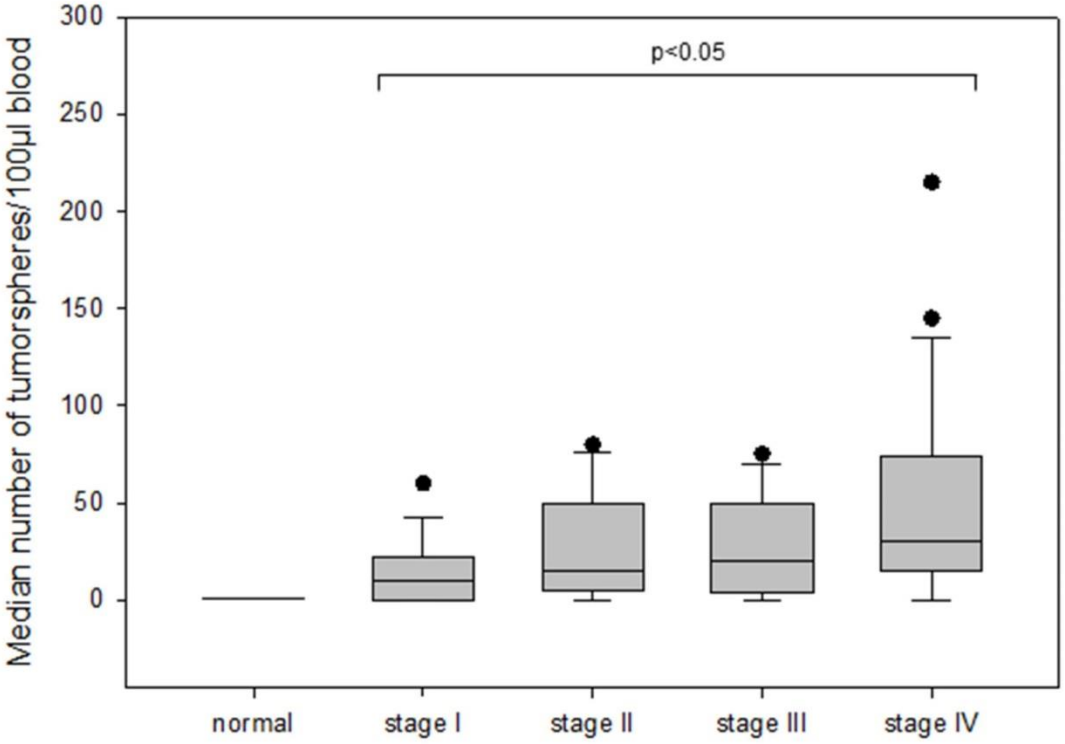

Figure 4: The number of tumorspheres in different stages of disease (I-IV) in breast cancer patients.

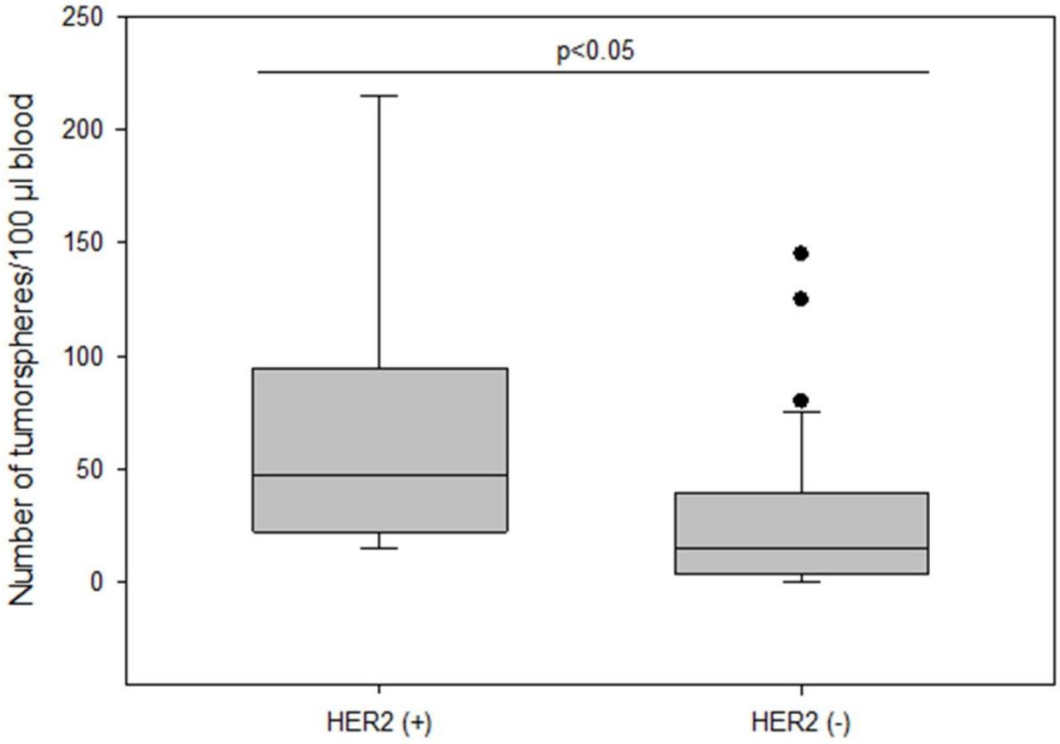

Figure 7: The number of tumorspheres and HER2 status in primary tumor.

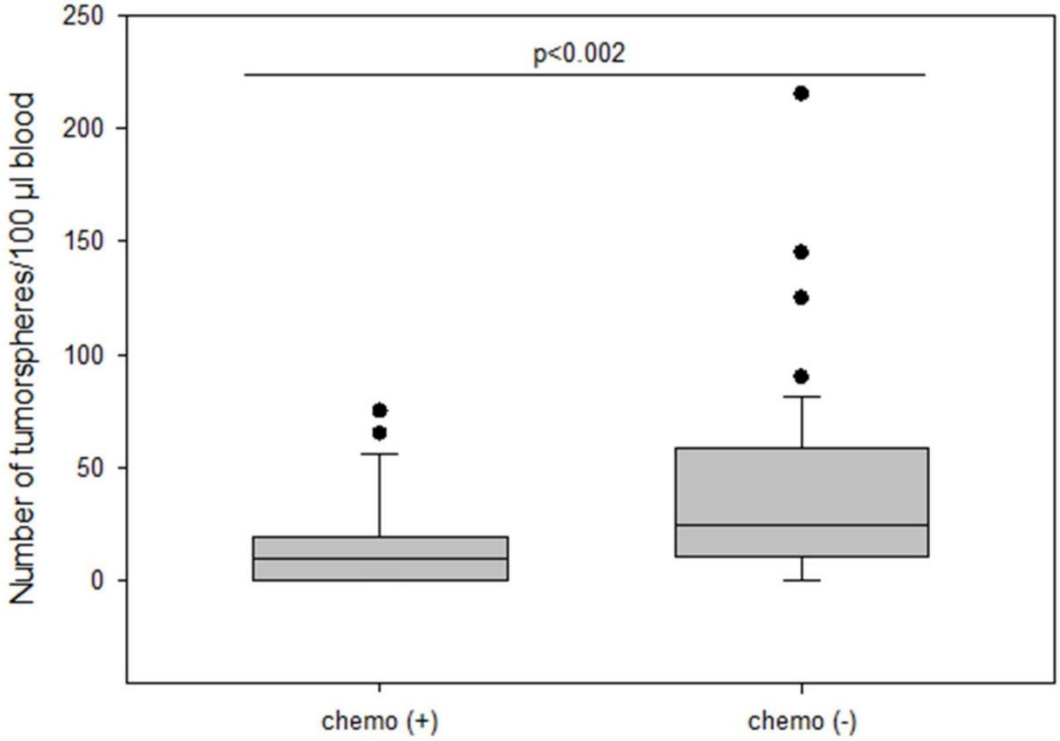

Figure 8: The number of tumorspheres in patients with and without chemotherapy.

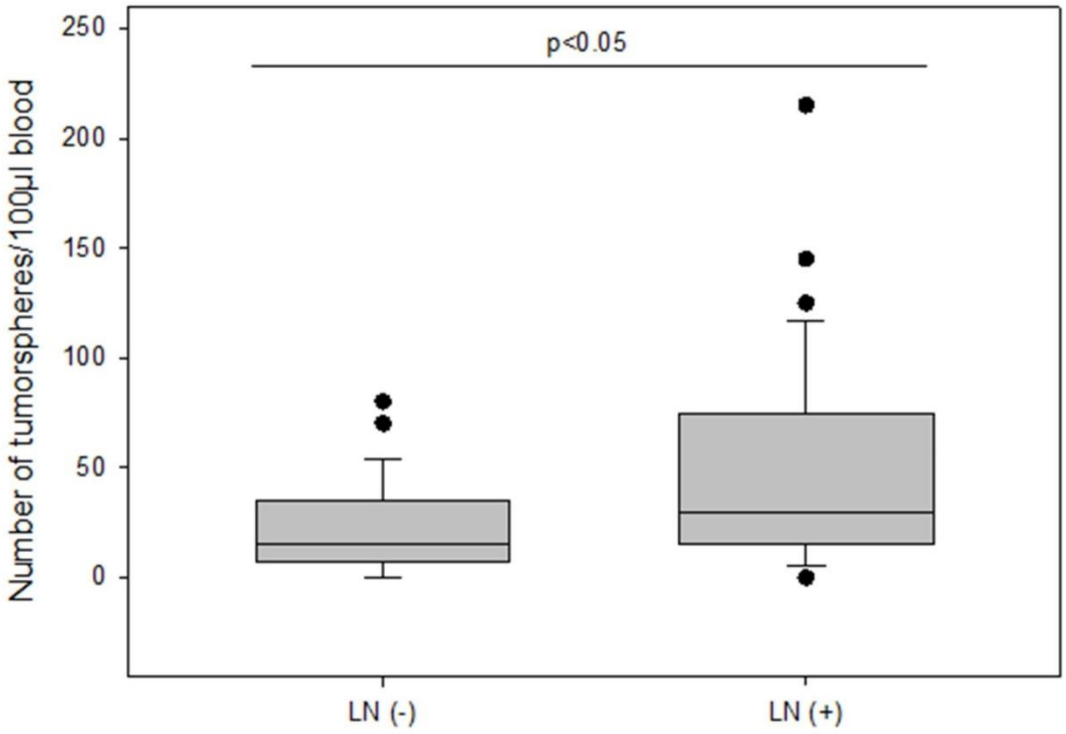

Figure 5: The number of tumorspheres without and with axillary lymph node metastasis.

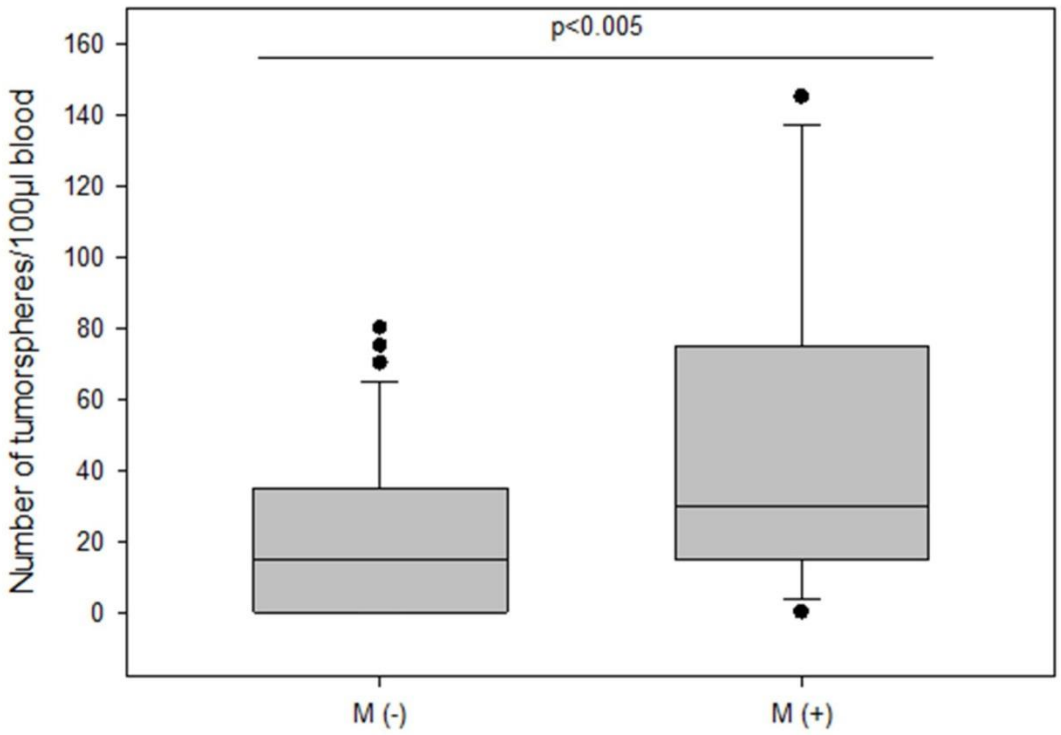

Figure 6: The number of tumorspheres in non-metastatic and metastatic patients.

of disease [22]. Sphere-forming efficiency may be due to autocrine/paracrine signals released by accompanying cells into the medium [23]. In our experiment morphologic and immunohistochemical analysis show that spheroids cultured from circulating tumor cells are bright, smooth edged and compact and are clearly different from irregular clumps of cells that also appeared at times in the culture (Figure 11). Furthermore, cultivation is performed without movement of culture flasks and leukocytes are dying during cell culture. Finally, we observed no sphere formation in healthy objects (negative control) which excludes the formation of tumorspheres as a result of aggregation of white blood cells.

Based on the type of growth, staining properties and genotypic characterization of the spheres this subpopulation had cancer stem cell (CSC) properties and

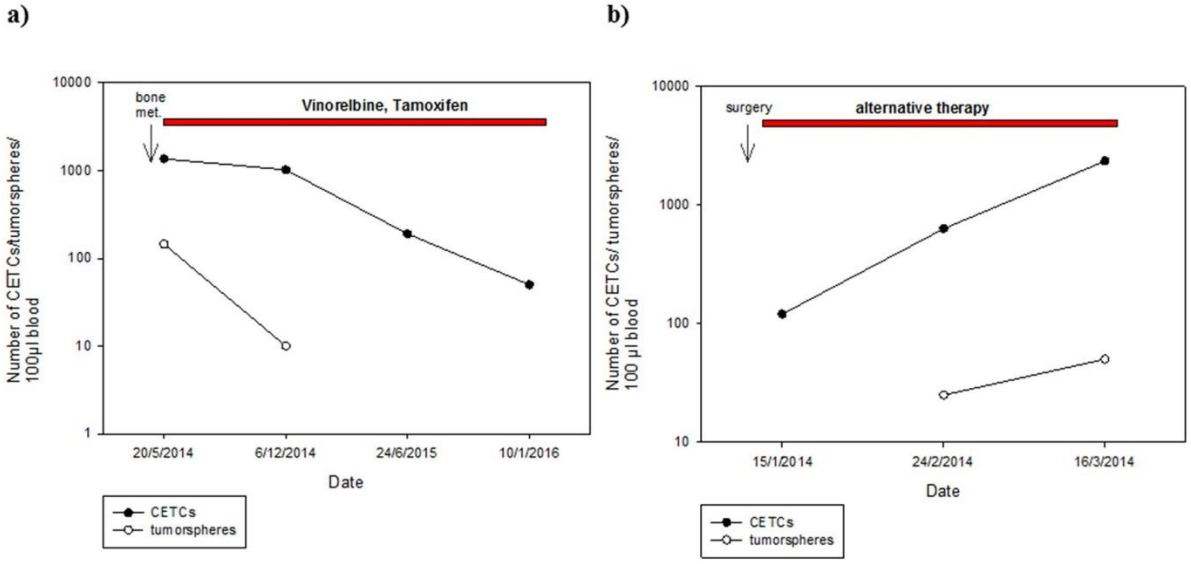

Figure 9: Exemplary courses of CETC and tumorsphere numbers from two individual patients with breast cancer. **A.** metastatic breast cancer patient (bone metastases) with decreasing CETC and tumorsphere numbers during therapy with Vinorelbine and Tamoxifen **B.** increasing CETC and tumorsphere numbers in breast cancer patient (stage III) without cytotoxic treatment.

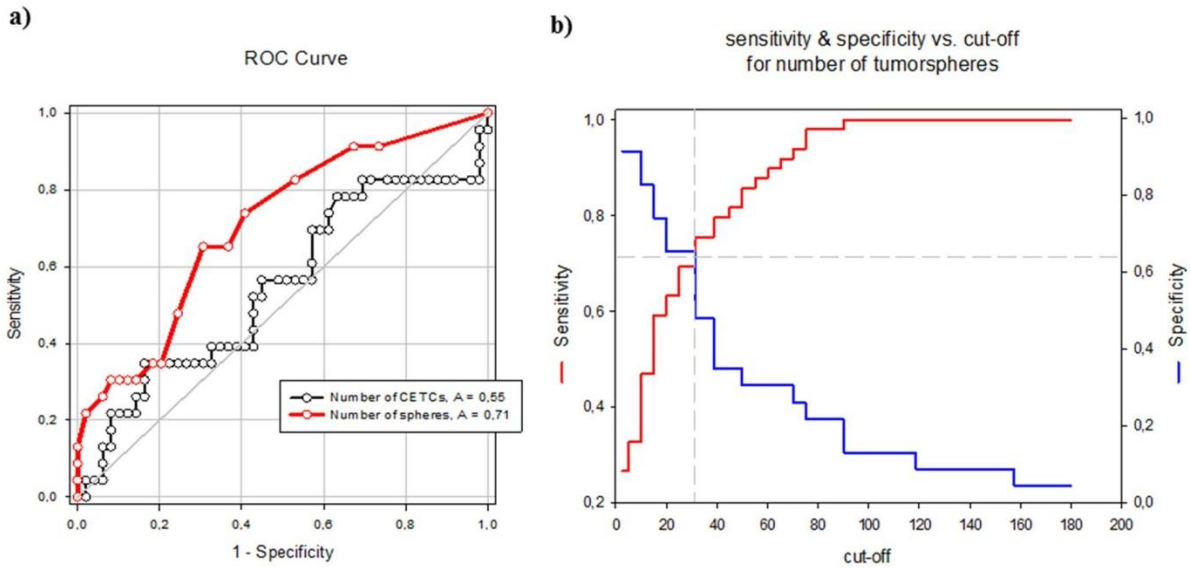

Figure 10: A. ROC curves for tumorsphere and CETC count to discriminate metastatic and non-metastatic breast cancer patients. **B.** Sensitivity and specificity values at selected cut-off points for number of tumorspheres.

these cells may be progenitors of metastases leading to relapse and treatment failure in patients with breast cancer.

Immunostaining showed both CD44 high/CD24 low expression and ALDH1 activity which are CSC markers. CD44 normally takes part in cell-cell and cell-matrix adhesion interactions, which is involved in cancer cell migration, proliferation and metastasis. Current experimental evidence indicates that the CD24 negative subpopulation might represent a more drug resistant phenotype [24]. It has been shown that breast cancer stem cells present high levels of ALDH1 [16]. Our findings are consistent with these results.

The expression of pluripotency- associated factors playing a crucial role in the development and malignant progression of various types of cancer including breast cancer [25], Aktas et al. could show that cells enriched for tumor suspected cells from metastatic breast cancer patients exhibited mRNA expression of ALDH1. These indirect indications from expression profiles led them to assume that a subpopulation among the very heterogenic circulating tumor cells display cancer stem cell characteristics [26]. We, here, demonstrate that CSC can be directly visualized by forming tumorspheres and Nanog, SOX2, OCT4, ALDH1, CD 133 and EpCAM was elevated in these tumorspheres relative to CETCs. Enumeration of tumorspheres strongly indicated that the number of spheres correlates with the aggressiveness of the tumor, e.g. lymph node involvement and metastasis. Patients with stage I had significantly less tumorspheres as compared to patients with distant metastasis. Comparing patients with and without metastatic disease revealed a tumorsphere count ≥ 30 spheres/100 µl blood as an independent predictor of the presence of metastatic disease (AUC-ROC, 0.72; $p<0.05$), growth of more than 30 spheres/100µl blood in patients with diagnosis of breast cancer warranting additional diagnostic imaging for possible distant metastases. As already evidenced in some studies dynamics of the number of CETCs is more important and prognostically relevant than single cell count analysis [4, 27, 28]. Single cell counting per se does not reflect the aggressiveness of tumor burden, especially in cases after surgery which leads to a temporary increase in cell numbers [29].

The number of tumorspheres was significantly higher in patients with HER2 positive tumors compared to HER2 negative tumors consistent with the finding of Korkaya et al. that HER2 overexpression increases the mammary stem cell population and also increases the capacity for sphere formation from tissue cultures. Furthermore, overexpression of HER2 in a series of human cancer cell lines increases the percentage of cells with ALDH activity, leading to increased amounts of spheres [30] suggesting that HER2 overexpression plays an important role in tumor growth, invasion and metastasis.

In the adjuvant situation where there is no residual disease present monitoring of CETCs has been shown to provide a valuable tool to routinely monitor response to therapy [4] and is currently the only available approach. We, here, show that as a sign of successful therapy also number of tumorspheres can decrease concurrently with CETCs during chemotherapy. In contrast, increasing numbers of CETCs and tumorspheres might warrant further diagnostic steps in order to timely change or

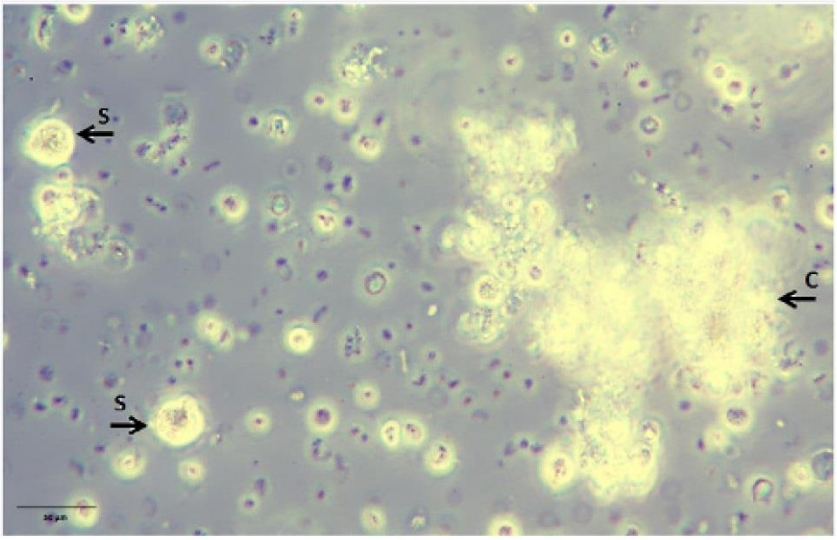

Figure 11: Non-adherent tumorspheres developed in suspension culture under stem cell-like growth conditions. Bright smooth-edged and compact spheres (S) reached 20 µm after 3 days of culture. Leucocytes aggregate to each other and form clumps (C) with an irregular outline.

initiate therapy. Knowing that not all patients benefit from chemotherapy, some patients might want to avoid chemotherapy. Our data, however, show that patients receiving no chemotherapy after surgery have increased levels of tumorspheres. Monitoring therapy using CETCs and spheroids might lead to new treatment considerations in personal tailoring of therapy. Finally, we found no relationship between the number of CETCs and presence of metastasis. The growth of tumorspheres was independent of the number of EpCAM positive circulating cells. So far, we do not know from which progenitor cells the tumorspheres arise because in two patients without EpCAM positive circulating cells we still observed EpCAM positive tumorsphere formation. It is extensively described that the phenotypical change of cancer cells known as epithelial-mesenchymal-transition (EMT) is a critical step in tumor invasion and metastasis. During this transition the expressions of epithelial markers such as EpCAM and cytokeratin is assumed to be downregulated and circulating tumor cells may become undetectable [31]. For this reason our new approach enables, without the necessity to rely on any markers and thus without selection and loss of cell populations, the identification of the most important subpopulation of CETCs with proliferative capacity and clonal expansion.

In conclusion, we are able to culture a subpopulation of circulating tumor cells and pheno- and genotypically characterize them for putative stem cell markers. We postulate that tumorspheres are a surrogate of distant metastasis in breast cancer patients. Further studies could provide new insights into the tumorigenic process and assess the potential of these spheroids in breast carcinomas as therapeutic target.

MATERIALS AND METHODS

72 patients with histologically confirmed breast cancer, 49 with primary breast cancer and 23 patients with metastatic disease were included in the study. Blood samples were drawn into normal blood count tubes with ethylene diaminetetraacetic acid (EDTA) for enumeration and cultivation of CETCs. The maintrac® approach was used for detection and quantification of CETCs, as reported previously [21]. In brief, 1 ml blood was subjected to red blood cell lysis using 15 ml of erythrocyte lysis solution (Qiagen, Hilden, Germany) for 15 min in the cold, spun down at 700 g and re-diluted in 500 ml of PBS-EDTA. 5µl of fluorescein-isothiocyanate (FITC)-conjugated mouse anti-human epithelial antibody (EpCAM) (Miltenyi Biotec GmbH, Germany) was added and incubated for 15 min in cold. Subsequently, the samples were diluted with 430 µl PBS-EDTA and stored over night at 4°C. A defined volume of the cell suspension and propidium iodide (Sigma-Aldrich, USA) was transferred to 96-wells plate (Greiner Bio-one, USA), evaluated with a fluorescence scanning microscope (ScanR, Olympus, USA) and visually examined for presence of fluorescence and cell morphology. Only vital CETCs were counted with positive EpCAM fluorescence, lacking of nuclear PI staining and with intact morphology (Figure 12). We used two types of quality controls for ensuring the consistent analysis of samples. Isotype control, as negative control to measure the level of non-specific background signal and fluorospheres (Flow-Check 770, Beckman Coulter, USA) for daily verification of optical components and detectors. The percentage of circulating cancer stem cells present in the population of circulating epithelial tumor cells was determined by observing the number of cells capable to clonally grow into tumorspheres: after erythrocyte lysis and one centrifugation step CETCs together with leukocytes were plated at a density of $2x10^5$ cells/ml in RPMI-1640 medium supplemented with L-glutamine, HEPES, penicillin/streptomycin and growth factors such as EGF, insulin and hydrocortisone. Cells were maintained at 37°C and 5% CO_2 without movement of the culture flasks. Fresh medium was added every five days and formation of tumorspheres was observed under an inverted light microscope (Primo Vert, Zeiss, Germany) at 40x magnification every 7 days. After 21 days tumorspheres were collected and prepared for immunostaining. Samples were centrifuged at 250 g for 7 min. The supernatant was discarded and the pellet resuspended with 500 µl PBS. 50 µl of cell suspension was transferred into 1.5 ml reaction tubes and prepared for the phenotypic characterization. Tumorspheres were incubated with 5 µl of fluorescein-isothiocyanate (FITC)-conjugated mouse anti-human EpCAM antibody (Miltenyi Biotec GmbH, Germany) and 5 µl of phycoerythrin (PE)-conjugated mouse anti-human CD-44 antibody (BD Bioscience, USA) or with 5 µl of phycoerythrin (PE)-conjugated mouse anti-human CD-24 antibody (BD Bioscience, USA) for 15 min in cold. The samples were subsequently diluted with 430 µl PBS-EDTA and then stored overnight at 4°C. 100 µl of cell suspension was transferred to 96-wells plates (Greiner Bio-one, USA). Analysis of red and green fluorescence of the tumorspheres was performed using a fluorescence scanning microscope (ScanR Olympus, USA). Tumorspheres were stained with propidium iodide to evaluate their viability prior to analysis. Finally, only vital tumorspheres with intact morphology were counted. ALDH1 activity of spheroids was determined using an ALDEFLUOR assay kit (Stem Cell Technologies™, Canada) according to the manufacturer´s protocol. In short, tumorspheres were suspended in ALDEFLUOR assay buffer containing ALDH1 substrate and incubated 45 min at 37°C. In parallel, a sample was treated with an ALDH1-specific inhibitor, as negative control for background fluorescence. Stained spheroids were analyzed with fluorescence scanning microscope (ScanR Olympus, USA). Moreover we examined 50 healthy donors aged from 20 to 53 years for determination of specificity and sensitivity and we observed no sphere formation in all cases.

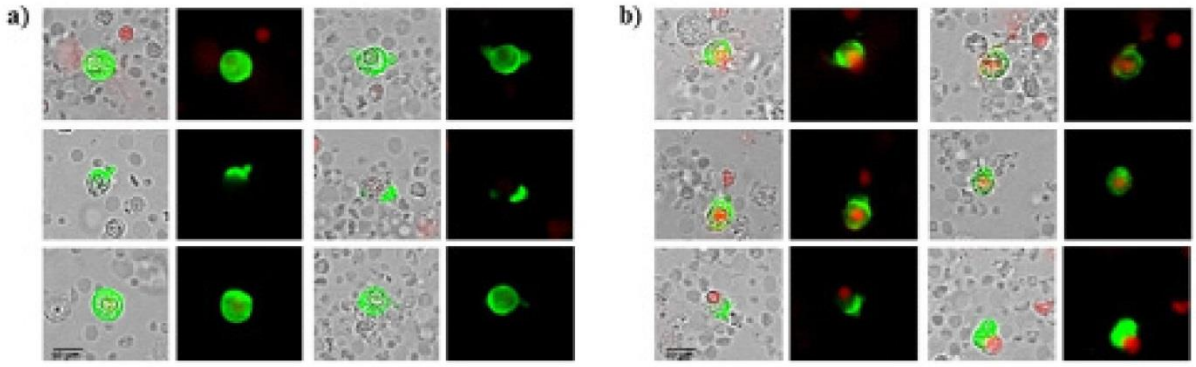

Figure 12: A, B. Examples of live and dead CETCs. A) Live CETCs have intact morphology with a well preserved membrane without PI staining in the nucleus. B) Dead CETCs show a positive PI staining because of loss of membrane integrity.

In some cases we performed dissociation of spheroids with StemPro®Accutase® (Gibco, USA) for 3 min at room temperature to obtain a second generation of tumorspheres.

For quantitative RT-PCR CETCs and tumorspheres were isolated individually using a semi-automated capillary approach and deposited one by one into micro cups. cDNA was prepared with CellAmp™ Whole Transcriptome Amplification kit (Takara Bio Inc./ Mobitec). Subsequently, PCR was carried out with Light Cycler® 480 SYBR Green I Master (Roche). The following primers were used in this study: *rpl13a*: AGC TCA TGA GGC TAC GGA AA (forward) and CTT GCT CCC AGC TTC CTA TG (reverse); *epcam*: GGG AAA TAG CAA ATG GAC ACA (forward) and CGA TGG AGT CCA AGT TCT GG-5 (reverse); *vimentin:* TCC GCA CAT TCG AGC AAA GA (forward) and ATT CAA GTC TCA GCG GGC TC (reverse); *nanog:* GGA TCC AGC TTG TCC CCA AA (forward) and TGC ACC AGG TCT GAG TGT TC (reverse); *oct4:* GGC CAC ACG TAG GTT CTT GA (forward) and ATA CCT TCC CAA ATA GAA CCC C (reverse); *sox2:* GCG GAA AAC CAA GAC GCT C (forward) and TCA TGT GCG CGT AAC TGT CC (reverse); *cd133:* GTC CTG GGG CTG CTG TTT AT (forward) and TCT GTC GCT GGT GCA TTT CT (reverse); *aldh1* CTG AGC CAG TCA CCT GTG TT (forward) and GGA CAG GTA AGT CTG GCG TG (reverse). Relative expression levels were calculated after normalization to the reference gene ribosomal protein L13a (*RPL13a)* by using the $\Delta\Delta C$T method.

Data were analyzed using Student´s t test for comparison of two variables or ANOVA for three and more variables. If the sample distribution was asymmetrical nonparametric tests (Mann Whitney U test for comparison two groups and Kruskal-Wallis for more groups) were performed. Relationships between parameters were evaluated using Pearson or Spearman´s rank correlation.

Diagnostic performance of tumorsphere count was assessed by constructing a ROC curve, and was evaluated by calculating the area under the ROC curve.

CONFLICTS OF INTEREST

The author declare no conflicts of interest.

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5.3 Publikation II

Chick Chorioallantoic Membrane (CAM) Assays as a Model of Patient-Derived Xenografts from Circulating Cancer Stem Cells (cCSCs) in Breast Cancer Patients

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Article

Chick Chorioallantoic Membrane (CAM) Assays as a Model of Patient-Derived Xenografts from Circulating Cancer Stem Cells (cCSCs) in Breast Cancer Patients

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Simple Summary: Circulating cancer cells—and in particular their very rare subpopulation, circulating cancer stem cells (cCSCs)—are responsible for recurrence and metastasis. In this study, we present a novel process in which patient-derived xenograft (PDX) can be harvested on chorioallantoic membrane (CAM) from circulating cancer stem cells. In our opinion, the CAM-based PDX model using circulating cancer stem cells can provide a fast, low-cost, easy-to-use, and efficient preclinical platform for drug screening, therapy optimization, and biomarker discovery.

Abstract: Background: cCSCs are a small subset of circulating tumor cells with cancer stem cell features: resistance to cancer treatments and the capacity for generating metastases. PDX are an appreciated tool in oncology, providing biologically meaningful models of many cancer types, and potential platforms for the development of precision oncology approaches. Commonly, mouse models are used for the in vivo assessment of potential new therapeutic targets in cancers. However, animal models are costly and time consuming. An attractive alternative to such animal experiments is the chicken chorioallantoic membrane assay. Methods: In this study, primary cultures from cCSCs were established using the sphere-forming assay. Subsequently, tumorspheres were transplanted onto the CAM membrane of fertilized chicken eggs to form secondary microtumors. Results: We have developed an innovative in vitro platform for cultivation of cCSCs from peripheral blood of cancer patients. The number of tumorspheres increased significantly with tumor progression and aggressiveness of primary tumor. The number of tumorspheres was positively correlated with Ki-67, Her2 status, and grade score in primary breast tumors. The grafting of tumorspheres onto the CAM was successful and positively correlated with aggressiveness and proliferation capacity of the primary tumor. These tumors pathologically closely resembled the primary tumor. Conclusions: The number of tumorspheres cultured from peripheral blood and the success rate of establishing PDX directly reflect the aggressiveness and proliferation capacity of the primary tumor. A CAM-based PDX model using cCSC provides a fast, low-cost, easy to handle, and powerful preclinical platform for drug screening, therapy optimization, and biomarker discovery.

Keywords: CAM-assay; cCSCs; tumorspheres; PDX; breast cancer

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1. Introduction

Breast cancer is the most common neoplasms among women, and it continues to be associated with a high mortality rate despite the increasing number of early diagnoses and improvement of the initial tumor cure rate [1]. Distant metastases remain the leading cause of death in breast cancer patients and they arise from hematogenous spread of circulating tumor cells (CTCs) from solid primary or metastatic tumor lesions [2–4]. Recent studies have provided strong support for the cancer stem cell hypothesis, which suggests that many cancers, including breast cancer, are driven by a subpopulation of cells that exhibit stem cell properties. These cells may mediate metastasis and, because of their resistance to radiotherapy and chemotherapy, contribute to disease recurrence and progression in cancer patients [5,6]. As a consequence, eradicating this subpopulation would be critical in order to achieve patient's cure [7,8].

Interestingly, some experimental findings have highlighted that a small subpopulation of CTCs displays CSCs features (tumor initiating capability), so that they can be considered as circulating cancer stem cells [9–11]. A number of non-invasive diagnostic assays are being developed in order to either diagnose patients with metastatic disease and/or to screen patients for relapse after treatment. A new diagnostic tool, developed in recent years by our team, involves measuring extremely rare circulating cancer stem cells in patients' blood to detect early-stage metastases or cancer recurrence in treated patients. This functional assay is based on the unique property of cancer stem cells to survive and grow in suspension culture [12]. cCSCs were able to form tumorspheres in vitro, confirming that this subpopulation of CTCs represent tumor initiating cells which can potentially grow into metastatic tumors in vivo. To assess tumorigenicity, breast cancer stem cells can be xenotransplanted into the subcutaneous area under the mammary fat pad of immunocompromised female mice [5]. Although they are associated with ethical and financial limitations, this assay is the 'gold standard' for assessing CSC tumorigenicity.

In contrast, the chick embryo chorioallantoic membrane (CAM) is a low cost, reproducible, and reliable model used to investigate several functional features of tumor biology—such as the ability of cells to be tumorigenic, to invade, and to metastasize into the embryo [13]. CAM is a highly vascularized extraembryonic membrane, thus providing rich nutrient conditions for the growth of human tumor cells. Cancer cells can be implanted easily, non-invasively, and in an immunodeficient environment because the chick embryo has an immature immune system in the early stages of development when the cells are implanted. Within days, tumor formation occurs and, in the case of aggressive tumors, metastasizing cells can colonize the embryo's organs via hematogenous metastasis. In human medicine, this model is widely used in preclinical oncology research to study the growth of many types of tumors, including breast cancer [14]. Moreover, it has also been used as a platform to analyze the values of anticancer drugs [15] and radiation therapy [16]. A recent important advance of the CAM assay is that patient tumor samples can be used to transplant on the CAM, enabling the establishment of a patient-derived CAM tumor [17].

In this manuscript, we describe, for the first time, the use of tumorspheres cultured from circulating cancer stem cells of breast cancer patients to establish patient-derived xenografts on the CAM membrane. These results allowed us to propose that the chick CAM model can be a new tool to evaluate breast CSC properties in vivo with the potential to be used in a personalized medicine context.

2. Material and Methods

2.1. Study Population

The patient population of this study consists of 75 women diagnosed with breast cancer in different stages of disease. Blood samples were drawn into normal blood count tubes with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant and processed within 72 h of collection in accordance with a recently published protocol [3,12,18]. The study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee of University Bayreuth (O 1305/1-GB).

2.2. Sphere-Forming Assays

Circulating cancer stem cells were cultured together with leukocytes in medium composed of RPMI-1640 supplemented with L-glutamine, HEPES, penicillin/streptomycin, and growth factors such as EGF, insulin, and hydrocortisone at a density of 2×10^5 cells/mL. Cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere, and the fresh culture medium was added once a week until cells started to form non adherent aggregates. The formation of spheres was confirmed under an inverted microscope (Zeiss, Jena, Germany) and after 14 days collected by gentle centrifugation for further experiments. Tumorspheres cultured from cCSCs were characterized using typical combination of markers for breast cancer stem cells. EpCAM positive tumorspheres were additionally stained for CD24 (clone ML5, mouse anti-human, BD Bioscience, Franklin Lakes, NJ, USA) and CD44 (clone 515, mouse anti-human, BD Bioscience, Franklin Lakes, NJ, USA) PE-conjugated antibody. To confirm tumor stem cell properties of tumorspheres, we used an aldehyde dehydrogenase isoform 1 (ALDH1) enzymatic assay, which quantifies the ALDH1 activity of tumorspheres. We used an ALDEFLUOR assay kit (Stem Cell TechnologiesTM, Vancouver, BC, Canada) according to the manufacturer's protocol. Tumorspheres expressing high levels of ALDH1 becoming brightly fluorescent could be easily distinguished from single cells because they develop a solid spherical formation with a diameter >50 μm.

2.3. CAM Xenografts Model from Circulating Cancer Stem Cells

In 10 cases of breast cancer patients, CAM xenograft experiments were performed. Briefly, fertilized chicken eggs were maintained in a humidified egg incubator (Grumbach, Asslar, Germany) at 37 °C. Protocols were approved by the Ethics Committee of the University Bayreuth. A small window was made in the shell on day 4 of chick embryo development under aseptic conditions. The window was resealed with adhesive tape and eggs were returned to the incubator until day 8 of chick embryo development. At day 8 the tumorspheres cultured from peripheral blood were mixed (1:1) with standard Matrigel (BD Biosciences) basement membrane matrix to prevent cell dispersion, in a total volume of 20 μL and directly seeded on the membrane of CAM through the small window created earlier (Figure 1).

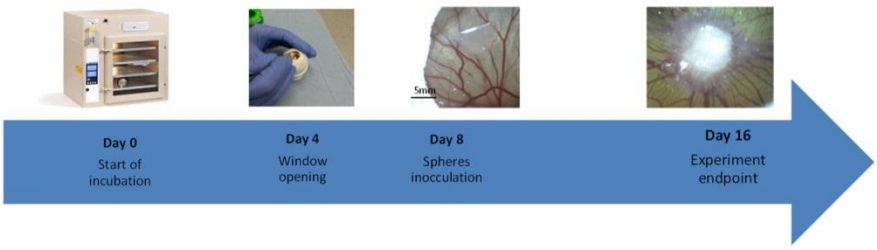

Figure 1. In vivo workflow: A timeline of chorioallantoic member (CAM). Fertilized eggs are incubated for 4 days, at which time a shell window is opened. At embryonic development day 8, tumorspheres are inoculated on top of the CAM. At day 16, the eggs are sacrificed, and the tumor growth is examined.

After completion of the study protocol, the embryo was sacrificed by decapitation. The CAM bearing the tumor was then excised with sterilized surgical scissors, fixed with 4% paraformaldehyde for 24 h, and embedded in paraffin.

2.4. Histopathology

Paraffin blocks were produced for each CAM tumor. The histological sections were deparaffinized, hydrated in xylene and graded alcohol series and then stained with H&E according to standard protocol. Histological images (40×, 100×, 200×, and 400×) were acquired by light microscope (Olympus BX43, Tokio, Japan) and digitized using a RGB video camera (Olympus DP 20, Tokio, Japan).

2.5. Graphs and Statistical Analyses

All statistical analyses were performed using SigmaPlot Version 13.0 (released 3 December 2017, Systat Software Inc., San Jose, CA, USA). A paired or unpaired Student's *t*-test was used for continuous variables, as appropriate. One-way ANOVA was performed to calculate the differences among multiple groups. A two-sided *p*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Clinicopathological Characteristics of Breast Cancer Patients

Clinicopathological information is summarized in Table 1. Twenty-six patients (34.7%) had T1, 33 (44%) T2, 9 (12%) T3, and 7 (9.3%) T4. Thirty-five patients (46.7%) had lymph node involvement. Sixteen patients (40%) had ER-positive and Her2-negative breast cancer, 16 (21%) had Her2-positive disease, of which 5 were also ER-positive, and 13 (17%) had triple-negative breast cancer (TNBC). The majority of breast cancer patients had non metastatic disease (52; 69.3%), while 30% of patients had metastatic breast cancer.

Table 1. Clinicopathological parameters of patients with breast cancer.

Clinicopathological Characteristics	Number of Patients	Median Number of Tumorspheres	*p*-Value
Age			
≤50 years	20	20	$p = 0.206$
>50 years	55	10	
Tumor size			
T1	26	7.5	
T2	33	12.5	$p = 0.318$
T3	9	25	
T4	7	30	
Lymph node			
positive	35	10	$p = 0.967$
negative	40	10	
Clinical stage			
I	13	5	
II	23	10	$p < 0.01$
III	16	10	
IV	23	32.5	
ER/PR status			
positive	62	15	$p = 0.955$
negative	13	20	
Her2-neu			
positive	16	35	$p < 0.05$
negative	59	10	
Grading			
1	14	0	
2	33	5	$p < 0.01$
3	28	32.5	
Ki-67 Index			
>15%	29	34	$p < 0.01$
<15%	46	8	
Metastasis			
positive	23	32.5	$p < 0.01$
negative	52	10	

3.2. Detection of Circulating Cancer Stem Cells

One of our aims was to detect circulating cancer stem cells using tumorsphere-forming assay. To determine the self-renewal and growth potentials of cCSCs, we maintained the suspension cultures. We were able to generate tumorspheres from peripheral blood in 58 (77.35%) cases. Tumorspheres cultured from cCSCs were very compact and the size increased with time, reaching a diameter of 50–100 µm after 21 days (Figure 2a). Tumorspheres were positive for EpCAM and CD 44, and negative or very low positive for CD24 (Figure 2b). The EpCAM and CD44 staining was very heterogeneous in the individual cells within individual tumorspheres. Most of the tumorspheres showed distinct fluorescence for ALDH1 (Figure 2c) typical for breast cancer stem cells.

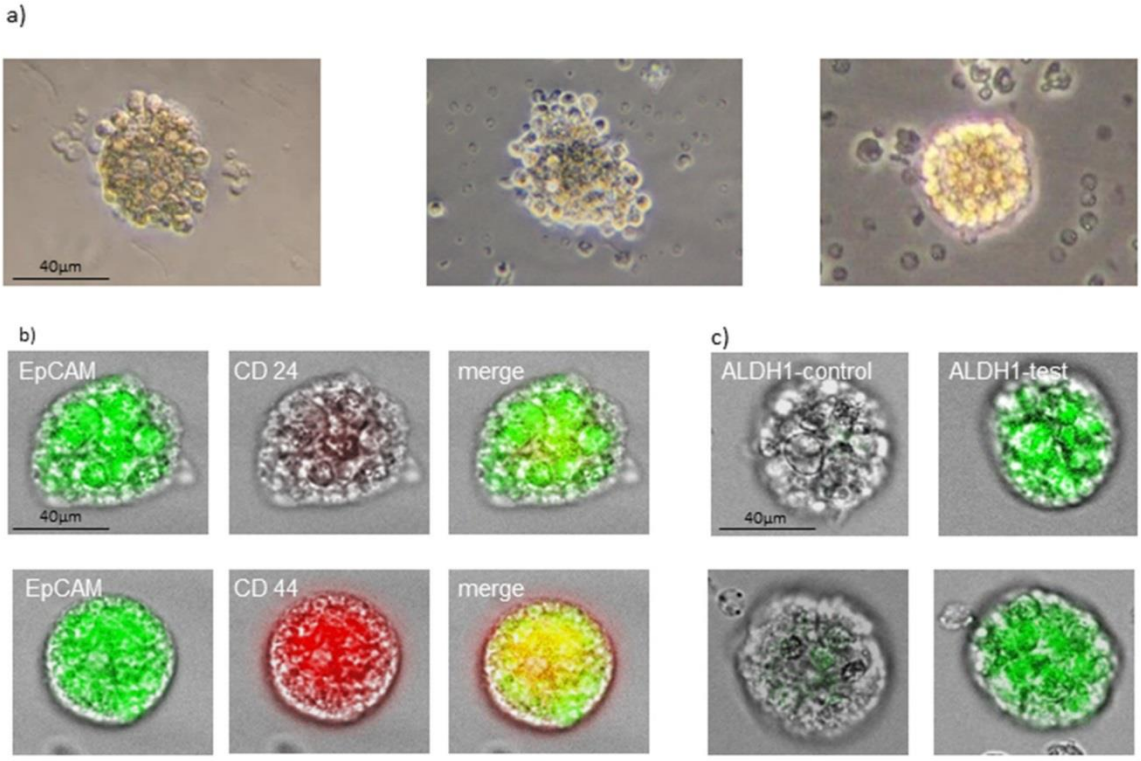

Figure 2. (**a**) Exemplary pictures of tumorspheres in light microscope. (**b**) Fluorescence staining of tumorspheres with cancer stem cell makers typical for breast cancer. (**c**) Expression of aldehyde dehydrogenase 1 (ALDH-1) in tumorspheres. Scale bar = 40 µm; magnification ×200.

3.3. Tumorspheres Count and Its Association with Clinicopathological Features

We correlated tumorsphere counts with clinicopathological features. The number of tumorspheres was significantly associated with presence and amount of distant metastasis and also aggressiveness of primary tumor. We found that patients with distant metastasis had statistically significantly more tumorspheres compared to patients without spreading to distant organs (median 32.5 vs. 10.0; $p < 0.01$) (Figure 3a). Furthermore, the highest level of tumorspheres was observed in the patient group with multiple metastases (median 61; $p < 0.001$) (Figure 3b). Additionally, our results indicate that the number of tumorspheres correlated significantly with the aggressiveness of the primary tumor. Patients with Her2-neu positive primary tumor had significantly higher levels of tumorspheres compared to patients with negative Her2-neu histology (median 35 vs. 10; $p < 0.05$) (Figure 3c). Additionally, the number of tumorspheres was positively correlated with the proliferation index Ki-67 and histological grade in primary breast tumor. By definition, tumors exhibiting a Ki-67 index of 15% or more were considered to be connected with worse prognosis. Based

on this cut-off criterion, 18 out of the 75 patients were shown to have primary tumors displaying highly proliferative potential. Patients with Ki-67 > 15% had significantly higher numbers of tumorspheres compared to those with Ki-67 < 15% (median 34 vs. 8; $p < 0.01$) (Figure 3d). With respect to differentiation status, we observed that patients with G3 primary tumors had significantly more tumorspheres compared to patients with G1 (median 32.5 vs. 0; $p < 0.01$) (Figure 4). In contrast, age, tumor size, lymph node and ER/PR status were not significantly associated with the number of tumorspheres.

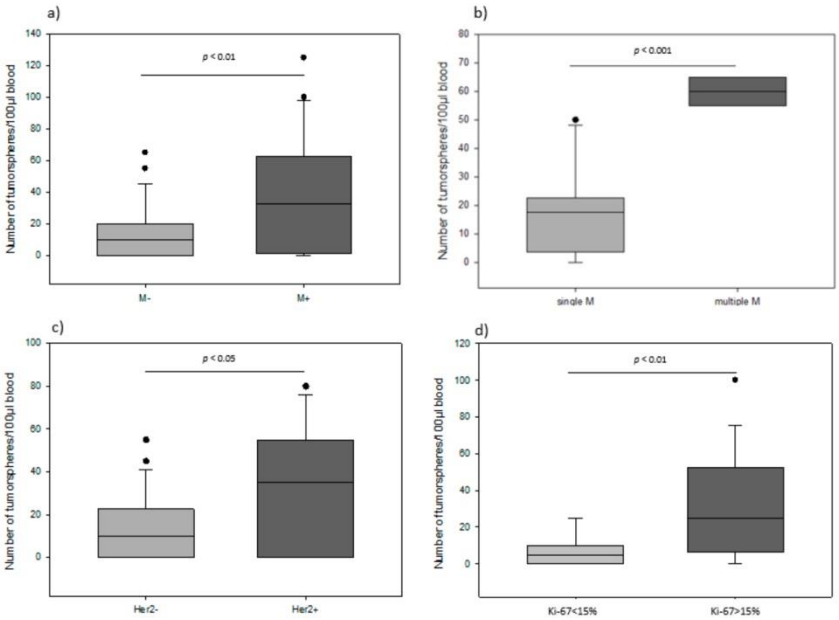

Figure 3. (**a**) Box plot analysis of the number of tumorspheres in patients without and with metastatic disease. (**b**) Box plot analysis showing the number of tumorspheres in patients with single and multiple metastases. (**c**) Box plot analysis of the number of tumorspheres in patients negative and positive for Her2-neu in primary tumor. (**d**) Box plot analysis showing the number of tumorspheres in patients with Ki-67 < 15% and >15% in primary tumor. The horizontal line in box-plot depicts median value. Dots specify outliers.

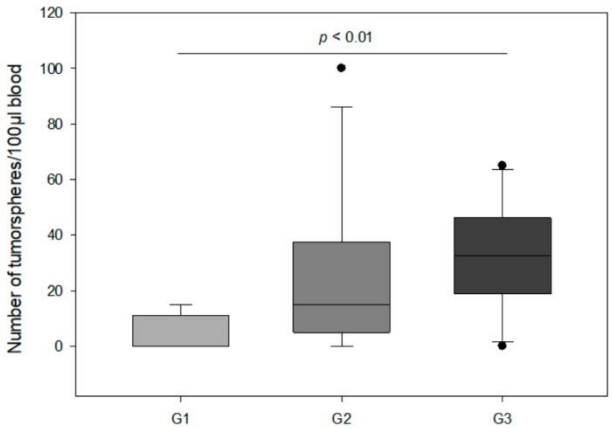

Figure 4. Box plot analysis of the number of tumorspheres according to the histologic tumor grade (G1—well-differentiated tumor; G2—moderately differentiated tumor; G3—poorly differentiated tumor). The horizontal line in box-plot depicts median value. Dots specify outliers.

3.4. Establishing a Breast Cancer Model from Tumorspheres Using the Chicken Egg CAM System

To establish a breast cancer chicken egg model, we used tumorsphere cultured from peripheral blood of breast cancer patients (n = 10). We were able to reproducibly generate tumors on CAM from 5 out of 10 breast cancer cases tested. Each patient was characterized separately in Table 2. In the CAM, tumors derived from tumorspheres were able to invade the CAM and to be vascularized (Figure 5a). Histological examination revealed that the CAM tumors contained extensive neoplastic tissue with a well-organized, preserved, and homogeneous structure, clearly distinguishable from the surrounding stroma and membrane border which resembled the morphology of the patient's primary breast tumor (Figure 5b). The success rate of tumor development on CAM membrane depended on the number of spheres and the aggressiveness of the primary tumor. Patients with a large number of tumorspheres and high Ki-67 index of primary tumor were more likely to develop tumors on the CAM membrane (Figure 6a,b). The estrogen receptor status did not have any influence on tumorsphere formation and CAM-tumor development.

Table 2. Characteristics of patients who underwent CAM experiment.

Patients	Grading	Ki-67	ER/PR	Her2-neu	Metastasis	Tumorspheres	Tumor on CAM
1	G3	40%	positive	positive	positive	80	present
2	G3	75%	positive	negative	negative	100	present
3	G3	65%	negative	negative	negative	50	present
4	G2	40%	positive	positive	negative	55	present
5	G3	30%	positive	positive	negative	20	present
6	G2	15%	positive	positive	negative	55	absent
7	G3	20%	positive	negative	negative	45	absent
8	G2	5%	positive	negative	negative	5	absent
9	G3	20%	positive	negative	negative	15	absent
10	G2	10%	positive	negative	negative	0	absent

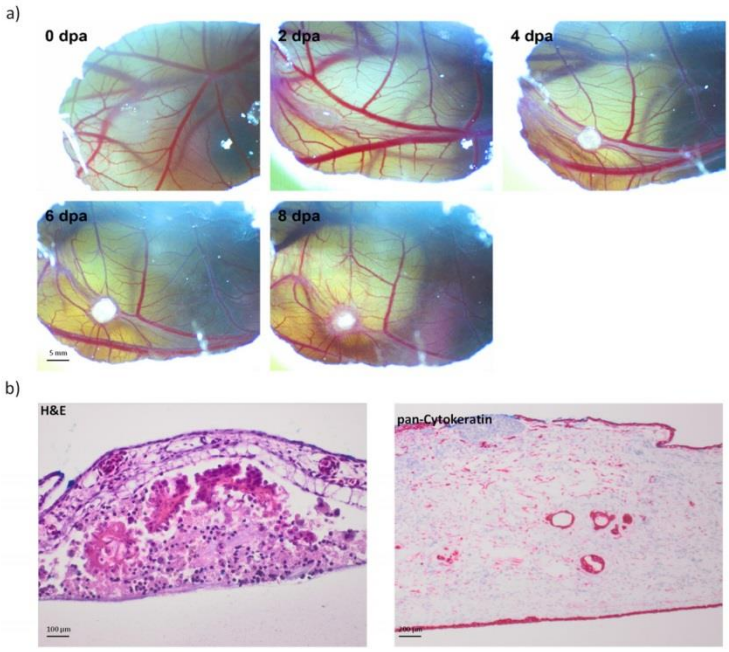

Figure 5. (**a**) Development of a microtumor from tumorspheres of a breast cancer patient on CAM membrane. Scale bar = 5 mm; magnification ×20. (**b**) H&E and pan-cytokeratin staining of a CAM xenograft developed from tumorspheres in light microscopy. Scale bar = 100 µm for H&E, scale bar = 200 µm for pan-cytokeratin; magnification ×200.

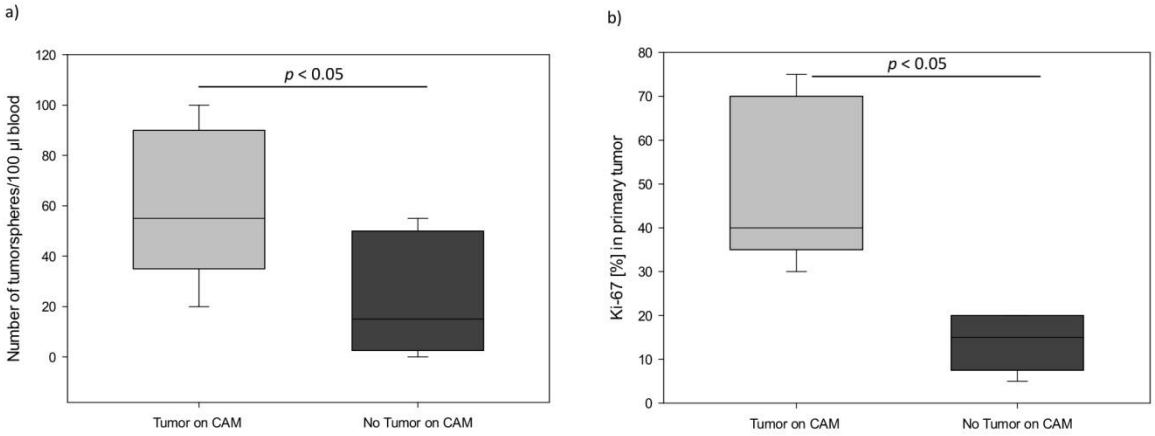

Figure 6. (**a**) Box plot analysis of the number of tumorpheres cultured from peripheral blood in patients who developed a tumor or not on the CAM membrane. (**b**) Box plot showing the Ki-67 proliferation index of primary tumor in patients with breast cancer, with or without tumor on CAM membrane. The horizontal line in box-plot depicts median value.

4. Discussion

Cancers exist in an extraordinary variety of types and subtypes, making each cancer individually unique. Tumors are heterogeneous and many cancer cell populations with different features are present. Among these tumor populations, cells with properties of stemness, commonly called CSCs, have been described and associated with more aggressive phenotypes [19]. There is strong evidence suggesting that cancer cells with stem properties selectively resist current cancer therapies, indicating the important role that CSCs play in tumor evolution, relapse, and metastasis [20,21]. Interestingly, some experimental findings have highlighted that a small population of CTCs also displays cancer stem cell characteristics (tumor initiating capability) [12,22,23]. Sphere-forming assays are well-described culture methods that have been used for stem cell isolation, identification, and enrichment from different tissues [24–27]. Starting materials for these cultures have been commercial cell lines, surgical resection specimens, and also peripheral blood with a subset of circulating cancer stem cells [11,12]. The role of cCSCs was investigated in several studies and the results indicated that cCSCs are linked to an unfavorable prognosis in various cancers [28–31]. In this report, we confirmed our previous findings that circulating cancer stem cells are able to generate tumorspheres and the number of tumorspheres was associated with presence of metastasis [12]. Patients with metastatic disease had more tumorspheres compared to the patients without metastases. Furthermore, patients with multiple metastases had the highest number of tumorspheres. These observations suggest that the number of spheres may reflect the aggressiveness of the tumor and provide additional information about the progression of the disease. We also observed that sphere-formation rates were positively correlated with Her2-neu, Ki-67, and grading of primary tumor. Patients with Her2-neu positive tumor had more tumorspheres in comparison to patients with Her2-neu negative status. Our results are consistent with those of Korkaya et al. [32] who showed that overexpression of Her2-neu increases the population of mammary stem cells as well as their ability to form spheres. Ginestier et al. [33] showed that, in human breast cancers, there was a correlation between Her2-neu amplification and cancer stem cells frequency as assessed by expression of the breast CSC marker ALDH-1.

Ki-67 is universally expressed in proliferating cells, and it is a predictive and prognostic marker for clinical practice in breast cancer patients [34]. The level of Ki-67 determines the categorization of low risk of recurrence and high risk of recurrence. Clinically, the most widely used cut-off for low-risk recurrence is a Ki-67 index below 15% [35]. In our study, the generation of tumorspheres was associated with Ki-67 status in the primary tumor.

Patients with Ki-67 >15% had more tumorspheres compared to patients with Ki-67 <15%. Cidado´s et al. [36] demonstrated that Ki-67 was required for maintenance of cancer stem cells but not cell proliferation. However, additional studies are needed to clarify the exact mechanism of how Ki-67 regulates CSC properties.

The histologic grade also is a prognostic factor for breast cancer, regardless of tumor size and the number of involved axillary lymph nodes [37]. In our population, patients with poorly differentiated primary tumor (G3) had significantly more tumorspheres as compared to patients with well differentiated tumor (G1). To our knowledge, there are no data in the literature with respect to the relationship between the number of cancer stem cells and the histologic grade in breast cancer. However, Mohanta et al. [38] showed that patients with poorly differentiated squamous cell carcinoma of the oral cavity had more CSCs, which correlated with poor prognosis in clinical settings.

The CAM assay is a frequently applied model to study cancer cell invasion and metastasis [13]. In our approach we sought to investigate the functional properties of tumorspheres. The CAM model has many advantages. It is cost effective, allows large scale screening and is an easily reproducible in vivo model [39,40]. We show here, for the first time, that tumorspheres cultured from peripheral blood in patients with breast cancer are able to induce tumors on CAM upon application. The efficacy of inoculation using surgical specimens varies widely, ranging from about 45% [41] to much higher rates of 70–80% [42–44] depending on the type of tumor. Baccelli et al. [45] identified a population of tumor cells circulating in blood from breast cancer patients that initiates metastasis in a xenograft assay, albeit at a much lower frequency in obtaining xenografts. As shown in the Table 2, five patients (50%) have successfully grown tumors on CAM and the success rate was associated with the number of tumorspheres. Moreover, we suppose that the inoculation rate is also dependent upon Ki-67 status and histologic grade of primary tumor, because only patients with high Ki-67 and low histologic grade have developed tumors on CAM membrane. Histopathological analysis was the key endpoint assay of our experiment, which confirmed that tumors grown on CAM had the original morphological profile of the patient's tumor. Given the high efficiency of patient derived tumor on CAM in a short 8-day period, it holds great promise as an in vivo platform to pursue pilot drug screening on an individual patient's tumor. Our model has an advantage over others because the biological material used for CAM-assay is easily and repeatedly available without high risk to the patient. A limitation of our study is the small number of patients who underwent CAM experiment, but further clinical validation for a large-size sample set remains to be done in future work.

Personalized medicine aims to provide cancer patients with tailored treatments, so it is necessary to use patient-derived tumor models to characterize possible treatment options. Currently, tumor organoids [46] or patient-derived xenograft mouse systems [47] are used for this purpose. Based on our success in growing patient derived tumors from tumor cells circulating in peripheral blood in the CAM assay, our model may provide an interesting alternative for investigating the individual tumor biology and a suitable platform for the testing of different drugs in personalized oncology.

5. Conclusions

The number of tumorspheres cultured from peripheral blood of cancer patients and the success rate of establishing patient derived xenografts on CAM directly reflect the aggressiveness and proliferation capacity of the primary tumor. The CAM-based PDX model using circulating cancer stem cells could provide an alternative step in translational cancer research to assess experimental and novel strategies for both diagnostic and therapeutic purposes.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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5.4 Publikation III

Sensitive detection of PD-L1 expression on circulating epithelial tumor cells (CETCs) could be a potential biomarker to select patients for treatment with PD-1/PD-L1 inhibitors in early and metastatic solid tumors

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Sensitive detection of PD-L1 expression on circulating epithelial tumor cells (CETCs) could be a potential biomarker to select patients for treatment with PD-1/PD-L1 inhibitors in early and metastatic solid tumors

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ABSTRACT

Background: The current cancer research strongly focuses on immune therapies, where the PD-1, with its ligands plays an important role. It is known that PD-L1 is frequently up-regulated in a number of different cancers and the relevance of this pathway has been extensively studied and therapeutic approaches targeting PD-1 and PD-L1 have been developed. We used a non-invasive, real-time biopsy for determining PD-L1 and PD-L2 expression in CETCs of solid cancer patients.

Methods: CETCs were determined from blood of 128 patients suffering from breast (72), prostate (27), colorectal (18) and lung (11) cancer. The number of vital CETCs and the expression of PD-L1 and PD-L2 were investigated using the maintrac® method.

Results: PD-L1 expressing CETCs were detected in 94.5% of breast, 100% of prostate, 95.4% of colorectal and 82% of lung cancer patients whereas only 75% of breast cancer patients had PD-L2 positive CETCs. In the PD-L1 and PD-L2 expressing patients the cell fraction of PD-L1 positive CETCs is significantly higher than the fraction of PD-L2 positive CETCs (54.6% vs. 28.7%; $p<0.001$). Breast cancer patients with metastatic disease had significantly more PD-L1 positive CETCs as compared to patients without metastasis (median 75% vs. 61.1%; $p<0.05$).

Conclusion: PD-L1 seems to be a major factor in immune evasion and is highly expressed on CETCs regardless of the type of cancer.Monitoring the frequency of PD-L1 positive CETCs could reflect individual patient's response for an anti-PD-1/PD-L1 therapy and may be a promising target of anticancer treatment.

INTRODUCTION

Metastatic disease is responsible for over 90% of cancer-related deaths. To date the characteristics of the primary tumor are used to predict the probability of tumor progression and metastatic relapse. However, the clonal landscape of the overall tumor burden is very heterogeneous and a single biopsy may fail to represent the whole cancer cell population. Furthermore, the biopsy is invasive and repeating the procedure is not always feasible due to safety concerns [1, 2].

Most of metastases are due to hematogenous dissemination of tumor cells from the primary tumor which starts at an early stage of cancer growth. Single tumor cells or cell clusters shed from the primary tumor travel to distant organ sites and can grow into metastatic

lesions [3]. Aggressive tumors may release thousands of cancer cells into the circulation but only a small part of them can survive and <0.01% eventually succeed in forming metastasis [2]. The detection of circulating tumor cells presents a technical challenge, because these cells are assumed to be rare. Most studies performed in the metastatic situation have shown a significant correlation between overall survival and the number of circulating tumor cells detectable with the respective approaches [4]. Using a nondissipative method avoiding cell loss [5] circulating tumor cell counts can be used as a marker for therapy response also in the adjuvant situation allowing continuous monitoring during treatment [6]. These cells can not only be followed over time but also further characterized at any time during the course of disease.

Therefore, using circulating tumor cells as a "liquid biopsy" holds great potential to better represent the actual composition of tumor cells with minimal risk for patients. It can be repeated frequently for real-time monitoring of cancer treatment and can give important information on therapeutic targets and drug resistance mechanisms [1, 2, 7, 8, 9].

In order to successfully evade immune surveillance, tumor cells use a variety of different strategies. One of them is the upregulation of surface programmed cell death ligand 1 (PD-L1, CD247, B7-H1) expression [10]. PD-L1 is a 40 kDa transmembrane protein that is expressed on activated immune cell types including natural killer cells, macrophages, myeloid dendritic cells, B cells, and vascular endothelial cells as well as numerous epithelial cells including cancers. The physiologic role of PD-L1 is to bind to the programmed cell death 1 receptor (PD-1) expressed on the surface of activated cytotoxic T cells [10, 11]. The PD-1/PD-L1 interaction serves as an important regulatory checkpoint against an excessive adoptive immune response to antigens and autoimmunity [10]. This binding causes inhibition of IL-2 production and T cell activation. The second ligand for PD-1 is PD-L2 (also known as B7-DC and CD273) but its role in modulating immune responses is less clear and only little information is available. Generally, PD-L2 is expressed at a lower level than PD-L1 but the relative affinity of PD-L2 to PD-1 is 2-6-fold higher than that of PD-L1 [12]. The expression of PD-L1 has been evaluated in a number of tumor types in different localizations like head and neck, lung, stomach, colon, pancreas, breast, kidney, bladder, ovary, cervix, as well as melanoma, glioblastoma, multipole myeloma, lymphoma, and various leukemias [11]. Although most of the analyses of PD ligand expression have focused on PD-L1 PD-L2 has also been reported to be upregulated in various tumors with distinct expression profiles such as certain B cell lymphomas and Hodgkin´s disease [13, 14]. Anti-PD-1 and anti-PD-L1 drugs which should restore anti-cancer immunity have been developed and are now available for clinical use. Numerous novel checkpoint-inhibitors are being tested now in clinical trials. Durable responses have been observed in different cancers including melanoma, renal, lung, prostate, and bladder carcinomas [15, 16].

To date, however, there is no reliable predictive biomarker for determining the response rate for a targeted PD-1/PD-L1 therapy. It has been shown that PD-L1 expression by tumor and/or infiltrating immune cells correlates with a therapeutic response [17]. The intra- tumor heterogeneity observed in both primary and metastatic sites, with significantly higher PD-L1 expression in metastatic sites, indicates that a single core biopsy might not be sufficient to determine PD-L1 expression. For this reason the primary tumor may not be an adequate surrogate for determining PD-L1 expression in metastatic sites [18]. These distant sites of disease represent aggressive subclones that were able to disseminate from the primary tumor and to escape immune destruction, therefore identifying PD-L1 on circulating tumor cells could be a new biomarker for better selecting patients for treatment with PD-1/PD-L1 antibodies [10, 18]. Because cells that are able to effectively evade cytotoxic T cells would have a greater selective advantage and likely contribute more to the progression of cancer disease. Effective immune eradication of these highly invasive cells through PD-L1 antibody therapy may be an effective strategy for arresting the progression of cancer [10]. The purpose of our study was to better characterize PD-L1/-L2 expression on circulating epithelial tumor cells (CETCs) in solid tumors which might contribute a new biomarker for targeted PD-1 and PD-L1 therapy.

RESULTS

For the development of an approach to detect PD-L1 or PD-L2 on CETCs the specificity of antibodies was determined. Therefore we analysed different cancer cell lines H820, Sk-Br-3, MCF-7 and SW-620 by using fluorescence scanning microscope. H820 was strongly positive for PD-L1 only with clones 29E.2A3 and MIH1 and completely negative with clone 130021 (Figure 1). The specificity of the staining was demonstrated by the fact that no signals were detected for the SW-620, MCF-7 and Sk-Br-3 cell lines or with isotype control antibody (Figure 2). To further evaluate to specificity of the antibody, Sk-Br-3 was treated with IFN-γ. However, in our hands, PD-L1 expression in Sk-Br-3 cells remained unchanged. Clone 29E.2A3 was selected for further investigations because lack of unspecific staining.

The patient characteristics according to PD-L1 expression are shown in Table 1. The median number of CETCs was 55/100µl blood (ranging from 5 to 805), 65/100µl blood (ranging from 5 to 905), 55/100µl blood (ranging from 5 to 650) and 40/100µl blood (ranging from 5 to 95) in breast, prostate, colorectal and lung cancer patients, respectively. As negative control we tested blood samples from 25 healthy controls and confirmed that none

of the samples were positive for CETCs. We found no statistically significant difference between the number of CETCs as well as PD-L1 positive CETCs and tumor entities. PD-L1 positive CETCs were observed in 68 breast (94.5%), 27 prostate (100%), 17 colorectal (94.5%) and 9 lung (82%) cases. Median percentage of PD-L1 positive cells among the CETCs was 68.9 (range: 0-100) in breast, 65.8 (range: 32-100) in prostate, 57 (range: 0-90.5) in colorectal and 55 (range 0-90) in lung cancer. The absolute number of PD-L1 positive CETCs did not correlate with any clinicopathological parameters except with the presence of distant metastasis and radiotherapy in breast cancer patients. Patients with metastatic disease exhibited a significantly higher fraction of PD-L1 positive CETCs as compared to patients without metastasis (median 75% vs 61.1%; $p<0.05$) (Figure 3).

Figure 4 shows a representative serial analysis of CETCs and PD-L1 expression in one exemplary primary metastatic breast cancer patient (cT2 cN2 M1 (liver); ER: +, PR: +, HER2/neu: 3+, Ki-67: 30%) who had received chemotherapy and now is under antibody and hormone therapy. She was treated with a combination of Ipilimumab and Nivolumab. After first administration of immune-checkpoint inhibitors (01-04/16) the remnant metastases in the liver were significantly reduced and the number of CETCs initially was at a very low level. The proportion of PD-L1 positive cells was above 80%. CETC numbers first decreased but subsequently we observed an increase in cell numbers. The patient then received the second dose of immunotherapy (09-10/16) leading to a sharp decrease in CETC numbers as well as the frequency of PD-L1 expression on these cells. During the following 6 months of follow-up without immunotherapy the number of CETCs persists at a low level but the percentage of PD-L1 positive CETCs increases continuously achieving 100% at the most recent analysis. During this time the metastases have remained stable.

30 (41.6%) patients received adjuvant radiotherapy, of which 20 patients were irradiated with standard regime (50 Gy in 25 fractions + 16 Gy boost). 10 patients older than 60 years obtained hypofractionated regime with 42.56 Gy in 16 fractions. Patients after radiotherapy (n=30) had a higher fraction of PD-L1 positive CETCs as compared to patients without radiotherapy (n=31) (median 77.4% vs 62.5%; $p<0.05$), regardless of radiation regime (Figure 5). Since inflammation occurring during radiation may stimulate PD-L1 expression on tumor cells we analysed CETCs from patients with a recent history of irradiation and observed the same phenomenon in our study.

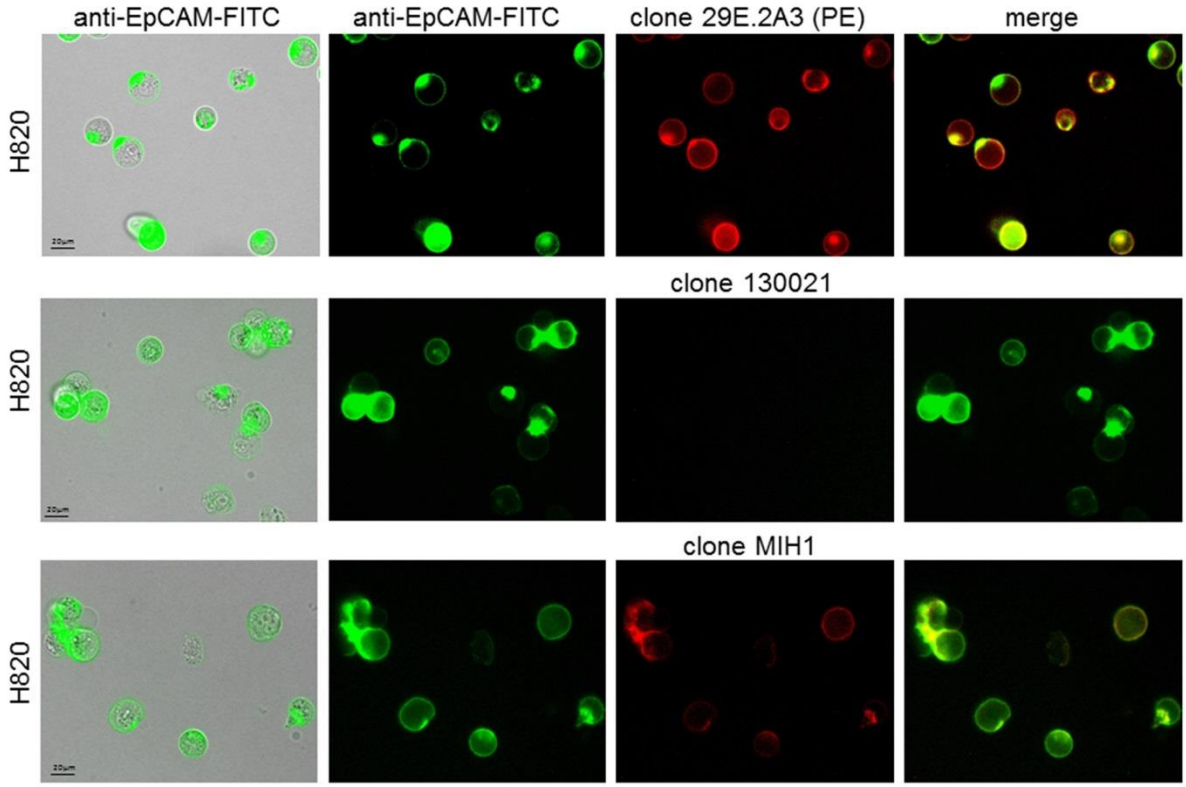

Figure 1: Specificity of different anti-human PD-L1 antibodies. H820 cell line was analyzed using three different anti-human PD-L1 MAb-PE clones: clone 29E.2A3, clone 130021, clone MIH1. The cells from the cell line H820 were positive for EpCAM (green) and PD-L1 (red) with clone 29E.2A3 and clone MIH1 and negative with clone 130021

We did not find any correlations between the numbers of PD-L1 positive CETCs and clinicopathological parameters in prostate, colorectal and lung cancer patients.

We, then, evaluated and compared the percentage of PD-L1 and PD-L2 positive CETCs in 28 breast cancer patients by performing co-expression analysis. The co-expression of PD-L1 and PD-L2 was confirmed in 82.1% of patients (Figure 6). In comparison with PD-L1, the percentage of PD-L2 positive CETCs was significantly lower (median PD-L1 54.6% vs median PD-L2 28.7%; $p<0.001$) (Figure 7) and did not correlate with any clinicopathological parameters. We found a substantial heterogeneity in PD-L1 and PD-L2 expression levels across the CETCs from the same patient at one time point (Figure 8a, 8b).

We next evaluated and compared the degree of concordance between PD-L1 expression and copy numbers in CETCs from the same patients in 13 breast cancer cases. PD-L1 amplified CETCs were detected in

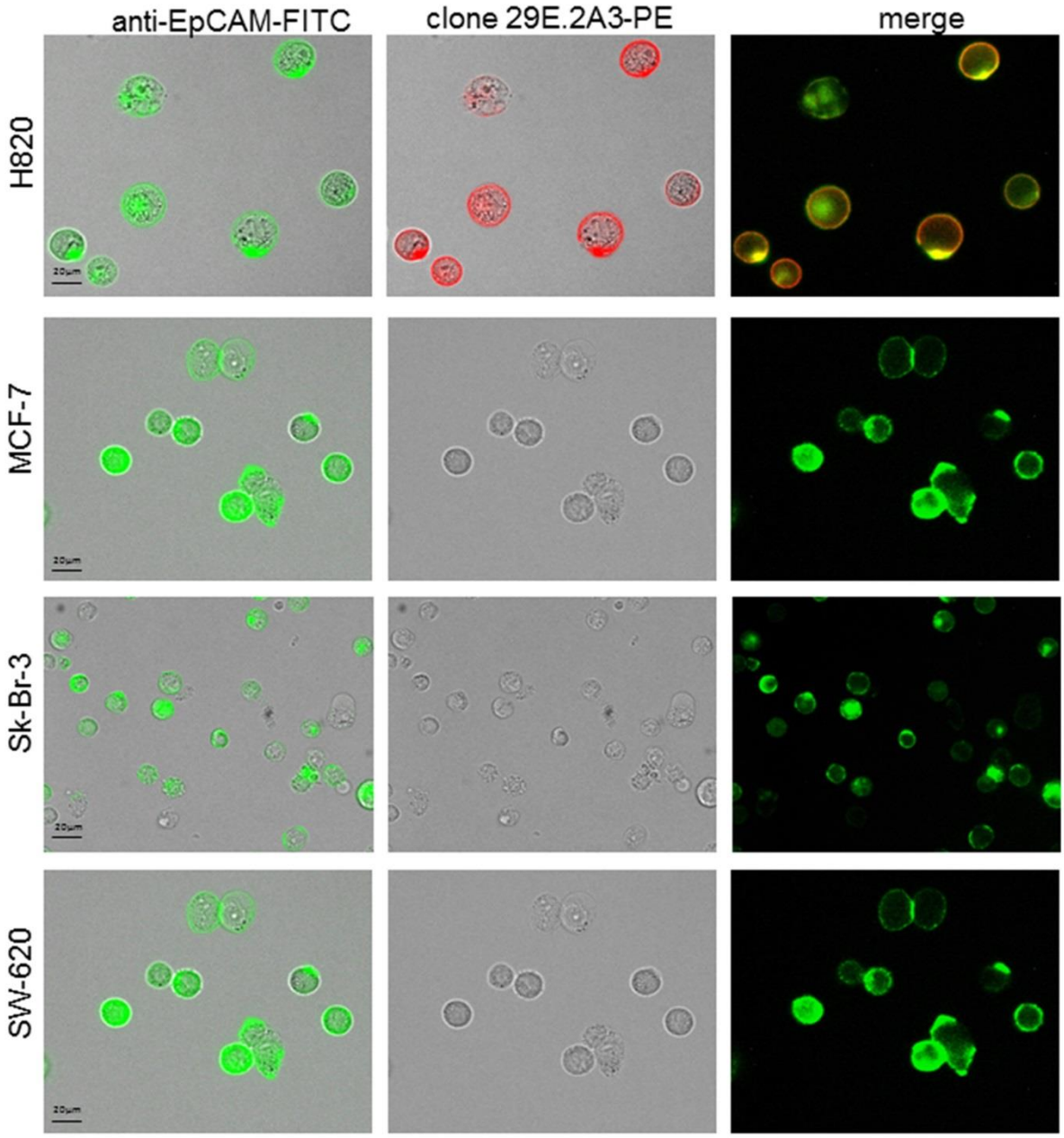

Figure 2: Specificity of clone 29E.2A3 with four different cell lines. H820 cells were positive for PD-L1 staining, whereas MCF-7, Sk-Br-3 and SW620 cells were completely negative.

Table 1: Characteristics of cancer patients and control group according to PD-L1 expression. (FEC= 5-fluorouracil, epirubicin, cyclophosphamide; EC= epirubicin, cyclophosphamide; TAC= docetaxel, doxorubicin, cyclophosphamide; HF=hypofractionated radiation therapy).

Clinicopathological Parameters	PD-L1 positive CETCs	PD-L1 negative CETCs	Median of PD-L1 positive CETCs (%)	p value
Breast cancer				
Gender				
Female	68 (94.5%)	4 (5.5%)		
Male				
Age				p>0.05
<50 years	19 (90.5%)	2 (9.5%)	70	
>50 years	49 (96%)	2 (4%)	67.9	
Tumor size				p>0.05
T1	38 (97.4%)	1 (2.6%)	75	
T2	13 (86.6%)	2 (13.4%)	64.3	
T3	7 (87.5)	1 (12.5%)	69.9	
T4				
n.a. (n=10)				
Lymph node metastasis				p>0.05
Positive	28 (96.5%)	1 (3.5%)	70	
Negative	25 (96%)	1 (4%)	76	
n.a. (n=17)				
Distant metastasis				p<0.05
Positive	17 (100%)	0 (0%)	75	
Negative	48 (88.9%)	6 (11.2%)	61.7	
n.a. (n=1)				
HER2 status				p>0.05
Positive	8 (88.9%)	1 (11.1%)	67.9	
Negative	42 (97.7%)	1 (2.3%)	70	
n.a. (n=20)				
ER status				p>0.05
Positive	53 (94.6%)	3 (5.4%)	73	
Negative	12 (100%)	0 (0%)	70	
n.a. (n=4)				
Chemotherapy				p>0.05
Adjuvant	38 (97.4%)	1 (2.6%)	71.4	
• FEC	19 (95%)	1 (5%)	76.6	
• EC	11 (100%)	0 (0%)	56.9	
• TAC	8 (100%)	0 (0%)	83.4	
Neoadjuvant (EC)	4 (100%)	0 (0%)	70	
No	27 (93%)	2 (7%)		

(*Continued*)

Clinicopathological Parameters	PD-L1 positive CETCs	PD-L1 negative CETCs	Median of PD-L1 positive CETCs (%)	p value
Endocrine therapy				p>0.05
Yes	44 (100%)	0 (0%)	72	
No	23 (92%)	2 (8%)	68.1	
n.a = 3				
Radiation				p<0.05
Yes	30 (100%)	0 (0%)	77.4	
• HF	10 (100%)	0 (0%)	79.5	
• Standard	20 (100%)	0 (0%)	70.7	
No	31 (100%)	0 (0%)	62.5	
n.a. (n=11)				

Clinicopathological Parameters	PD-L1 positive CETCs	PD-L1 negative CETCs	Median of PD-L1 positive CETCs (%)
Prostate cancer			
Age			
<60 years	6 (100%)	0 (0%)	61
>60 years	21 (100%)	0 (0%)	73.4
Stage			
I	3 (100%)	0 (0%)	75
II	4 (100%)	0 (0%)	72.1
III	4 (100%)	0 (0%)	42.6
IV	12 (100%)	0 (0%)	68.1
n.a. (n=4)			
Lymph node metastasis			
Positive	8 (100%)	0 (0%)	68.8
Negative	9 (100%)	0 (0%)	81.1
n.a. (n=10)			
Distant metastasis			
Positive	15 (100%)	0 (0%)	65
Negative	11 (100%)	0 (0%)	66.7
n.a. (n=1)			
Chemotherapy			
Yes	6 (100%)	0 (0%)	71.8
No	15 (100%)	0 (0%)	69.5
n.a (n=6)			
Radiation			
Yes	6 (100%)	0 (0%)	70.25
No	17 (100%)	0 (0%)	66.7
n.a. (n=4)			

(*Continued*)

Clinicopathological Parameters	PD-L1 positive CETCs	PD-L1 negative CETCs	Median of PD-L1 positive CETCs (%)
Colorectal cancer			
Gender			
Female	9 (100%)	0 (0%)	60
Male	8 (89%)	1 (11%)	55
Age			°
<60 years	6 (86%)	1 (14%)	60
>60 years	11 (100%)	0 (0%)	56
Stage			
I	1 (100%)	0 (0%)	56
II	2 (100%)	0 (0%)	46.5
III	3 (100%)	0 (0%)	55
IV	9 (90%)	1 (10%)	63.3
n.a. (n=2)			
Lymph node metastasis			
Positive	10 (91%)	1 (9%)	66.6
Negative	5 (100%)	0 (0%)	56
n.a. (n=2)			
Distant metastasis			
Positive	9 (90%)	1 (10%)	15
Negative	8 (0%)	0 (0%)	12.5
Chemotherapy			
Yes (FOLFOX)	10 (100%)	0 (0%)	66
No	6 (85.7%)	1 (14.3%)	58
n.a. (n=1)			

Clinicopathological Parameters	PD-L1 positive CETCs	PD-L1 negative CETCs	Median of PD-L1 positive CETCs (%)
Lung cancer			
Gender			
Female	2 (67%)	1 (33%)	56.6
Male	7 (88%)	1 (12%)	55.5
Age			
<60 years	1 (33%)	2 (67%)	44.6
>60 years	8 (100%)	0 (0%)	66.8
Stage			
I	0 (0%)	1 (100%)	0
II	1 (100%)	0 (0%)	55
III	2 (100%)	0 (0%)	41.6
IV	5 (83%)	1 (17%)	78.6
n.a. (n=1)			

(*Continued*)

Clinicopathological Parameters	PD-L1 positive CETCs	PD-L1 negative CETCs	Median of PD-L1 positive CETCs (%)
Distant metastasis			
Positive	4 (80%)	1 (20%)	78.6
Negative	4 (66.7)	2 (33.3%)	47.8
Chemotherapy			
Yes	2 (100%)	0 (0%)	64.3
No	4 (57%)	3 (43%)	33.3
n.a. (n=2)			
Control group			
Gender			
Female	0 (0%)	10 (100%)	
Male	0 (0%)	15(100%)	
Age			
<30 years	0 (0%)	8 (100%)	
>30 years	0 (0%)	17 (100%)	

all examined cases (Figure 9). The percentage of amplified CETCs ranged from 62 to 96% with median 75% and was significantly associated with PD-L1 expression (r=0.84, P<0.001) in the examined patients (Figure 10).

DISCUSSION

The PD-1/PD-L1 axis is a key suppressor of the cytotoxic immune response permitting cancer progression and metastasis and blockade of this pathway is a new promising therapeutic approach in oncology [13]. Clinical trials testing anti-PD-1 or anti-PD-L1 drugs have shown promising results with durable responses in different cancers [14]. Attention is now focused on the identification of a predictive biomarker to select patients who will actually benefit from a PD-1/PD-L1 blockade.

As there is no standard immunohistochemical technique, reports about the frequency of PD-L1 positivity

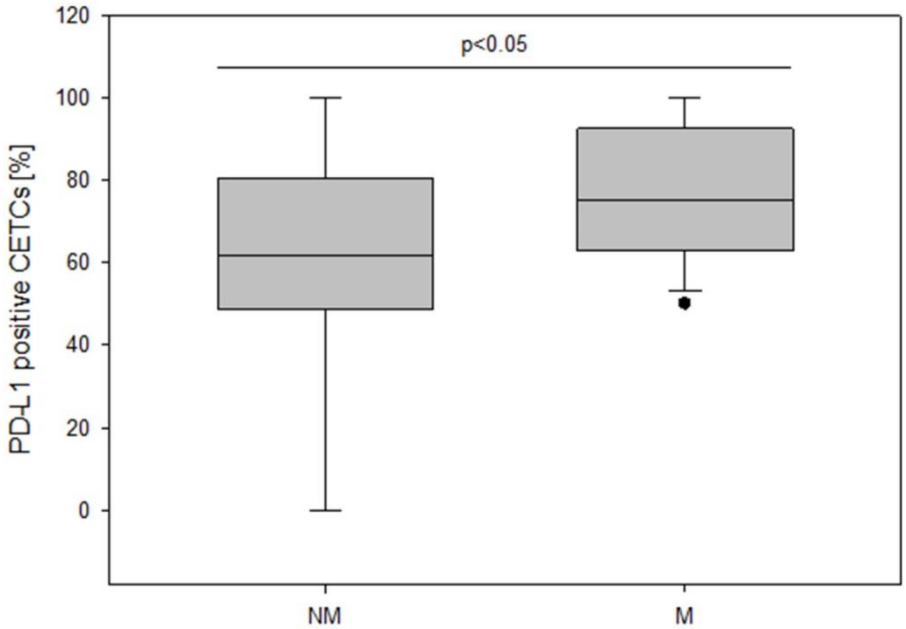

Figure 3: The frequency of PD-L1 positive CETCs (%) in non-metastatic and metastatic breast cancer patients.

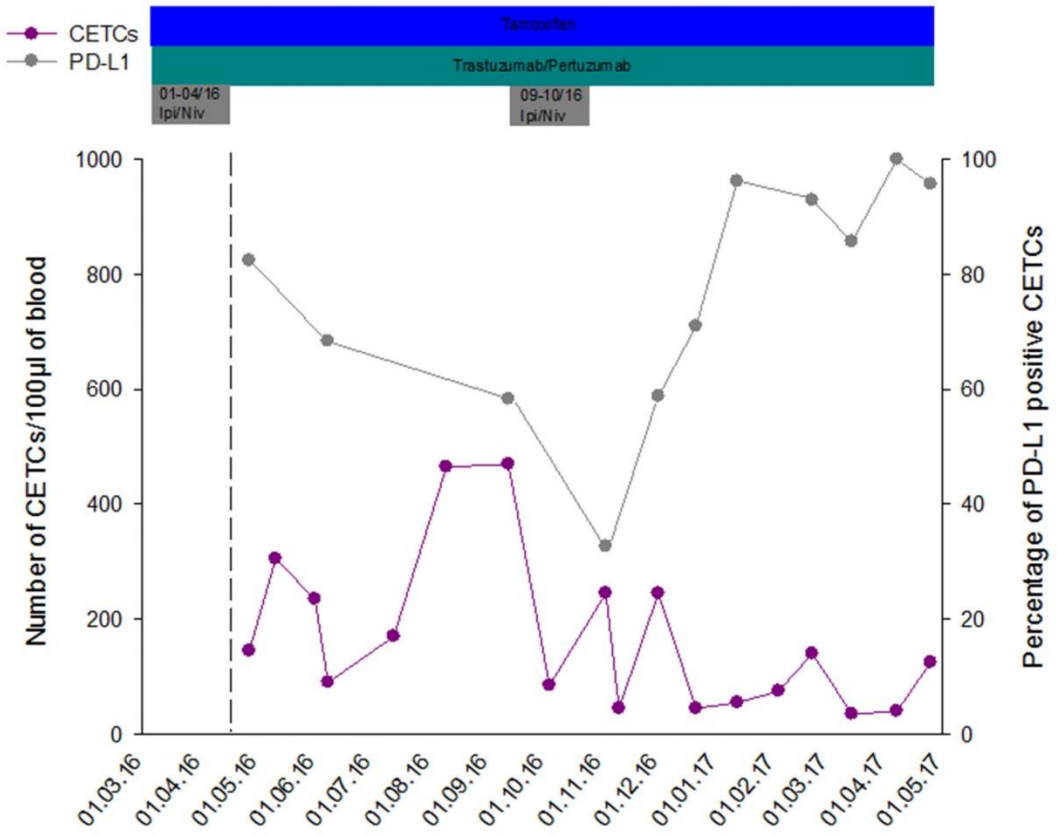

Figure 4: Exemplary course of number of CETCs and frequency of PD-L1 positive CETCs in one primary metastatic breast cancer patient during combined therapy with Nivolumab (Niv) and Ipilimumab (Ipi).

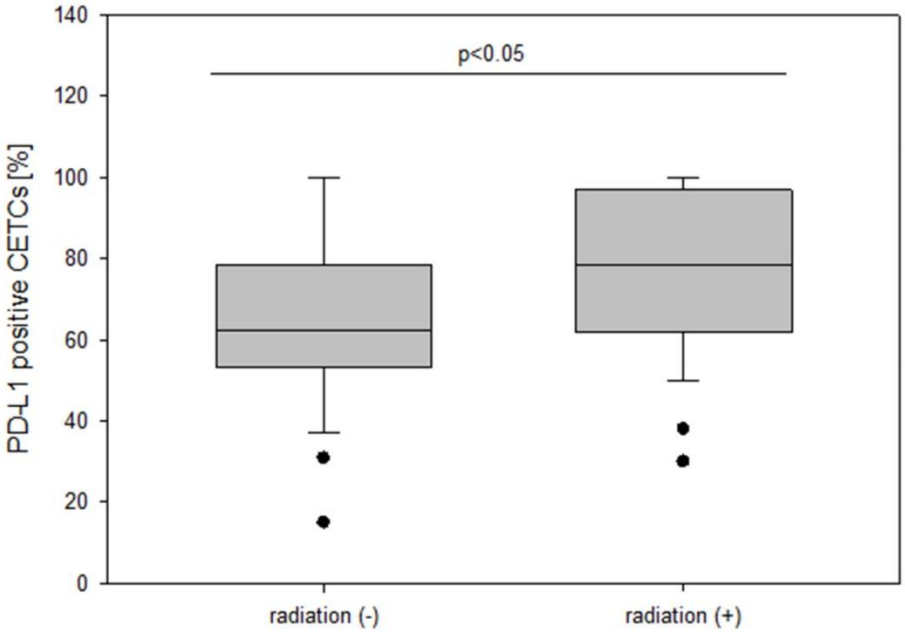

Figure 5: The frequency of PD-L1 positive CETCs (%) in breast cancer patients with and without radiation.

in formalin fixed paraffin embedded (FFPE) breast cancer tissue sections varies widely in the literature. Ghebeh et al. [19] and Muenst et al. [20] reported very similar results with PD-L1 positivity in 34% and 23.4% of patients, respectively. A very recent study analyzing 192 specimens showed that PD-L1 expression was present in 56.6% of breast cancer cases [21]. In contrast, Ali et al. analyzed 3916 breast tumors and found that PD-L1 was expressed in only 1.7% of the total cases [22]. These differences may be due to different methods and antibodies applied and to the fact, that surface antigens often are altered or destroyed by the fixation procedures [23, 24].

In contrast, circulating tumor cells, which are the precursors of metastatic disease, are accessible and can be detected in a comparable way as other blood cells [25].

Tumor cells that are invasive and able to effectively evade cytotoxic T cells would have a greater selective advantage and likely contribute more to the progression of cancer disease. Very little data is available for PD-L1 expression on these cells and its significance in circulating tumor cells. For this reason we investigated PD-L1 expression on circulating epithelial tumor cells in breast, prostate, colorectal and lung cancer patients.

For the establishment of the PD-L1 assay on CETCs we tested different cancer cell lines (MCF-7, Sk-Br-3, SW620 and H820) and different clones of PD-L1 antibodies (clone 29E.2A3, clone 130021 and clone MIH1). Clone 29E.2A3 reacted positively only with H820 cells. In contrast to Mazel et al, we found no specific PD-L1 staining with clone 130021 neither with the H820 nor with the Sk-Br-3 cell line even after IFNγ treatment. However, with clone 29E.2A3 the most specific and unambiguous staining was observed in the H820 but not in the Sk-Br-3 cell line. The latter showed minimal binding in two other studies [26, 27]. Therefore we selected the H820 cell line as positive control and clone 29E.2A3 for the current study.

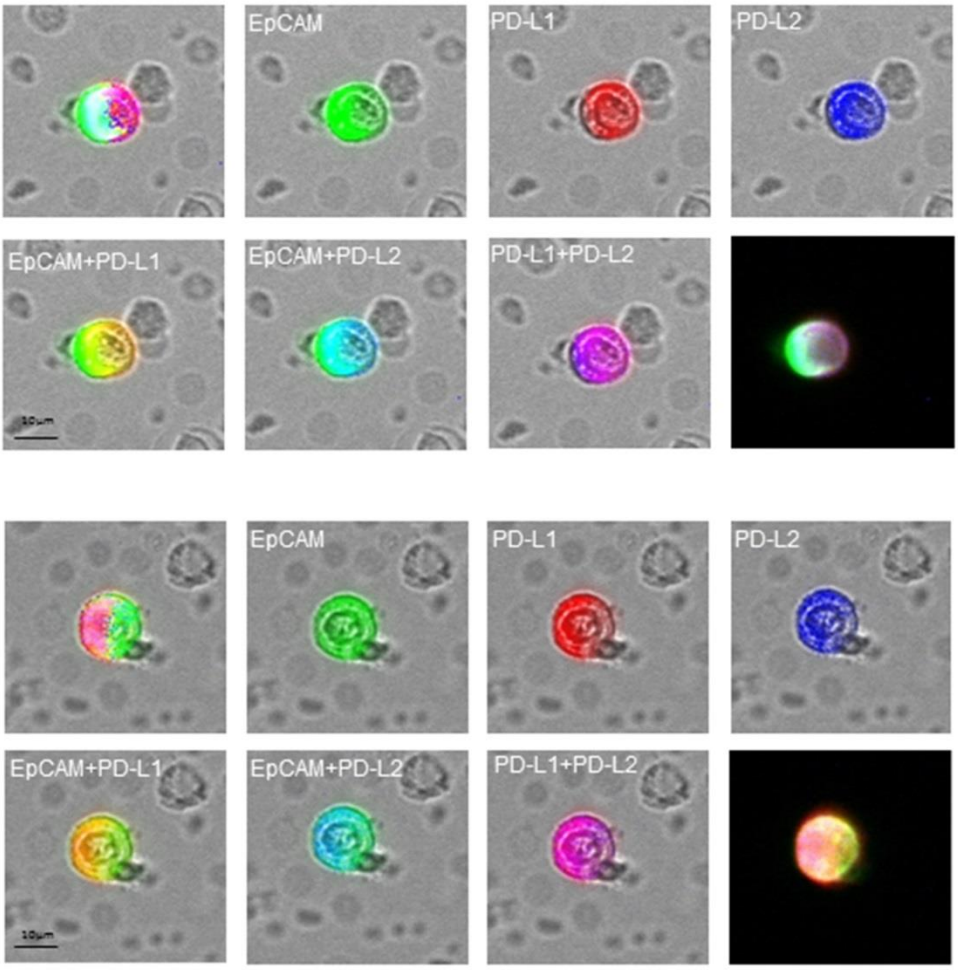

Figure 6: Fluorescence co-localization of EpCAM (green), PD-L1 (red) and PD-L2 (blue) on the CETCs in two representative results.

We found neither an association between the number of CETCs and different cancer types nor between the fraction of PD-L1 positive CETCs and different cancer types. In our approach 94.6% of breast cancer patients had PD-L1 positive CETCs although in different proportions. Mazel et al. using the CellSearch system found PD-L1 positive circulating tumor cells in 11/16 metastatic breast cancer patients (68.8%). The fraction of PD-L1 positive circulating tumor cells varied from 0.2-100% in individual patients [27], which is consistent

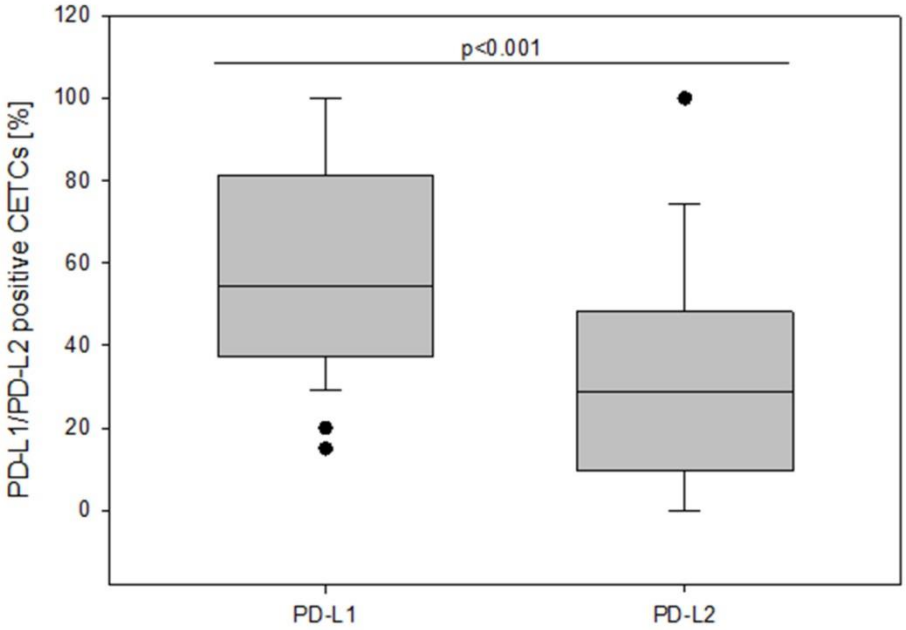

Figure 7: The frequency of PD-L1 and PD-L2 positive CETCs (%) in breast cancer patients.

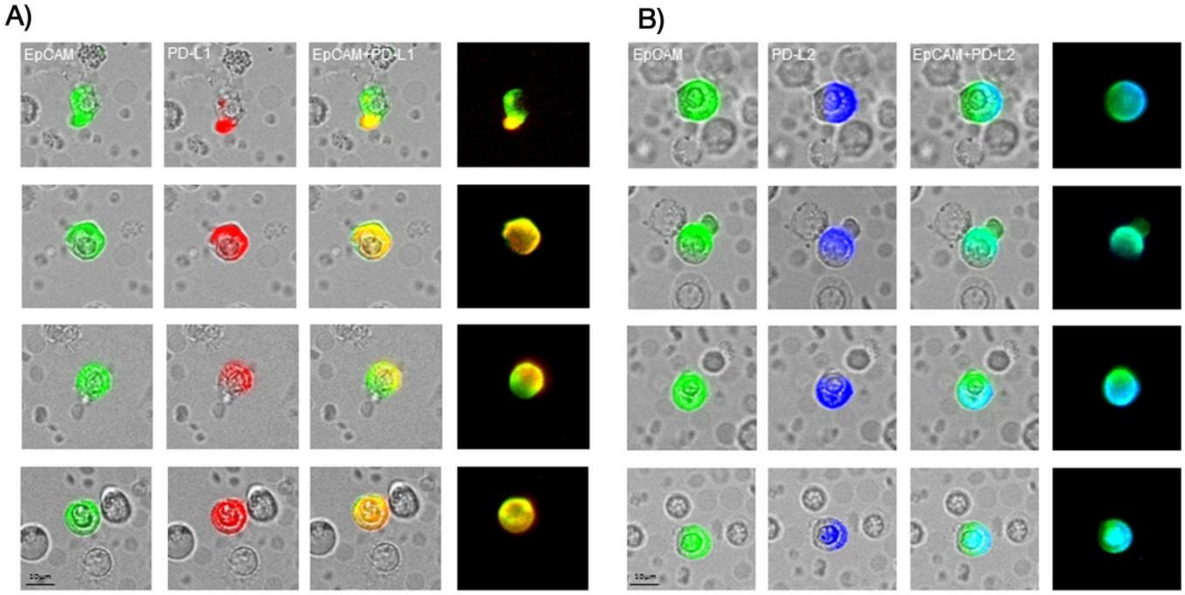

Figure 8: Illustrative CETCs pictures of double antibody staining for a) EpCAM (green) and PD-L1 (red) and b) EpCAM (green) and PD-L2 (blue). The expression of PD-L1 and PD-L2 is very heterogeneous and intensity of fluorescence varies strongly across CETCs from the same patients at one time point.

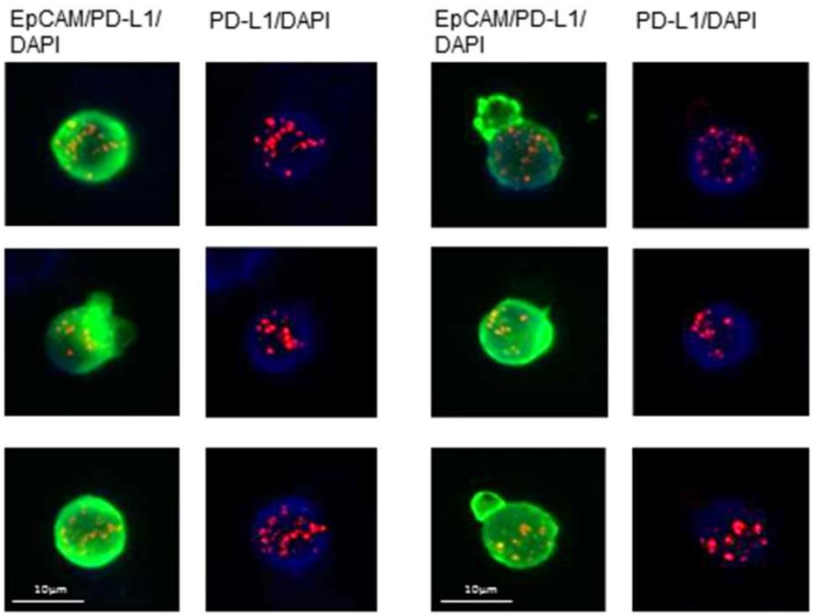

Figure 9: Illustrative examples of fluorescence in situ hybridization (FISH) for PD-L1 in CETCs from breast cancer patients.

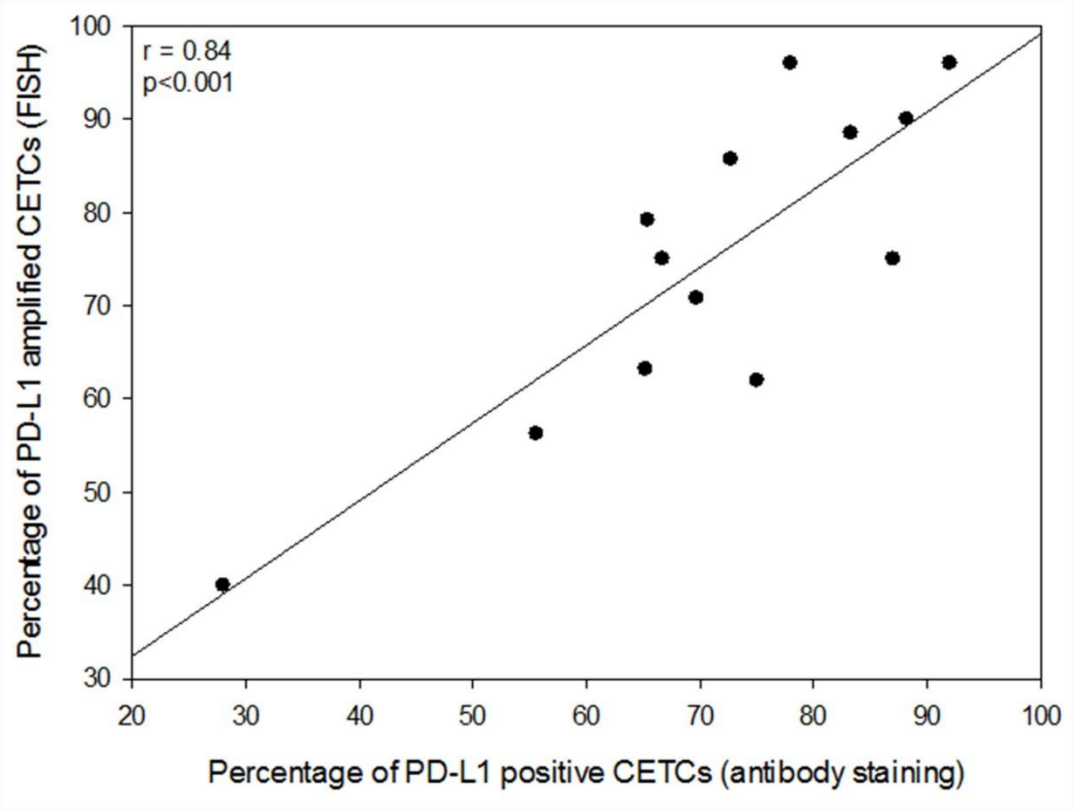

Figure 10: Correlation between the frequency of PD-L1 positive CETCs (antibody staining) and the frequency of PD-L1 amplified CETCs (FISH).

with our results (PD-L1 positive CETCs ranged from 0 to 100%). However, in contrast to the results of Mazel et al. we were able to detect PD-L1 positive CETCs also in patients without metastases. This may allow treatment decisions already in the adjuvant situation [28]. The frequency of PD-L1 positive patients and the expression on CETCs was high as compared to results from tumor tissue. Apart from the better accessibility of surface antigens in circulating cells (as depicted above) a possible explanation for this discrepancy is the fact that tumor cells circulating in the blood are continuously in contact with T-lymphocytes. Upon tumor antigen recognition T cells produce interferon gamma, which through the interferon gamma receptor leads to beneficial antitumor effects, such as increased antigen presentation, increased production of chemokines and direct tumor growth arrest and apoptosis. However, interferon gamma pathway also leads to an adaptive increase in PD-L1 expression

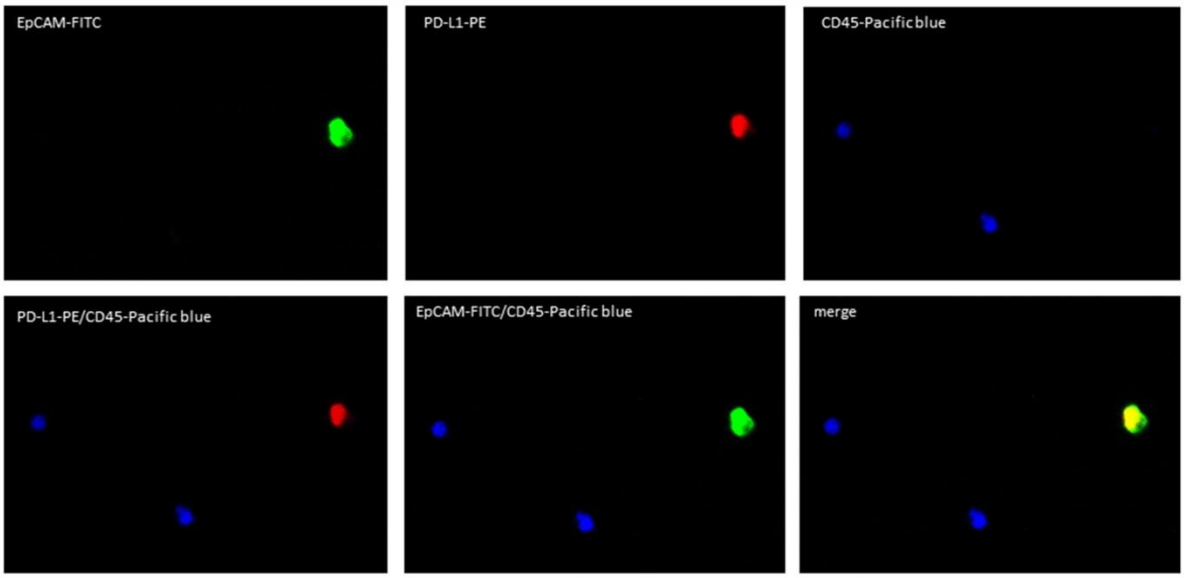

Figure 11: Fluorescence microscope images of PD-L1 positive CETCs. CETC is positive for EpCAM and PD-L1 and strictly negative for CD45.

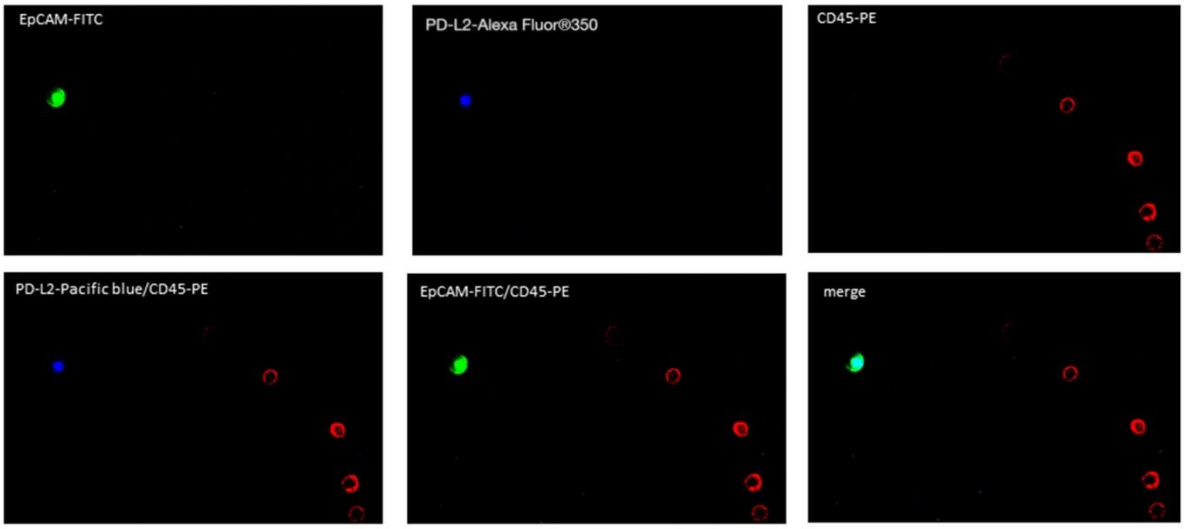

Figure 12: Fluorescence microscope images of PD-L2 positive CETCs. CETC is positive for EpCAM and PD-L2 and strictly negative for CD45.

on the tumor cells resulting in escape of T cell cytotoxic effects [29]. This may be a reason why the frequency of PD-L1 positive CETCs in our analyses is significantly higher compared to tumor tissue. So far the discordance between PD-L1 status on CETCs and corresponding tumor tissue was not investigated but will be important for future assessment. Intratumoral heterogeneity, small sample size, lack of standardization and the fact that PD-L1 up-regulation is a dynamic biomarker might limit the interpretation of solid tumor biopsies and could lead to false negative results depriving patients from treatment that might benefit them. Additionally, other factors like cancer type, stage of cancer analyzed and treatment history can influence the results [13, 16, 30]. Furthermore, repeatable tissue biopsies are not feasible because it is an invasive, technically challenging procedure carrying risks to the patient. In contrast, liquid biopsy through the accessible and fairly non-invasive approach might allow for a dynamic characterization of PD-L1 expression on CETCs and serial monitoring the response to treatment [31, 32]. We, here, show that as a sign of successful immunotherapy, the total number of CETCs declined and the fraction of PD-L1 positive CETCs was significantly reduced. After discontinuation of checkpoint inhibitors the percentage of PD-L1 positive CETCs increased continuously and has achieved 100%. Taken together, immune-checkpoint inhibitors were able to eliminate PD-L1 positive CETCs from the peripheral blood of this breast cancer patient. It has been shown that persistence of PD-L1 positive circulating tumor cells correlates with poor prognosis and might reflect a mechanism of therapy escape [30]. Results regarding PD-L1 expression in tumor tissue and overall survival (OS) or disease free survival (DFS) are contradictory and the status of PD-L1 can either correlate with poor prognosis, better prognosis or show no correlation with prognosis at all. Muenst et al. postulated that PD-L1 expression is a negative prognostic factor of poor outcomes in breast cancer [20]. In contrast, Reiss et al. suggested that PD-L1 could be a good prognostic biomarker for OS in breast cancer [33]. In our study patients with metastatic disease had higher numbers of PD-L1 positive CETCs compared to patients without distant metastasis. Baptista et al. noticed that PD-L1 expression in tumor samples was significantly correlated with recurrence at distant sites [21]. Here we show that the number of PD-L1 positive CETCs correlates with the aggressiveness of tumor.

In the adjuvant situation chemo- and radiotherapy are the major components of cancer treatment but many patients get local recurrence or metastasis. The association of PD-L1 expression and obtaining radiotherapy found in our study correlates well with the known inflammatory effect of radiotherapy. Dovedi et al. showed that fractionated radiotherapy is responsible for an increased IFNγ production by $CD8^+$ T cells mediating up-regulation of PD-L1 expression on tumor cells. Additionally, there is a strong correlation between PD-L1 expression on tumor cells and lymphocytic infiltration not only among tumors but also within regional sites in a tumor [13, 34, 35].

We were surprised to observe that 100% of prostate cancer patients had PD-L1 positive CETCs. Also in prostate tumor tissue PD-L1 expression seems to be elevated in comparison to other tumor types. Gevensleben et al. detected PD-L1 expression in 61.7% of primary prostate cancers [36]. Massari and colleagues recent study showed that PD-L1 was expressed in 50% of castration-resistant prostate adenocarcinoma [37].

The results reporting PD-L1 expression in colorectal cancer are highly diverse. Whereas Masugi et al. [38] found that 89% of colorectal carcinomas exhibited high tumor PD-L1 expression Lee et al. and Rosenbaum et al. reported on low levels of PD-L1 expression in colorectal cancer (5% and 9%, respectively) [39, 40] again possibly due to differences in methods applied. The frequency of patients with PD-L1 positive CETCs in our approach was 94.5% and thus fits rather to the former results of Masugi et al.

Also in NSCLC reports on PD-L1 expression varies highly ranging from 7.4% to 72.7% [41]. With respect to circulating tumor cells in patients with advanced NSCLC Nicolazzo et al. found that 95% of patients had a subpopulation of PD-L1 positive of PD-L1 positive [31] similar to our results of 82% in NSCLC patients.

Detection of copy number variants which, differently to the difficulties in antibody-dependent approaches, is independent of most fixation procedures can be an alternative method to IHC. The PD-L1 gene is located on chromosome 9p24.1 and the amplification of this gene locus has been reported in lymphomas [42], triple negative breast cancer [43] and NSCLC [44]. The up-regulation of proteins may be due to an increase in copy numbers and the overexpression of PD-L1 is frequently observed in PD-L1 amplified cases in such tumors. Information on the PD-L1 copy number status was lacking in CETCs so far. Here, we showed for the first time that PD-L1 copy numbers were increased and PD-L1 copy number gains were associated with PD-L1 expression on CETCs.

The role of PD-L2 in evading the immune system is not fully understood. Comparing the expression level of both PD-1 ligands on CETCs in breast cancer patients we observed that frequency of cells with PD-L2 expression was significantly lower compared to the frequency of PD-L1 expression. Therefore PD-L2 may play only a marginal role in immunotherapy. This is in agreement to previous studies which found that in comparison to PD-L1, PD-L2 expression was observed less frequently in tissue samples. PD-L2 expression may be relatively restricted to macrophages, dendritic cells, and fibroblasts [12, 44]. To the best of our knowledge this is the first report on PD-L2 determination on circulating tumor cells.

Breakthrough therapy with checkpoint inhibitors in the treatment of cancer may gain even more importance in

the near future. Drugs inhibiting PD-L1 and PD-1 exhibit a favorable toxicity profile, but so far treatment is applied only to a subset of patients. There is a need to identify reliable biomarkers to predict response to these therapies and to facilitate patient selection [18]. Taken together, the high frequency of PD-1 ligand expression by circulating epithelial tumor cells provides an important rationale for the capacity of antibody blockade of this pathway already in the adjuvant situation to enhance immune response. Furthermore, analysis of CETCs for PD-L1 expression could be useful for therapy stratification and monitoring response to therapy. Additionally, during course of therapy serial tests could allow the detection of early resistance development.

MATERIALS AND METHODS

Blood collection and processing

Peripheral blood (7.5 ml) from altogether 128 patients with breast (72/56%), prostate (27/21%), colorectal (18/14%) and lung (11/9%) cancer in different stages of disease was drawn into normal blood count tubes with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant and processed within 48 hours of collection. In parallel, healthy control blood samples were collected from 25 female and male donors aged from 20-40 years. In patients with primary breast cancer (n=55) the sampling of peripheral blood was carried-out 6-12 weeks after end of standard therapy (tumor resection, adjuvant chemotherapy, adjuvant radiotherapy). In patients with local or distant recurrence the blood was collected prior to treatment of recurrent disease.

maintrac®

For CETC enumeration and further characterization the maintrac® approach was used, as reported previously [5]. Briefly, 1 ml blood was subjected to red blood cell lysis using 15 ml of erythrocyte lysis solution (Qiagen, Hilden, Germany) for 15 min in the cold, spun down at 700 g and re-diluted in 500 µl of PBS-EDTA. 5µl of fluorescein-isothiocyanate (FITC)-conjugated anti-human epithelial cell adhesion molecule antibody (EpCAM) (clone HEA-125, Miltenyi Biotec GmbH, Germany) at a final concentration of up to 10^7 cells/100 µl cell suspension were added and incubated for 15 min in cold. The corresponding isotypic control for EpCAM (Mouse $IgG1_K$ FITC, Miltenyi Biotec GmbH, Germany) was used at the same final concentration. The samples were subsequently diluted with 430 µl PBS-EDTA. A defined volume of the cell suspension and propidium iodide (PI) (Sigma-Aldrich, USA) was transferred to wells of ELISA plates (Greiner Bio-one, USA). Analysis of red and green fluorescence of the cells was performed using a Fluorescence Scanning Microscope, ScanR, (Olympus, Hamburg, Germany), enabling detection and relocation of cells for visual examination of EpCAM positive cells. For data analysis we used the ScanR Analysis software (Olympus, Hamburg, Germany). Vital CETCs were defined as EpCAM-positive cells, lacking in CD45-/PI-staining and with intact morphology, and only these cells were counted. We used fluorospheres (Flow-Check 770, Beckman Coulter) for daily verification of optical components and detectors of the microscope, which are required to ensure the consistent analysis of samples.

Cell lines

H820 lung cancer cells which were used as a positive control for PD-L1 analysis were obtained from the American Type Culture Condition (ATCC, Manassas, USA). Three different clones of anti-PD-L1 antibodies were tested: (1) clone 29E.2A3 (BioLegend, San Diego, USA), (2) clone 130021 (R&D Systems, Minneapolis, USA), (3) clone MIH1 (eBioscience, San Diego, USA) (Figure 1). SW620 colorectal cancer cells, MCF7 and Sk-Br-3 breast cancer cells were used as a negative control and were obtained from the CLS cell lines service (Eppenheim, Germany) (Figure 2). H820 cells were grown in RPMI-1640 medium with 5% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Waltham, USA), SW620 and Sk-Br-3 cells were grown in Dulbecco´s modified Eagle´s medium with 4,5g/L glucose, 2mM L-glutamine (Gibco, Thermo Fisher Scientific, Waltham, USA) and 10% FBS. Cells were maintained at 37°C in 5% CO_2. MCF-7 cells were grown in Minimum Essential Eagle ready-to-use medium (CLS cell lines service (Eppenheim, Germany). For immunofluorescence analysis cells were detached from cell culture flasks using StemPro® Accutase® Cell Dissociation Reagent (Gibco, Thermo Fisher Scientific, Waltham, USA) washed and stained for PD-L1 with the same protocol like a patient sample.

PD-L1/-2 analysis

The analyses of PD-L1 and PD-L2 expression on the CETCs were performed with an extended maintrac® approach. For PD-L1 expression analysis we used an anti-human PD-L1 phycoerythrin (PE)-conjugated antibody (clone 29E.2A3, BioLegend, San Diego, USA) at a final concentration of 0.2 µg/ml and for PD-L2 we used an anti-human PD-L2 Alexa Fluor® 350 conjugated antibody (clone 176611, novus biologicals, Littleton, USA) at a final concentration of 2 µg/ml. The corresponding isotypic controls for PD-L1 (Mouse IgG2b PE, BioLegend, San Diego, USA) and PD-L2 (Mouse IgG2b Alexa Fluor® 350, Novus biologicals, Littleton, USA) were used at the same final concentration. Finally, cells were visually inspected looking for a green, red and blue surface staining, but also a well-preserved nucleus (Figure 8a, 8b). For excluding expression of PD-L1/PD-L2 on hematopoetic cells we additionally

performed staining with EpCAM-FITC, PD-L1-PE/PD-L2-Alexa Fluor® 350 and CD45-Pacific blue/CD45-PE antibodies (Figure 11, 12). The results for PD-L1 and PD-L2 were calculated as percentage of total number of CETCs.

Fluorescence-in-situ-hybridization (FISH)

For FISH analyses the *PD-L1* gene was tested by using a dual fluorescence kit (CD274(PD-L1)/CEN9q FISH Probe, abnova, Taiwan) containing the *CD274* (*PD-L1)* gene (9p24, directly labeled with Texas Red) and *CEN9q* (9q21, labeled with FITC). Patient cells were transferred onto Poly-L-Lysin coated slides. Before hybridization slides were fixed with 4% paraformaldehyde for 10 min and treated for 10 min with proteinase K at room temperature. In a next step, cells were denatured for 5 min at 72°C in 70% formamide - 2 x standard saline citrate solution, air dried and dehydrated in 70%, 85% and 96% ethanol. After overnight hybridization at 37°C in a humidified chamber, slides were washed, air dried and counterstained with 0.2 µM DAPI in an anti-fade solution. At least 20 nuclei per sample were counted. CETCs were positive for PD-L1 amplification when more than 3 PD-L1 signals in one cell were counted. The final results for PD-L1 amplification were calculated as percentage of 20-30 visually expected EpCAM positive cells.

CONFLICTS OF INTEREST

We declare that we have no conflicts of interest.

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5.5 Publikation IV

B7-H3 on circulating epithelial tumor cells correlates with the proliferation marker, Ki-67, and may be associated with the aggressiveness of tumors in breast cancer patients

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B7-H3 on circulating epithelial tumor cells correlates with the proliferation marker, Ki-67, and may be associated with the aggressiveness of tumors in breast cancer patients

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Abstract. Circulating epithelial tumor cells (CETCs) in peripheral blood are a prerequisite for the development of metastases. B7-H3 is an important immune checkpoint member of the B7 family and inhibits T-cell mediated antitumor immunity. Its expression is associated with a negative prognosis and a poor clinical outcome. Based on the clinical success of inhibitory immune checkpoint blockade, monoclonal antibodies (mAbs) against B7-H3 appear to be a promising therapeutic strategy. The proliferation biomarker, Ki-67, is used as a prognostic factor for breast cancer and reflects the proliferative potential of the tumor. In order to better understand the role of B7-H3 and Ki-67 in cancer development, in this study, we used a real-time biopsy for determining both biomarkers on CETCs in breast cancer patients. Blood from 50 patients suffering from breast cancer was analyzed for CETCs and the expression of B7-H3 and Ki-67 using the maintrac® method. B7-H3 expression on CETCs was found in 82% of the patients. The frequency of B7-H3- and Ki-67-positive CETCs was significantly higher in patients who had received radiation therapy compared to patients who had not received irradiation. B7-H3-positive CETCs seemed to be more aggressive as the percentage of B7-H3-positive CETCs correlated with the percentage of cells positive for the proliferation marker, Ki-67 ($r=0.72$ $P<0.001$). A significant association between the Ki-67 and B7-H3 expression level on the CETCs and nodal status was observed. On the whole, the findings of this study indicate that breast cancer patients have detectable CETCs with a high frequency of B7-H3 expression regardless of the stage of the disease. B7-H3 seems to be an important factor in immune evasion and may thus be a promising target for anticancer therapies. Radiation may lead to an upregulation of B7-H3 expression on CETCs, which could be a possible mechanism of acquired radio-resistance.

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Key words: B7-H3, circulating epithelial tumor cells, Ki-67, breast cancer

Introduction

Breast cancer is the most frequently diagnosed malignancy in women (1). Despite improved methods of early detection and diagnosis, as well as usually effective clinical management, breast cancer is the second leading cause of cancer-related mortality due to recurrent metastatic disease (2). Of note, 90% of breast cancer-related deaths are not due to the primary tumor, but due to metastatic disease. Current models of metastasis support the hypothesis that cells can detach from the primary tumor and move to distant sites via the blood vessels and lymphatic system (3). For this reason, new methods are necessary for the detection and treatment of the residual tumor cells in order to prevent metastasis (4). A large number of studies have documented disseminated tumor cells in the bone marrow or circulating tumor cells in the peripheral blood from patients with most types of epithelial cancer (5-10). The detection and characterization of tumor cells in the peripheral blood have various potential applications in oncology (11). In the early stage of the disease, the enumeration and characterization of circulating tumor cells can potentially help to monitor the effect of systemic therapy, detect an early relapse of malignancy, predict the risk for metastatic disease and, thus, help to improve prognosis (12,13).

In the more advanced stages of the disease, circulating tumor cells may provide prognostic information and aid the physician in monitoring the response to treatment (14,15). Moreover, circulating tumor cells may represent characteristics of the residual tumor, inform about the sensitivity to anticancer drugs and, thus, can be used for a personalized anticancer therapy (16). The phenotypic characterization of tumor cells circulating in the blood has the potential to improve the current understanding of metastasis formation and immune modulation. They can be used as a real-time liquid biopsy in a variety of human cancers and may play a major role in helping to administer a targeted therapy.

B7-H3 is a surface antigen against which a targeted therapy can be envisioned. It is a type I transmembrane protein and an important immune checkpoint member of the B7 ligand family, for which the receptor(s) have not yet been identified. Its expression is induced on immune cells, particularly antigen-presenting cells (17). It is assumed that B7-H3 is involved in the inhibition of T-cells. On the other hand, it has been found that B7-H3 has also stimulatory immunological functions (18,19).

B7-H3 protein has been detected in several cell lines (18,20) and numerous studies have described B7-H3 expression in human malignancies (17,21-27). Apart from immune evasion, B7-H3 plays a role in cancer progression, including invasion and migration, angiogenesis and gene regulation (19). The proportion of the expression is associated with both a negative prognosis and a poor clinical outcome in patients (17). The blocking immune checkpoints, such as CTLA-4, programmed cell death protein 1 (PD-1) and its ligand, PD-L1, has shown clinical benefit in patients with different tumor entities. Due to its comparable role in immune evasion, B7-H3 has become an interesting target for novel immunotherapeutic treatments (17,28-30).

Ki-67 (also known as MKI67) is a cellular marker that is tightly linked to the cell cycle. The fact that Ki-67 is universally expressed among proliferating cells and is absent in quiescent cells has led to the further evaluation of Ki-67 as a marker of proliferation (31). Although little is known about the exact function of the protein in dividing cells, Ki-67 is expressed during the G1, S and G2 phases of the cell cycle with a peak during mitosis and it is absent in the G0 phase (32,33). There is a strong association between the proportion of Ki-67-positive cells and tumor size, aggressiveness, the level of angiogenesis and the survival of patients. Patients with breast cancer and a Ki-67 index >15% have a poor prognosis associated with a shortened disease-free and overall survival (34). On the other hand numerous studies indicate a positive correlation between the percentage of proliferating cells and the response to preoperative treatment with chemotherapy. The higher the level of Ki-67, the more pronounced the sensitivity of breast cancer to neoadjuvant therapy (35). The detection of Ki-67 on circulating epithelial tumor cells (CETCs) may be clinically more informative than the examination of total CETC numbers as it allows for the quantification of proliferative and non-proliferative subpopulations among the CETCs.

The aim of this study was to evaluate B7-H3 and Ki-67 expression on CETCs in breast cancer patients which may contribute to a better understanding of the immune escape mechanisms of these cells. Identifying the proliferative subpopulation of CETCs may serve as a useful tool with which to predict the aggressiveness of cancer.

Patients and methods

Peripheral blood (7.5 ml) from 50 breast cancer patients in different stages of disease was drawn into blood count tubes with ethylenediamine-tetra-acetic acid (EDTA) as an anticoagulant and processed within 48 h of collection. Medical records were reviewed for determination of ER/PR and HER2 status in the primary tumor or metastatic biopsy upon diagnosis. The primary tissue was processed in the corresponding hospitals according to the ASCO-CAP guidelines. In parallel, healthy control blood samples were collected from 12 male and 8 female donors aged between 20-40 years. The sampling of peripheral blood was carried out 6-12 weeks after the end of standard therapy (tumor resection, adjuvant chemotherapy and adjuvant radiotherapy). In patients with local or distant recurrence, blood was collected prior to the treatment for recurrent disease. All patients and healthy volunteers gave their informed consent to participate in the study, which was approved by the Ethics and Scientific Committees of the University of Jena (Jena, Germany).

For CETC enumeration and further characterization, the maintrac® approach was used, as reported previously (36). Briefly, 1 ml blood was subjected to red blood cell lysis using 15 ml of erythrocyte lysis solution (Qiagen, Hilden, Germany) for 15 min at 4˚C spun down at 700 x g and re-diluted in 500 ml of PBS-EDTA. Subsequently, 5 μl of fluorescein-isothiocyanate (FITC)-conjugated anti-human epithelial cell adhesion molecule antibody (EpCAM, dilution 1:4, clone HEA-125, cat. no. 130-113-203, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) at a final concentration of up to 10^7 cells/100 μl cell suspension were added and incubated for 15 min at 4˚C. The corresponding isotypic control for EpCAM (Mouse IgG1K FITC, Miltenyi Biotec GmbH) was used at the same final concentration. The samples were subsequently diluted with 430 μl PBS-EDTA. A defined volume of the cell suspension and propidium iodide (PI; Sigma-Aldrich, St. Louis, MO, USA) was transferred to wells of ELISA plates (Greiner Bio-One, Monroe, NC, USA). The analysis of red and green fluorescence of the cells was performed using a Fluorescence Scanning Microscope, ScanR, (Olympus, Tokyo, Japan), enabling the detection and relocation of cells for the visual examination of EpCAM-positive cells. For data analysis, we used the ScanR Analysis software (Olympus). Vital CETCs were defined as EpCAM-positive cells, lacking in nuclear PI staining and with intact morphology, and only these cells were counted (Fig. 1). We used fluorospheres (Flow-Check 770, Beckman Coulter, Brea, CA, USA) for the daily verification of optical components and detectors of the microscope, which are required to ensure the consistent analysis of samples.

The analyses of B7-H3 and Ki-67 expression on the CETCs were performed with an extended maintrac® approach. For B7-H3 expression analysis, we used an anti-human B7-H3 phycoerythrin (PE)-conjugated antibody (dilution 1:10, clone MIH42, cat. no. 351002, BioLegend, San Diego, CA, USA) at a final concentration of 0.9 μg/ml and for Ki-67 we used an anti-human Ki-67 phycoerythrin (PE)-conjugated antibody (dilution 1:10, clone Ki-67, cat. no. 350503, BioLegend) at a final concentration of 0.1 μg/ml. The corresponding isotype controls for B7-H3 (Mouse IgG1 PE, cat. no. 400101, BioLegend) and Ki-67 (Mouse IgG1 PE, cat. no. 400111, BioLegend) were used at the same final concentration. Finally, the cells were visually inspected looking for a green and red surface staining, but also a well-preserved nucleus (Fig. 2). For excluding the expression of B7-H3/Ki-67 on hematopoetic cells, we additionally performed staining with EpCAM-FITC, B7-H3-PE/Ki-67-PE and CD45-Pacific blue (clone J.33, dilution 1:1, cat. no. A74763, Beckman Coulter, Krefeld, Germany) antibodies (Fig. 3). The results for B7-H3 and Ki-67 were calculated as a percentage of the total number of CETCs.

MCF-7 and Sk-Br-3 (data not shown) breast cancer cells, which were used as positive controls for Ki-67 analysis, were obtained from the CLS Cell Lines Service (Eppenheim, Germany). The Sk-Br-3 cell line was grown in Dulbecco's modified Eagle's medium with 4,5 g/l glucose, 2 mM L-glutamine (Gibco/Thermo Fisher Scientific, Waltham, MA, USA) and 10% FBS. The cells were maintained at 37˚C in 5% CO_2. The

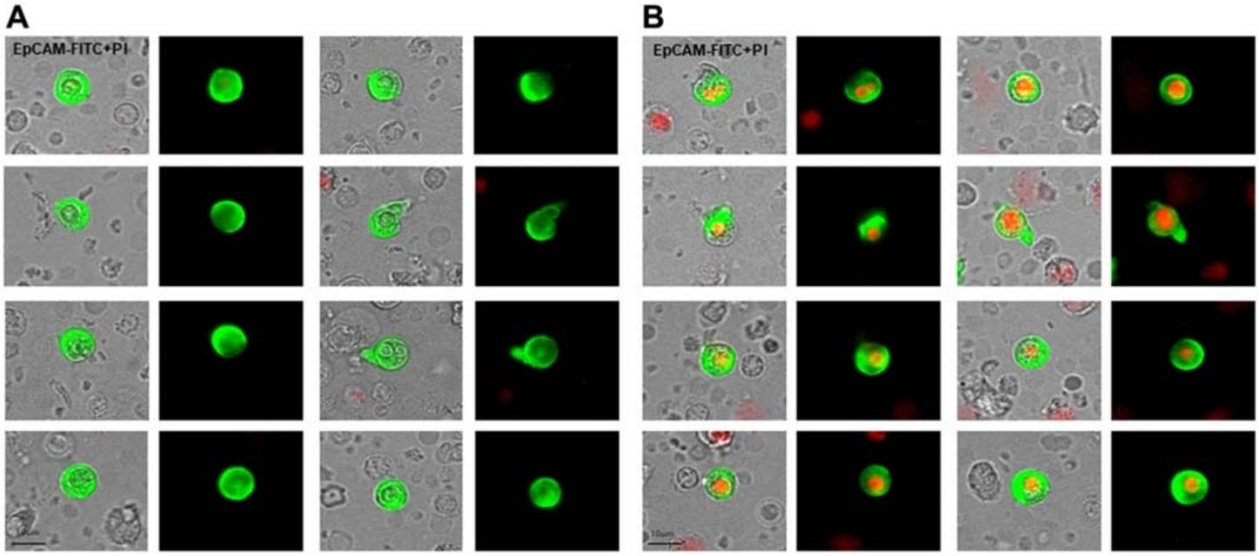

Figure 1. Illustrative images of live and dead CETCs. (A) The live CETCs with a green EpCAM surface staining, a well preserved morphology, but no red intracellular staining by PI are shown. EpCAM-fluorescence varies strongly between the individual cells. (B) Images representing dead CETCs exhibiting red intracellular staining by PI. CETCs, circulating epithelial tumor cells.

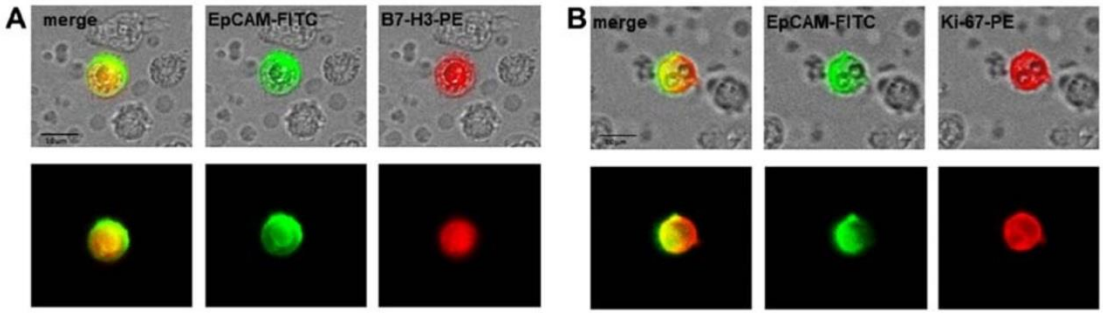

Figure 2. Illustrative CETC images of double antibody staining for (A) EpCAM (green) and B7-H3 (red) and (B) EpCAM (green) and Ki-67 (red). The yellow color is a result of the mixture of green and red due to co-expression at the same site of EpCAM and B7-H3 or Ki-67. CETCs, circulating epithelial tumor cells.

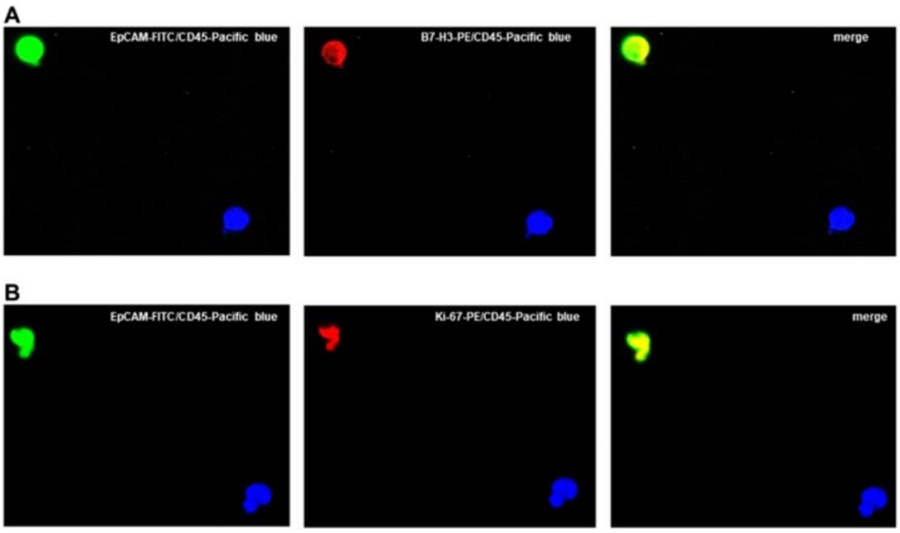

Figure 3. Fluorescence microscope images of (A) B7-H3- and (B) Ki-67-positive CETCs. CETC is positive for EpCAM, B7-H3 or Ki-67 and strictly negative for CD45. The yellow color is a result of the mixture of green and red due to co-expression at the same site of EpCAM and B7-H3 or Ki-67. CETCs, circulating epithelial tumor cells.

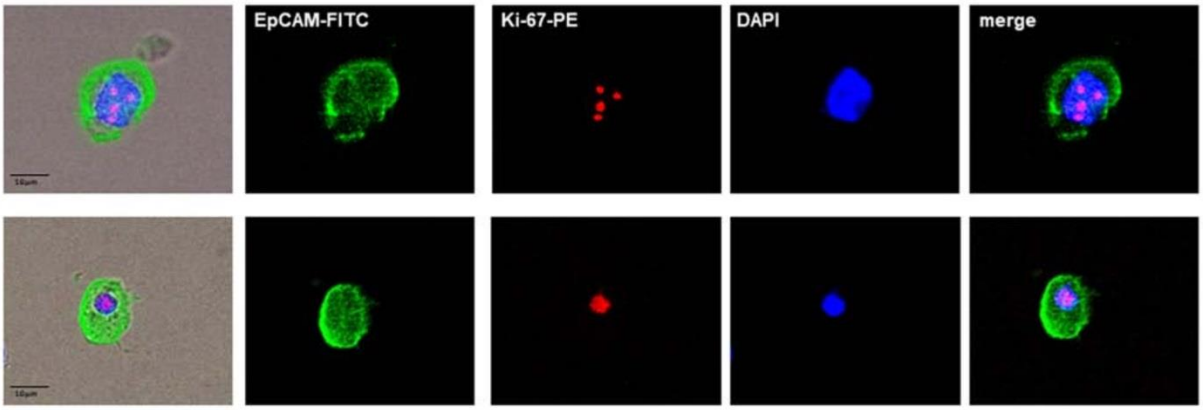

Figure 4. Two examples of Ki-67 staining on the MCF-7 cell line.

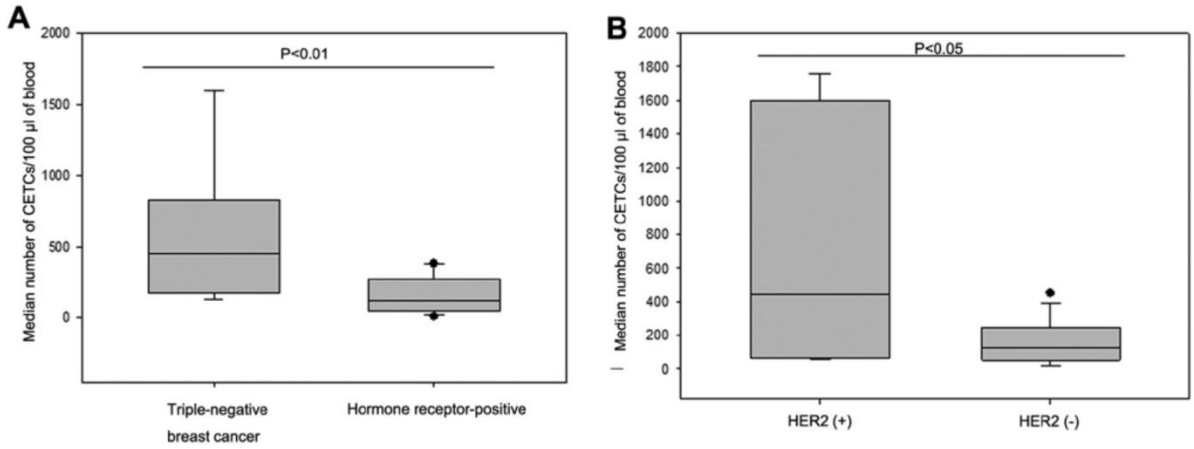

Figure 5. (A) Median number of CETCs in patients with triple-negative and hormone receptor-positive primary tumors; and (B) median number of CETCs in patients with HER2-positive and HER2-negative primary tumors. CETCs, circulating epithelial tumor cells; HER2, human epidermal growth factor receptor 2.

MCF-7 cells were grown in minimum essential medium Eagle ready-to-use medium (CLS Cell Lines Service). For immunofluorescence analysis, the cells were detached from the cell culture flasks using StemPro® Accutase® Cell Dissociation Reagent (Gibco/Thermo Fisher Scientific) washed and stained for Ki-67 with the same protocol as the patient samples (Fig. 4).

Statistical analysis. Statistical analysis was performed using the software programs SigmaPlot version 13.0 (Systat Software Inc., Chicago, IL, USA) for Windows. Comparisons between variables were performed using a Student t-test for normal distributed variables or Mann-Whitney Rank Sum Test for not normally distributed variables. Correlation analysis was carried out was calculated with Pearson's correlation coefficient. The significance level was set at P<0.05.

Results

A total of 50 patients with histologically confirmed breast cancer were enrolled in this study. Out of these, 25 (50%) patients had T1; 8 (16%) patients had T2 and 11 (22%) patients had T3/4 tumor size. The primary tumors were histologically positive for estrogen receptor (ER) and progesterone receptor (PR) in 30 patients (60%) and positive for human epidermal growth factor receptor 2 (HER2) in 11 patients (22%). In total, 15 (30%) patients were in stage I; 8 (16%) were in stage II; 15 (30%) and 6 (12%) were in stage III and IV, respectively. The age of the patients ranged from 32 to 78 years (median, 59 years). The median number of CETCs was 145 per 100 μl of blood (range, 10-1,760). Patients with triple-negative breast cancer (TNBC; n=7) had significantly more CETCs as compared to hormone receptor-positive patients (n=30; median 450 vs. 125; P<0.01) (Fig. 5A). Additionally, patients with primary HER2-positive tumors (n=11) had significantly more CETCs as compared to patients with HER2-negative tumors (n=33; median 445 vs. 125; P<0.05) (Fig. 5B). No statistically significant differences in CETC numbers were observed according to tumor size and lymph node/ distant-metastasis (Table I). As a negative control, we tested blood samples from 20 healthy controls and confirmed that none of the samples were positive for CETCs (data not shown). B7-H3-positive CETCs were observed in 43 patients (86%). The percentage of B7-H3-positive CETCs ranged from 0 to 80% (median, 35%). An association between hormone

Table I. Characteristics of cancer patients according to B7-H3 and Ki-67 expression.

Clinicopathological characteristics	Number of patients (%) with CETCs	P-value	Number of patients (%) with CETCs positive for B7-H3	P-value	Number of patients (%) with CETCs positive for Ki-67	P-value
Age (years)		P=0.164		P=0.745		P=0.583
≤50	10 (20)		10 (23)		9 (20)	
>50	40 (80)		33 (77)		36 (80)	
Tumor size		P=0.876		P=0.168		P=0.315
T1	25 (50)		20 (47)		21 (47)	
T2	8 (16)		7 (16)		8 (18)	
T3/4	11 (22)		10 (23)		10 (22)	
n.a.	6 (12)		6 (14)		6 (13)	
Lymph node status		P=0.424		P=0.189		**P<0.05**
Positive	23 (46)		21 (49)		22 (49)	
Negative	21 (42)		16 (37)		17 (38)	
n.a.	6 (12)		6 (14)		6 (13)	
Metastasis		P=0.253		P=0.616		P=0.568
Positive	6 (12)		6 (14)		6 (13)	
Negative	38 (76)		31 (72)		33 (74)	
n.a.	6 (12)		6 (14)		6 (13)	
ER status		**P<0.05**		P=0.178		**P<0.05**
Positive	30 (60)		23 (53)		25 (56)	
Negative	14 (28)		14 (33)		14 (31)	
n.a.	6 (12)		6 (14)		6 (13)	
PR status		**P<0.05**		P=0.178		**P<0.05**
Positive	30 (60)		23 (53)		25 (56)	
Negative	14 (28)		14 (33)		14 (31)	
n.a.	6 (12)		6 (14)		6 (13)	
HER2 status		**P<0.05**		P=0.412		**P<0.05**
Positive (2+/3+)	11 (22)		10 (23)		11 (25)	
Negative (0/1+)	33 (66)		27 (63)		28 (62)	
n.a.	6 (12)		6 (14)		6 (13)	
Stage		P=0.181		P=0.404		P=0.453
I	15 (30)		10 (23)		11 (25)	
II	8 (16)		8 (19)		8 (18)	
III	15 (30)		13 (30)		14 (31)	
IV	6 (12)		6 (14)		6 (13)	
n.a.	6 (12)		6 (14)		6 (13)	
Radiotherapy		P=0.523		**P<0.05**		**P<0.05**
Yes	22 (44)		22 (51)		22 (49)	
No	26 (52)		19 (44)		21 (47)	
n.a.	2 (4)		2 (5)		2 (4)	

Bold print denotes the statistically significant P-values.

receptor-status in primary tumors and the percentage of B7-H3-positive CETCs was observed. Patients (n=14) with tumor tissue negative for ER/PR had a significantly greater number of B7-H3-positive CETCs as compared to patients (n=30) with a positive ER/PR status (median 50% vs. 26%; P<0.05) (Fig. 6A).

Ki-67-positive CETCs were detected in 45 patients (90%) and the percentage ranged from 0-100 (median, 45%).

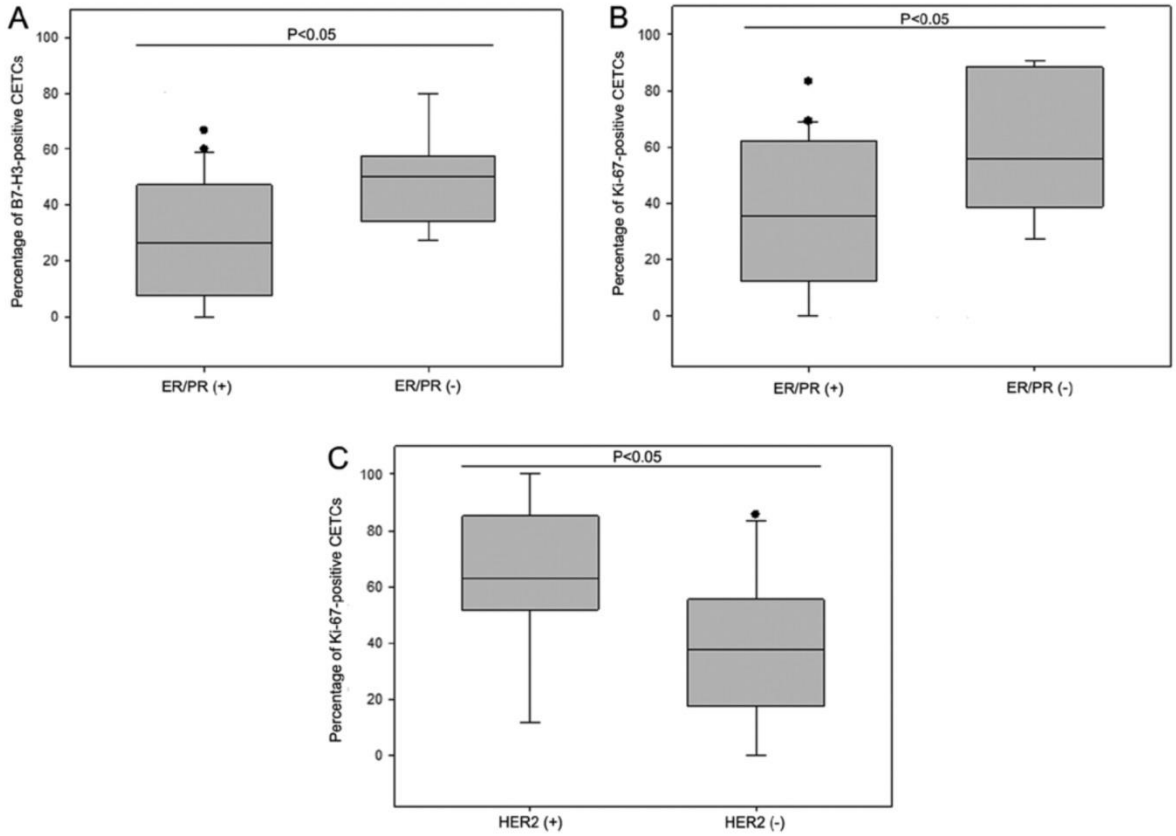

Figure 6. (A) The percentage of B7-H3-, and (B) Ki-67-positive CETCs in patients with HR-positive and HR-negative tumor tissues, and (C) the percentage of Ki-67-positive CETCs in patients with HER2-positive and HER2-negative primary tumors. CETCs, circulating epithelial tumor cells; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

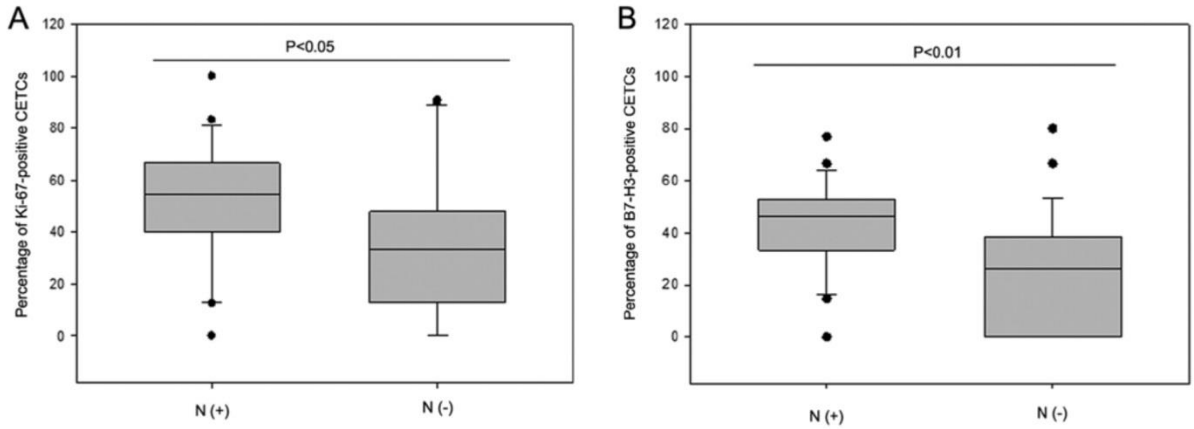

Figure 7. The percentage of (A) Ki-67-, and (B) B7-H3-positive CETCs in patients with positive and negative lymph nodes. CETCs, circulating epithelial tumor cells.

Furthermore, the percentage of Ki-67-positive CETCs was significantly associated with the ER/PR and HER2 status in the primary tumor. Patients (n=14) with hormone receptor-negative tumors had a greater number of Ki-67-positive CETCs than patients with hormone receptor-positive tumors (n=30) (median, 56% vs. 35%; P<0.05) (Fig. 6B). In addition, patients (n=11) with a HER2-positive primary tumor had a greater number of Ki-67-positive CETCs than patients (n=33) with a HER2-negative primary tumor (median, 63% vs. 38%; P<0.05) (Fig. 6C). An association was observed between the percentage of Ki-67 positive CETCs and the lymph node status. Patients (n=23) with positive lymph nodes had a significantly greater number of Ki-67-positive CETCs than patients (n=21) with negative lymph nodes (median, 55%

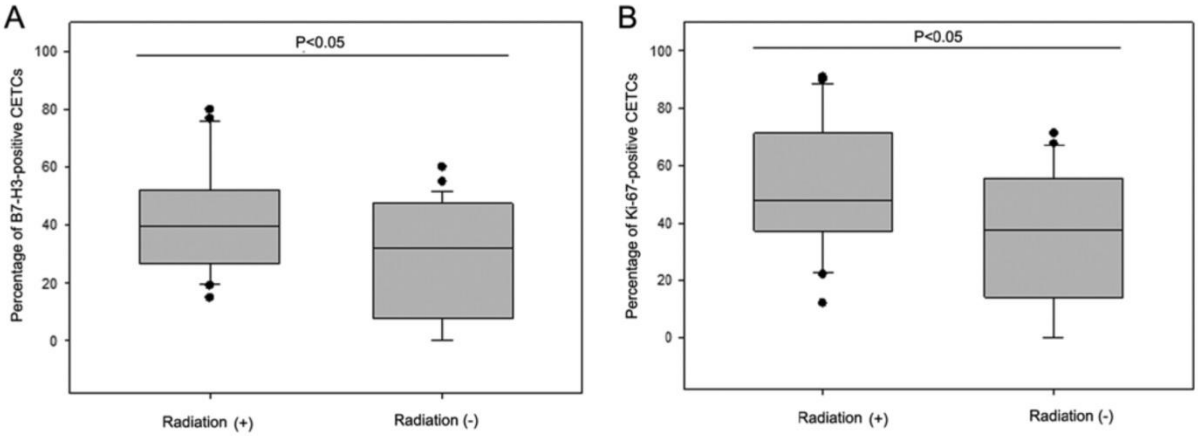

Figure 8. Percentage of (A) B7-H3-, and (B) Ki-67-positive CETCs in patients with and without radiotherapy. CETCs, circulating epithelial tumor cells.

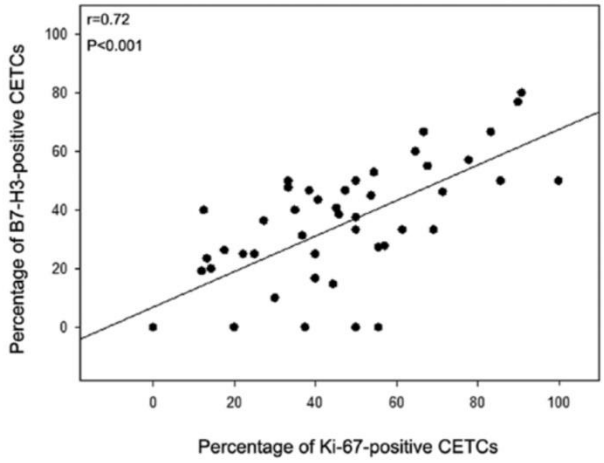

Figure 9. Correlation between the frequency of B7-H3- and the frequency of Ki-67-positive CETCs. CETCs, circulating epithelial tumor cells.

vs. 33%; P<0.05) (Fig. 7A). Additionally an association was observed between the percentage of B7-H3-positive CETCs and the lymph node status (median, 46% vs. 26%; P<0.01) (Fig. 7B).

Patients (n=22) who had received radiotherapy had a higher fraction of B7-H3- and Ki-67-positive CETCs as compared to patients (n=26) without radiotherapy (median B7-H3, 40% vs. 23%, P<0.05; median Ki-67, 48% vs. 38%, P<0.05) regardless of the radiation regimen (Fig. 8). Comparing the percentage of B7-H3-positive CETCs with the percentage of Ki-67-positive CETCs, both biomarkers significantly correlated with each other (r=0.721; P=0.001) (Fig. 9). Statistical analysis revealed that the highest association was observed between the percentage of B7-H3- and Ki-67-positive CETCs (P<0.001) (Fig. 9), followed by lymph node positivity (P=0.013) (Fig. 7) and the administration of radiation (P=0.038) (Fig. 8A). All other parameters were not significant.

Subsequently, we evaluated and compared the percentage of B7-H3- and Ki-67-positive CETCs in 20 breast cancer patients by performing co-expression analysis. The co-expression of B7-H3 and Ki-67 was confirmed in 90% of patients. Fig. 10 shows two typical cell galleries of CETCs from one patient which show that these cells have a parallel expression of both, B7-H3 and Ki-67 on their surface. The percentage of B7-H3- and Ki-67-positive CETCs ranged from 23-75% with a median of 35% (data not shown).

Discussion

The analysis of circulating tumor cells is essential for understanding the vascular spread of cancer to distant sites and for being able to make use of these cells for real-time and non-invasive tumor monitoring. A number of techniques have been developed over the past 20 years to detect, isolate and characterize circulating tumor cells (37). Circulating tumor cell analysis may play an important role as a 'liquid biopsy', which will allow physicians to follow changes of the systemic part of the disease over time, enabling adjustment of treatment and thus a promising new diagnostic tool for patients suffering from e.g., breast cancer (36). The majority of the procedures have, however, been hampered by the paucity of these cells recovered by the different approaches particularly those using magnetic bead enrichment after a fixation step (38), which impedes statistical analysis of the circulating tumor cells (39). Apart from mere quantitative analysis, current research on circulating tumor cells is focusing on the identification of novel diagnostic and therapeutic biomarkers expressed by these cells, such as B7-H3 and Ki-67 on tumor cells circulating in the blood, which allows for the determination of the aggressiveness of the residual tumor load, and can thus contribute to the determination of prognosis. They reflect the biological properties of the remnant tumor left in the body after previous interventions, which are crucial for the further development of the disease and can provide starting points for targeted treatment.

The association between the total number of circulating tumor cells and immunohistochemistry in breast cancer is controversial. Punnoose *et al* reported lower numbers of circulating tumor cells in TNBC as compared to luminal subtypes (40). By contrast, Peeters *et al* found no significant association between immunohistochemically defined subtypes

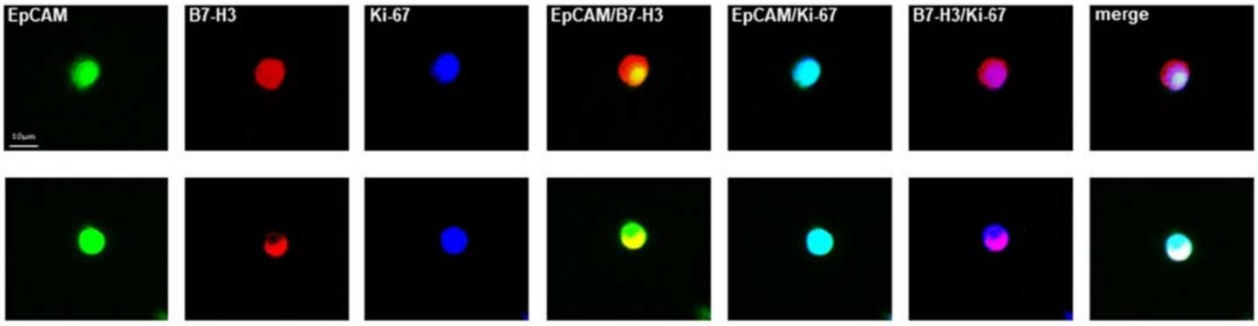

Figure 10. Fluorescence co-localization of EpCAM (green), B7-H3 (red) and Ki-67 (blue) on the CETCs in two representative results from one patient.

and circulating tumor cell numbers, but very high cell counts of >80 circulating tumor cells in 7.5 ml blood were found more frequently in patients with Luminal A and TNBC metastatic tumors (41). In this study, using a non-enrichment approach, the number of CETCs was significantly higher in patients with TNBC consistent with the clinical findings that this type of tumor is more aggressive and has an increased potential to be invasive, to migrate and to metastasize. Additionally, we observed a significant difference in the absolute CETC count in patients with HER2-positive tumors who had higher CETC counts as compared to patients with HER2-negative tumors. Our results are contradictory to the results by Liu *et al* (42) and Giordano *et al* (43) who reported a lower circulating tumor cell count in HER2-positive subgroups. These discrepancies may be explained by the different methods used for the enumeration of circulating tumor cells.

Due to the growing interest in immunotherapy, the analysis of B7-H3 expression in the primary breast tumor and on the circulating tumor cells may be crucial. B7-H1, known as PD-L1, is one of the most studied targets in present clinical trials. There is a high homology between B7-H3 and B7-H1; therefore, the blockade of both these molecules is highly feasible (17,18). Different antibodies which inhibit B7-H3 are currently being investigated in clinical trials [e.g., 8H9: NCT01099644, NCT01502917, and NCT00089245; enoblituzumab (MGA271): NCT01391143] (19). The present study was designed to demonstrate for the first time, at least to the best of our knowledge, B7-H3 expression in connection with Ki-67 on CETCs in patients with breast cancer. We were able to show that B7-H3 was expressed on a fraction of CETCs in the majority (82%) of breast cancer patients. Comparably, Maeda *et al* (18) detected B7-H3 expression in 92% of breast cancer tissues at various levels and Sun *et al* found that B7-H3 expression was present in 80.55% of breast cancer tissues compared to 16.48% in normal breast tissues (28). Arigami *et al* demonstrated a strong expression of B7-H3 in 39% of breast cancers and the expression was related to the progression of primary breast cancer to axillary lymph nodes (30). In gastric cancer, Arigami *et al* found that B7-H3 expression can be a useful blood marker for predicting tumor progression (44).

On the single cell level, on average of 30% of the CETCs expressed B7-H3 in our patient population. There is little data with respect to the expression of B7-H3 on circulating tumor cells in breast cancer patients and none regarding the extent of expression on these cells, at least to the best of our knowledge. Arigami *et al* (30) only reported on mRNA expression for B7-H3, but this did not allow for the calculatation at the single tumor cell level.

This study suggests that B7-H3 expression in breast cancer is significantly higher in hormone receptor-negative patients, whereas Liu *et al* and Sun *et al* found no association between B7-H3 positivity in breast tumor tissue and ER or PR status, histological subtype or differentiation (23,45). The association between B7-H3 on CETCs and hormone receptor status may contribute to explain the worse prognosis of patients with hormone-negative tumors.

The proliferation biomarker, Ki-67, is established as a prognostic factor for breast cancer. The proportion of dividing cells reflects the proliferative potential of the tumor. The expression of Ki-67 in healthy breast tissue is very low (<3%). By contrast, in breast cancer tissues, Ki-67 is often overexpressed (33,46). Müller *et al* using the CellSearch approach reported no expression of Ki-67 in the circulating tumor cells detected in the 47 examined patients (47). This may be due to the low frequency and low number of circulating tumor cells detected by their approach. By contrast, Spiliotaki *et al* (48), using a different approach, found Ki-67 positive cells in 27.5% out of 40 circulating tumor cell positive patients. Kallergi *et al*, using the same approach as Spiliotaki *et al*, observed Ki-67 positive circulating tumor cells in 51.7 and 44% of patients with early and metastatic breast cancer, respectively (49). In this study, we found positive staining for Ki-67 on the CETCs in 45 of 50 patients (90%). This difference may be explained by the different technologies used to detect circulating tumor cells.

Furthermore, we also found an association between a higher frequency of Ki-67-positive CETCs and a negative ER/PR and positive HER2 status. Similar results were observed in breast cancer tissue by Nishimura *et al*, where a higher Ki-67 index was significantly associated with a larger tumor, younger age, positive lymph nodes, a higher nuclear grade, a negative ER/PR status, p53 overexpression and a positive HER2 status (50). In addition, they reported that a high Ki-67 index in breast cancer tissue was significantly associated with positive lymph nodes (50). In this study, the expression of Ki-67 on CETCs was statistically significantly associated with a positive lymph node status. Furthermore, comparable to the findings of Arigami *et al*, the percentage of B7-H3-positive CETCs was significantly associated with a positive lymph node status (30).

Liu *et al* found an association between B7-H3 expression in tumor tissue and a positive lymph node metastasis (45). B7-H3 overexpression in CETCs may thus be important for tumor progression and invasiveness.

Post-surgery adjuvant therapies, such as chemo- and radiotherapy are essential for patients with breast cancer; however, many patients suffer from local recurrence or metastasis. In this study, we observed that patients who have received radiotherapy had an upregulated expression of B7-H3 on CETCs. This may be due to inflammatory processes occurring during radiotherapy. Maeda *et al* demonstrated that B7-H3 was induced by inflammatory cytokines in dendritic cells and monocytes (18). Additionally, Sun *et al* demonstrated that the stimulation of hepatocellular carcinoma cells with interferon-γ *in vitro* led to a significant upregulation of B7-H3 expression (51). Subsequent radiotherapy resistance observed in breast cancer patients may be due to the upregulation of B7-H3 in CETCs. Combination therapies with B7-H3 blockade may in the future be able to overcome radio-resistance. Of note, in this study, we found that not only B7-H3, but also Ki-67 were more highly expressed in the CETCs in patients who had received radiotherapy. Although there are data available about changes in Ki-67 index during radiotherapy in breast tumor tissue (52), a comparison between tumor tissue and circulating tumor cells has not been made to date, at least to the best of our knowledge. It is well known that a high Ki-67 index in tumor tissue is associated with lower disease-free and overall survival rates in breast cancer (50).

In samples with a >50% fraction of B7-H3 expression and Ki-67 expression, a correlation between B7-H3 and Ki-67 can be assumed in individual CETCs. The exact physiological and pathological function of B7-H3 and particularly its role in the development and progression of human cancers remains unclear, as both stimulatory and inhibitory properties have been described (53). B7-H3 may serve as an inhibitor of anti-tumor immunity. Thus, proliferation may induce B7-H3 and at the same time protect CETCs from destruction by self-reactive T lymphocytes. Understanding the mechanisms through which B7-H3 can be induced in and is association with the proliferation of CETCs may, in the future, contribute the designing of suitable drugs for breast cancer therapy.

The limitations of the study include the small patient sample size and the preponderance of patients in early stage of breast cancer. On the other hand, the possibility of detection of circulating tumor cells also in this patient population may be of advantage as they are still in a situation where a cure is possible.

In conclusion, the analysis of the properties of CETCs offers a low invasive, easy-to-repeat, real-time 'liquid biopsy' approach, which reflects the actual aggressiveness of the tumor. Furthermore, we demonstrate for the first time, at least to the best of our knowledge, that B7-H3 and Ki-67 are expressed on the CETCs in patients with breast cancer. The B7-H3 pathway regulates the innate and adaptive immunity and promotes cancer cell aggressiveness through various immunological functions. Therefore it could become a unique and interesting target for future cancer immunotherapies.

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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

MP and KP designed the study. MP and DSS performed the experiments. MP, DSS and UP analyzed the data. MP and DSS wrote the paper in consultation with KP and UP. All authors discussed the results and contributed to the final manuscript.

Ethics approval and consent to participate

All patients and healthy volunteers gave their informed consent to participate in the study, which was approved by the Ethics and Scientific Committees of the University of Jena (Jena, Germany).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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5.6 Manuskript I

Evidence of platelet-covered circulating tumor cells in peripheral blood of breast cancer patients

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Evidence of platelet-covered circulating tumor cells in peripheral blood of breast cancer patients

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Keywords: circulating tumor cells, platelets, breast cancer

Abstract

1) Background: Solid tumors are notorious for their ability to form lethal metastases. Development of distant metastases is a result of the primary tumor shedding cells that travel via the blood to distant sites. Platelets are known to specifically enhance tumor cells' survival in the bloodstream. To study the interplay of platelets with circulating tumor cells, we implemented our approach to label circulating epithelial tumor cells and platelets.

2) Methods: Blood samples were collected from 36 breast cancer patients avoiding fixation and processed at 3 timepoints following blood collection using the maintrac approach. Samples were additionally stained with anti-CD36 antibody to visualize platelets.

3) Results: We observed at 0h post-blood draw platelet aggregates strictly attached to single cells that did not stain with anti-EpCAM antibody. After keeping blood samples at room temperature for 24h platelet aggregates could still be detected, but the anti-EpCAM antibody became accessible to the underlying cells. CETCs were detected in 94% patients on day 1 post-blood draw. At 48h following initial blood drawing, platelets had almost completely disappeared from the cell surface.

4) Conclusion: Our results suggest that platelets play a key role in masking circulating tumor cells. Masking may explain the difficulties in detection of these cells and prevention of their elimination by the immune system.

Introduction

Platelets are one of the components of blood that are involved in wound healing and hemostasis by forming a blood clot. Recent studies have suggested that platelets have a crucial role in enhancing the survival of circulating tumor cells in the bloodstream and aggravating cancer metastasis [1, 2]. As early as 1865, a link between cancer and activation of the coagulation system was recognized [2–4]. Global platelet hyperactivity can be observed in almost all cancer patients, but thromboembolic disease does not occur with equal frequency in all neoplasms. Activation of coagulation and malignancy-associated thrombosis is one of the most common clinical manifestations in cancer patients, associated with poor prognosis and shortened survival rates [5–7]. Platelets adhere to circulating tumor cells (CTCs) with receptor-ligand bonds and the attached platelets facilitate adhesion of the CTC to the vessel wall [2, 8–10]. Platelets abundantly express CD36, a protease-resistant surface glycoprotein IV scavenger receptor that contributes to platelet adhesion and collagen dependent thrombus stabilization and enables their visualization in fluorescence microscopy [11, 12]. Besides, circulating tumor cells induce platelet activation, which promotes tumor angiogenesis through the release of proteases and phospholipids [13, 14]. These aggregates have a higher probability of becoming trapped in microvasculature, giving tumor cells more time to extravasate into adjacent tissues [15]. Platelets protect the tumor cells in the bloodstream from shear stress and immunological elimination, thus increasing CTC survival [6]. However, this protective shield may also be the reason why antibodies do not reach the surface antigens of tumor cells. This observation may also explain why targeted anti-EpCAM antibody therapy had limited efficacy in the phase III studies [16–18]. Simultaneously, platelet-covered circulating tumor cells are difficult to detect immediately after blood sampling. We show proof-of-principal that an interaction exists between circulating tumor cells (CTCs) and platelets which is the cause of the difficulty in detecting them in freshly collected blood samples.

Material and Methods

The study is designed as a biology driven, proof of principal translational research experiment to investigate the interaction between CTCs and platelets at 3 different time points after blood collection. All specimens were collected into tubes containing the anticoagulant EDTA and were processed using the maintrac® method directly (0h), 24h, and 48h after blood draw. Blood samples were stored at room temperature until sample processing.

36 patients with breast cancer in different stages of disease that had presented at our center (Transfusion Center Bayreuth) were recruited to the study. Informed consent was obtained for all donors and all experiments were performed in compliance with the relevant laws and institutional guidelines (Ethics committee of Bavarian Medical Association). In addition to breast cancer patients, blood samples were collected from 5 healthy female donors. Blood samples were processed as described earlier with the maintrac® method [19]. Shortly, red blood cells were lysed and an immunohistochemistry approach was used to identify CTCs. Cells in the remaining cell suspension were stained with a fluorescein-isothiocyanate (FITC)-conjugated anti-human epithelial cell adhesion molecule antibody (EpCAM) (clone HEA-125, Miltenyi Biotec GmbH, Germany). Subsequently, the samples were transferred to wells of ELISA plates (Greiner Bio-one, USA) and stained additionally with propidium iodide (PI) (Sigma-Aldrich, USA) for the discrimination between live and dead cells. Samples were measured using a Fluorescence Scanning Microscope, ScanR, (Olympus, Hamburg, Germany), enabling detection of cells for visual examination of EpCAM positive cells.

For identification of platelet-covered CTCs we used a secondary antibody staining with a Phycoerythrin (PE)-conjugated anti-human CD36 antibody (clone 5-271, Biolegend, San Diego, USA). The staining was performed identical as the EpCAM staining. Thus, we were able to detect platelets (red staining) together with CTCs (green staining) in parallel.

Results

In 36 patients that had presented at our center (Transfusion Center Bayreuth, TZB), CTCs were detected in none of the patients directly after blood draw. After storing the samples for 24h at room temperature, more than 5 cells/100µl cell suspension were detected in 94% of the patients. No CTCs were observed in the control group with any cancer diagnosis, neither directly, nor 24 h after blood draw.

Typically, in the freshly collected patient samples (0 h) we observed platelet aggregates attached to single cells that did not stain with the anti-EpCAM antibody (Figure 1a). These clogged unstained tumor suspected cells were surrounded by cells to which platelets were not attached. The dense aggregation of platelets to the suspected tumor cells inhibited their staining with the anti-EpCAM antibody.

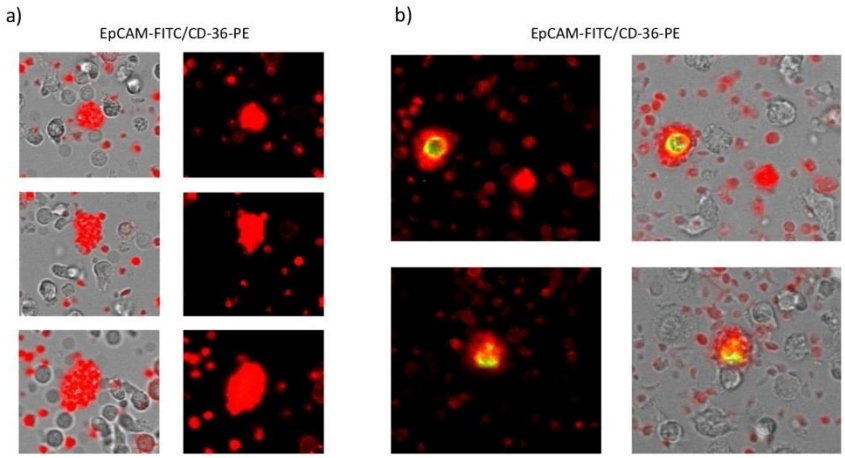

Figure 1: Antibody staining with anti-EpCAM-FITC (green) and anti-CD36-PE antibody (red). a) Immunofluorescence images in freshly collected patient samples (0h). No EpCAM staining could be detected in all patients directly after blood collection. Platelet-covered CTCs could be observed that did not stain with anti-EpCAM antibody. b) Immunofluorescence images 24 hours after blood collection. Cells were stained in the same manner as at time 0h. EpCAM positive cells could be detected after 24 hours of storage. The density of attached platelets decreased after 24 hours, resulting in binding and thus detection of EpCAM-positive cells.

After the blood samples were kept at room temperature for 24 hours, numerous platelet aggregates could still be detected, but the underlying cells became accessible to the anti-EpCAM antibody. The decrease in the density of attached platelets on the cells and the concomitant increase of platelets in the background could indicate that the platelets detached from the CTCs making the antigens accessible to the anti-EpCAM antibody (Figure 1b).

At 48 h following initial blood drawing, platelets had almost completely disappeared from the cell surface and almost no platelet-covered CTCs could be observed (Figure 2).

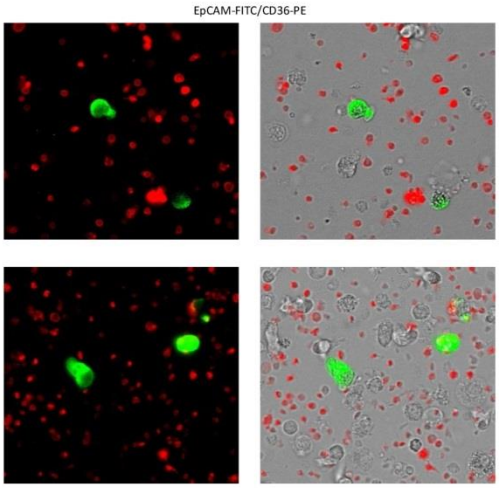

Figure 2: Antibody staining with anti-EpCAM-FITC and anti-CD36-PE antibody. At 48h after blood collection CETCs were positive for EpCAM (green). Platelets (red) disappeared from the surface of CETCs and were present unbound in the cell environment.

Numbers of EpCAM-positive cells remained stable between 24-48h (Median 24h 7 CTCs/100µl cell suspension vs. Median 24h 8 CTCs/100µl cell suspension), as reported previously (Figure 3) [19]. Clinicopathological features were not correlated with the presence of platelet-covered CTCs. The intensity of EpCAM staining increased during individual days.

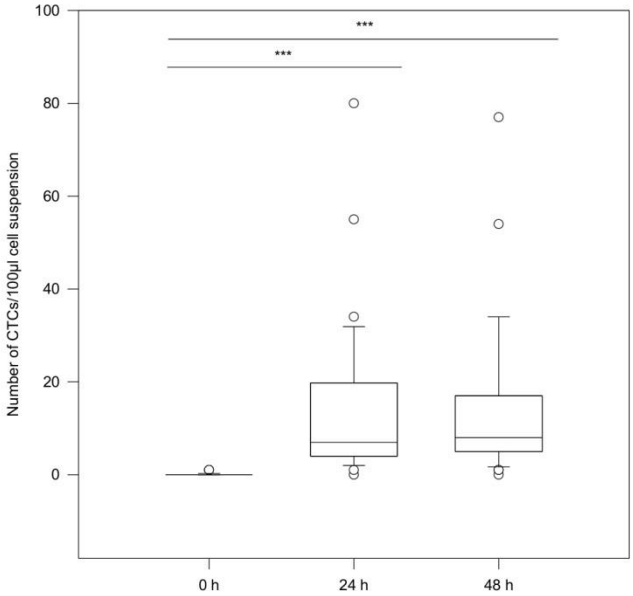

Figure 3: Box plot analysis of the number of CTCs according to the 3 different time points (0h, 24h and 48h). No EpCAM positive CTCs could be detected in freshly collected blood samples. After resting the blood sample for 24-48h at room temperature EpCAM positive cells were observed and the number of CTCs retained constant.

Additionally, in 10 patients we used different blood tubes for blood collection which contained fixative substances preventing detachment of platelets from tumor cells in the blood sample. Thus, surface antigens were permanently covered with platelets and inaccessible for EpCAM staining at any time during the analysis.

Discussion

In most solid malignant tumors, no metastases are present at diagnosis but still, up to 50% of patients suffer relapse, which is assumed to be due to cells shed either directly into the circulation or via the lymphatic system. Lymph nodes are no barrier to dissemination [20]. The key questions are when and how dissemination occurs, how disseminated cells survive, and what induces them to form metastases. In blood, tumor cells are not readily detectable. In fact, we have noted that in freshly drawn unfixed blood samples, circulating tumor cells become detectable only after the sample rests at room temperature overnight or is treated with a surfactant [21], indicating that their surface molecules initially are somehow masked.

A critical step for distant metastasis formation is survival of cancer cells in the bloodstream. The main threats to circulating tumor cells are shear stress and natural killer (NK) cytotoxic cells. It has been reported that platelets protect circulating tumor cells [22], which correlates with the tumor cells' abilities to aggregate platelets [23]. Indeed, platelets are known to bind benign exfoliated epithelial cells that enter the circulation (e.g. upon wounding). Whereas normal epithelial cells may not be capable to proliferate, and seem to become eliminated after days or weeks [24] circulating tumor cells persist [25] when covered with platelets [16]**.** Covering the surface of tumor cells with platelets may serve as a shield against immune assault because the effect of anti-tumor attacks mediated by NK cells is primarily based on the direct interaction with circulating tumor cells [26, 27]. Platelets that cover CTCs also prevent antibodies from accessing the tumor cell surface and it is likely the reason for the inability to detect these cells in freshly drawn blood. This evasion of circulating tumor cells from immune recognition may also be the reason why anti-EpCAM- antibodies have, so far, not been successful in treating epithelial tumors [17]. Drugs that theoretically would be effective may not be able to access the clogged cells, an important consideration in adjuvant or neoadjuvant systemic chemotherapy.

The present study was able to show that the tumor cells in the blood form aggregates with platelets, which serve as a protective shield against shear forces in the blood and against elimination by the immune system [8]. This underscored the evidence from the literature of a close interaction between tumor cells and platelets. At the same time, tumor cell-induced platelet aggregates make it difficult to detect circulating tumor cells immediately after blood collection [6]. Recently, several antiplatelet drugs are in the preclinical and clinical studies showing its new use in cancer prevention, reducing metastasis and mortality in many cancer types [28–30]. We also hypothesized that targeting the interaction between platelets and tumor cells may represent a novel and promising antitumor strategy to prevent metastasis.

Conclusion:

In this work, we could show that circulating tumor cells are covered by platelets in cancer patients. We show proof-of-principle that coated circulating tumor cells exist in significant numbers directly after blood draw thereby impeding their visualization with antibody fluorescence staining. Storing blood samples overnight at room temperature caused platelets to detach from circulating tumor cells, which allowed antibody binding and their detection. Further studies are needed to determine the clinical significance of platelet-coated circulating tumor cells.

Declarations

Funding: Not applicable.

Conflicts of interest: The authors declare no competing interests.

Data availability: The datasets generated and/or analyzed during the study are not publicly available due to preservation of privacy but are available from the corresponding author on reasonable request.

Code availability: Not applicable

Authors´contributions: Conceptualization, K.P., D.S., M.P.; Methodology, D.S., M.P. and K.P.; Validation, D.S., and M.P.; Investigation, D.S., M.P., E.S.; Writing-Original Draft Preparation, K.P., D.S., M.P.; Writing-Review & Editing, K.P., M.P.; Visualization, D.S. and M.P.; Supervision, K.P. and D.S.; Project Administration, D.S., K.P., M.P.

Ethics declarations: All subjects have provided written informed consent before participation in the study which has been approved by the Ethics Committee of the Bavarian Medical Association.

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5.7 Publikation V

Influence of adjuvant radiotherapy on circulating epithelial tumor cells and circulating cancer stem cells in primary non-metastatic breast cancer

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Original Research

Influence of adjuvant radiotherapy on circulating epithelial tumor cells and circulating cancer stem cells in primary non-metastatic breast cancer

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ABSTRACT

Background: : There is an unmet need to identify biomarkers that directly reflect response to adjuvant radiotherapy (RT). Circulating epithelial tumor cells (CETCs) represent the liquid component of solid tumors and are responsible for metastatic relapse. CETC subsets with cancer stem cell characteristics, circulating cancer stem cells (cCSCs), play a pivotal role in the metastatic cascade. Monitoring the most aggressive subpopulation of CETCs could reflect the aggressiveness of the remaining tumor burden. There is limited data on the detection and monitoring changes in CETC and cCSC numbers during RT in early breast cancer.
Methods: : CETC numbers were analyzed prior to, at midterm and at the end of RT in 52 primary non-metastatic breast cancer patients. Hormone receptor status was determined in CETCs prior to and at the end of RT. For the identification of cCSCs cell suspensions from the peripheral blood of patients were cultured *in vitro* under conditions favoring growth of tumorspheres.
Results: : Hormone receptor status in CETCs before RT was comparable to that in primary tumor tissue. Prior to RT numbers of CETCs correlated with aggressiveness of primary tumors. cCSCs could be successfully identified and monitored during RT. Prior to RT patients treated with neoadjuvant chemotherapy had significantly higher numbers of CETCs and tumorspheres compared to patients after adjuvant chemotherapy. During RT, the number of CETCs decreased continuously in patients after neoadjuvant chemotherapy but not after adjuvant chemotherapy.
Conclusion: : Monitoring the number of CETCs and the CETC subset with cancer stem cell properties during RT may provide additional clinically useful prognostic information.

Introduction

Breast cancer is the most commonly diagnosed malignancy in women and accounts for 30% of all cancers [1]. The management of breast cancer has changed significantly in the last few decades. It is often diagnosed at an early, potentially curable stage and treatment has become more effective [2]. Treatment of local disease includes surgery, often followed by adjuvant radiotherapy. Neoadjuvant or adjuvant systemic treatment options include chemotherapy, endocrine therapy, biologic therapy or a combination of these. Multimodal treatment is guided by TNM classification and risk stratification including histology, hormone receptor status, human epidermal growth factor receptor 2 (HER2) status, histologic grade and proliferation index. Recently, in addition to these clinical and pathological features, molecular characterization and in selected cases additional gene expression assays of the tumor are used to determine the potential benefit from systemic therapy [3]. Radiotherapy after breast conserving surgery (BCS) or mastectomy is an important component of therapy as it significantly reduces local recurrence rates and breast cancer mortality [4–8]. In contrast to chemotherapy, indication for radiotherapy mostly relies on clinical and pathological features. Since the implementation of radiotherapy only 2.5% of women with primary breast cancer experience locoregional, but 20% to 30% still experience distant relapse without previous local relapse whatever the treatment undertaken and patients may die from their disease [1,9,10]. In women with early-stage breast cancer, surgery is supposed to completely remove detectable macroscopic disease; however, microscopic tumor foci might still remain in the conserved breast or chest wall. Current tumor staging procedures as well as high resolution imaging technologies are not sensitive enough to detect micro-metastases or early tumor cell dissemination.

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Cells originating from primary tumors, locoregional recurrences, or metastases, which circulate in the peripheral blood and possess antigenic and genetic tumor-specific characteristics are so called circulating tumor cells [11]. The mechanism of metastasis formation from solid tumors is not fully understood, but recent evidence indicates that the key event in tumor progression is the dissemination of cells from the primary tumor at an early stage of the disease [11].

Circulating tumor cells represent the liquid component of solid tumors and indicate the presence of residual disease. This minimal residual disease could lead to locoregional recurrence or distant metastases, or both [12,13]. In metastatic disease they have been proposed to identify subpopulations of patients who are most likely to respond to a given therapy and for development of improved individualized therapies [14,15]. It was shown that the number of circulating tumor cells in metastatic breast cancer patients is an independent predictor for progression and overall survival [16,17]. Even in low-risk, non-metastatic patients circulating tumor cells may become prognostic of imminent metastasis and survival [18,19]. By means of maintrac®, a non-dissipative method avoiding cell loss [20], trajectories of circulating epithelial tumor cell (CETC) counts can be used as a marker for therapy response in the adjuvant situation allowing for continuous monitoring during treatment [21]. Monitoring the number of circulating tumor cells is superior to conventional imaging as a method for evaluating the response to different therapies, but has not yet been applied to assess the outcome of radiotherapy, especially in early breast cancer [18,22–28].

Solid tumors consist mainly of differentiated cells which have limited or no self-renewal abilities. Although cancer stem cells may be difficult to define definitively, growing evidence suggests that a tumor consists of a small subpopulation of undifferentiated cells within a tumor that have the ability to grow clonally and self-renew [9]. They are responsible for tumor initiation, tumor progression, metastasis and most importantly for recurrence after treatment, caused by resistance to conventional therapies [9,29]. Also circulating tumor cells seem to harbor a subpopulation of cancer stem cells, the so called circulating cancer stem cells (cCSCs), which are essential for metastatic spread [30,31]. We have shown that also a subpopulation of cells in the peripheral blood is capable to expand clonally and correlates strongly with the likelihood of the presence of metastasis in breast cancer patients [31,32].

So far, there is only limited data [33] about monitoring the number of CETCs and the number of cCSCs during radiotherapy, especially in early-stage breast cancer. We have now investigated the number of CETCs, and the number of cCSCs, prior to, midterm and at the end of adjuvant radiotherapy after breast conserving therapy. Hormone receptor and HER2 status of CETCs were also investigated and correlated to those of the primary tumor tissue.

Methods

The study is designed as a biology driven, translational research trial to investigate the feasibility of identifying and characterizing circulating epithelial tumor cells, as well as circulating cancer stem cells from whole blood samples prior to radiotherapy, at midterm of the radiotherapy series and at the end of radiotherapy using the maintrac® method.

Inclusion criteria

52 breast cancer patients treated at the University Hospital Jena were enrolled between April 2016 and March 2019. This study was conducted according to the Declaration of Helsinki and was approved by the local ethics committee (No. 0921-08/02) and registered in the German Clinical Trials Register (DRKS00011840). Breast cancer patients were enrolled after giving written informed consent if they met the following eligibility criteria: female patients aged ≥ 18 years with histologically proven primary non-metastatic invasive breast carcinoma (stages I-IIIA). Breast conserving surgery had to be completed at the University Hospital Jena with the curative-intended adjuvant radiotherapy of the breast/chest wall +/- lymphatic drainage had to be foreseen.
The allowed dose concepts were as follows:

1 hypofractionated therapy (5 × 2.66 Gy/week) to a total absorbed dose of max. 42.56 Gy or
2 normofractionated therapy (5 × 1.8 Gy or 5 × 2.0 Gy/week) to a total absorbed dose of 50.4 Gy or 50.0 Gy.

If indicated, an additional boost to the tumor bed was given within 5 × 2.0 Gy/week to total dose 16.0 Gy.
Exclusion criteria for the study were the presence of distant metastases, prior malignancies within 10 years of the breast cancer diagnosis or previous radiotherapy.

Study procedures

For CETC analysis, peripheral blood samples were collected (2 × 7.5 ml) into EDTA-tubes (ethylenediaminetetraacetic acid) as an anticoagulant and sent to the Laboratory in Bayreuth for analysis. Peripheral blood samples were obtained from all patients at three time points: at the same day of the first session of radiotherapy (pre RT), in the middle of the radiotherapy series (mid RT) (after 8 sessions for hypofractionated RT and after 14 sessions for normofractionated RT) and on the last day of the radiotherapy series (end RT). In addition to breast cancer patients, blood samples were collected from 10 healthy female donors aged 20-45 years for control.

Immunoflourescence assay for identification of CETCs (maintrac® method)

Samples were maintained at room temperature and processed within 72 h after blood collection. For counting and further characterization of CETCs, we used the maintrac® approach, as reported previously [34]. In short, after red blood cell lysis, an immunocytochemistry approach was used to identify CETCs. Cells in the remaining cell suspension were stained in a 1.5 ml reaction tube with a fluorescein-isothiocyanate (FITC)-conjugated anti-human epithelial cell adhesion molecule antibody (EpCAM) (clone HEA-125, Miltenyi Biotec GmbH, Germany) at a final concentration of up to 10^7 cells/100 µl. The corresponding isotypic control for EpCAM (Mouse IgG1K FITC, Miltenyi Biotec GmbH, Germany) was used at the same concentration to determine and subtract background levels of staining. The samples were subsequently transferred to wells of ELISA plates (Greiner Bio-one, USA) and stained additionally with propidium iodide (PI) (Sigma-Aldrich, USA) for the discrimination between live and dead cells. Analysis of red and green fluorescence of the cells was performed using a Fluorescence Scanning Microscope, ScanR, (Olympus, Hamburg, Germany), enabling detection and relocation of cells for visual examination of EpCAM positive cells. For data analysis, we used the ScanR Analysis software (Olympus, Hamburg, Germany). Vital CETCs were defined as EpCAM-positive cells with intact morphology, but lacking nuclear PI staining. Exclusively these cells were counted for the analysis (Fig. 1). We used fluorospheres (FlowCheck 770, Beckman Coulter) for daily verification of optical components and detectors of the microscope, which are required to ensure the consistent analysis of samples.

Secondary antibody staining

The secondary antibody stains of CETCs were performed as described previously in order to compare EpCAM positive cells with basal histological characteristics of the primary tumor [34,35]. Cell suspensions were stained as described above. EpCAM positive cells were additionally stained for estrogen (ER) and progesterone (PR) receptors. For ER receptor staining we used an anti-human ER PE-conjugated antibody (clone E115, abcam, Cambridge, USA) at a final cell concentration of up to 10^7 cells/100µl cell suspension (Fig. 2a). For the PR staining we used an Alexa Fluor®594 conjugated PR antibody (clone YR85, abcam,

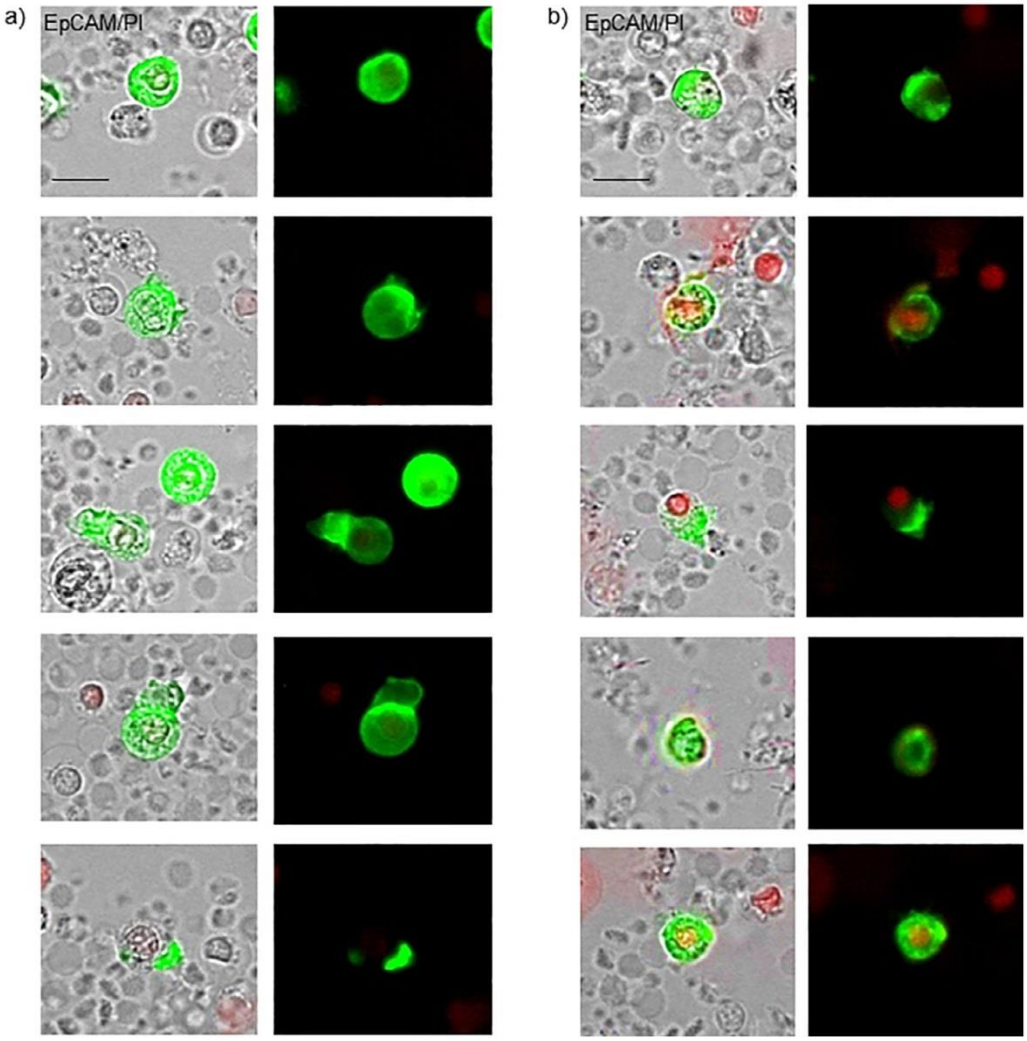

Fig. 1. Exemplary cell galleries of (a) vital and (b) dead CETCs from one patient. (a) vital CETCs are positive for EpCAM (green) and negative for PI (red) and have an intact morphology. (b) Dead cells are either positive for EpCAM and PI or only positive for EpCAM and negative for PI because of the destroyed cell membrane. Scale bar: 10µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Cambridge, USA) at a final concentration of 2µg/ml (Fig. 2b). Finally, cells were visually inspected for green and red surface staining, and additionally well-preserved nucleus and the percentage of double positive CETCs for the respective staining was determined. The isotype controls for ER and PR (rabbit IgG PE, abcam, Cambridge, USA) were used at the same concentrations.

Fluorescence-in-situ-hybridization (FISH) of human epidermal growth factor receptor 2 (HER2)

For the investigation of HER2 gene amplification on chromosome 17 in 20 patients prior to RT, a fluorescence-*in-situ-hybridization* assay was performed with the PathVysion HER2 DNA Probe Kit. This kit includes a control probe specifically against the alpha satellite DNA sequence in the centromeric region of chromosome 17 (*chromosome enumeration probe* 17, 17q11.1-q11.1 CEP 17, green fluorescence). In addition, this kit includes the probe, which is specific for the HER2 gene locus (*locus specific identifier*, 17q11.2-q12 LSI, orange fluorescence). Patient cells, which were previously labeled with the anti-EpCAM-FITC antibody (see above) were transferred onto Poly-L-Lysin coated slides. Slides were fixed with 4% paraformaldehyde before hybridization for 10 min and treated for further 10 min with proteinase K at room temperature. Afterwards, the cells were denatured for 5 min at 72°C in 70% formamide/2x standard saline citrate solution, air dried and dehydrated in 70%, 85% and 96% ethanol. Slides were hybridized overnight at 37°C in a humidified chamber, washed, air dried and counterstained with 0.2 µM DAPI in an anti-fade solution. Twenty nuclei per sample were analyzed. CETCs were positive for HER2 amplification when more than 3 HER2 signals in one cell were counted (Fig. 2c). Finally, the results for HER2 amplification were calculated as percentage of 20–30 visually inspected EpCAM positive cells.

Analysis of stem cell properties of cCSCs

For the cell culture of cCSCs, we used the stemtrac approach as described previously [31]. For the identification of the population of circulating cancer stem cells capable to grow clonally into tumorspheres *in vitro*, we cultured CETCs together with leukocytes at a density of 2×10^5 cells/ml for up to 3 weeks in RPMI-1640 supplemented with L-glutamine, HEPES, penicillin/streptomycin and growth factors such as EGF, insulin and hydrocortisone. Cells were maintained at 37°C and 5% CO_2 without movement of the culture flasks. Depending on the morphology of the tumorspheres observed in the culture flask under an inverted light microscope (Primo Vert, Zeiss, Germany) at 40x magnification, tumorspheres were collected and prepared for immunostaining analogous to the maintrac® staining. Tumorspheres were stained with an

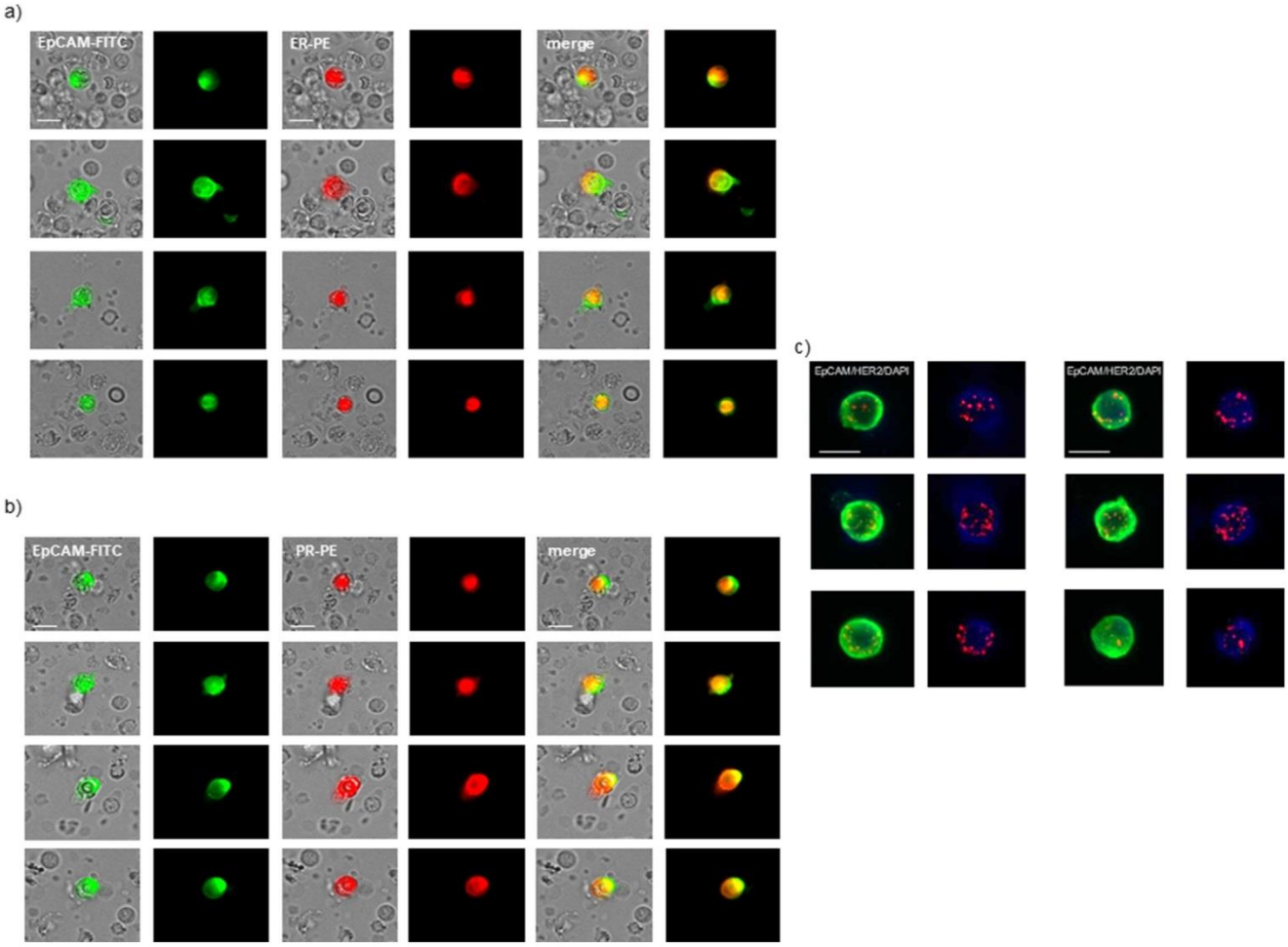

Fig. 2. Typical CETC images of double antibody staining for (a) EpCAM (green) and ER (red) and (b) EpCAM (green) and PR (red) from one patient. (c) Typical cell gallery of HER2 amplified CETCs from one patient. Cells are positive for EpCAM (green), and DAPI (blue). Cells are HER2 amplified (red signals). Scale bar 10μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

anti-EpCAM antibody at the same concentration as for the CETCs and afterwards measured with the same fluorescence microscope as used for the CETCs. Tumorspheres cultured from cCSCs could easily be distinguished from single or aggregated cells because they develop a solid spherical formation. This assay can be used to estimate the percentage of cancer stem cells present in a population of tumor cells.

For the characterization of cultured tumorspheres from cCSCs we used the typical combination of markers for breast cancer stem cells. EpCAM positive tumorspheres were additionally stained for CD24 (clone ML5, mouse anti-human, BD Bioscience, USA) and CD44 (clone 515, mouse anti-human, BD Bioscience, USA) PE-conjugated antibody [31].

Aldehyde dehydrogenase isoform 1 (ALDH1) activity is a universal marker for the identification and isolation of cancer stem cells. To confirm tumor stem cell properties of isolated cells, we used an ALDH1 enzymatic assay [31], which quantifies the ALDH1 activity of tumorspheres. We used an ALDEFLUOR assay kit (Stem Cell Technologies™, Canada) according to the manufacturer´s protocol. Tumorspheres expressing high levels of ALDH1 become brightly fluorescent. The number of such tumorspheres was counted.

Statistical methods

Statistical analysis was performed using the software program SigmaPlot version 13.0 (Systat Software Inc., Chicago, USA) for windows. Based on the antidromic behavior of CETC numbers during RT patients were dichotomized into 2 groups: patients with increasing (n=25) and decreasing (n=27) CETC numbers during RT. Comparisons between the variables were performed with the independent sample t test (dichotomous variables) or ANOVA (variables with more than two categories), taking into account the possibility of using nonparametric tests. The screening criterion used was a p-value of <0.05.

Results

CETCs and clinicopathological features

Peripheral blood samples from healthy volunteers (n=20) were used as a negative control and processed analogous to the samples from breast cancer patients. In these samples no CETCs were found and no tumorsphere formation *in vitro* was observed during cultivation.

Patient characteristics of all patients with primary breast cancer are shown in Table 1. CETCs were compared with the primary tumor tissue with regard to the hormone receptor expression and HER2 status. ER and PR status in CETCs were evaluable in all patients prior to and at the end of RT. The HER2 status in CETCs was determined in a subset of 20 patients prior to RT. Most patients with positive ER, PR or HER2 receptors expressed in the primary tumor tissue (immunohistochemical > 1%), had also ER (89% of patients) (Fig. 3a), PR (70% of patients) (Fig. 3b) and HER2 (100% of patients) (Fig. 3c) positive CETCs. Most

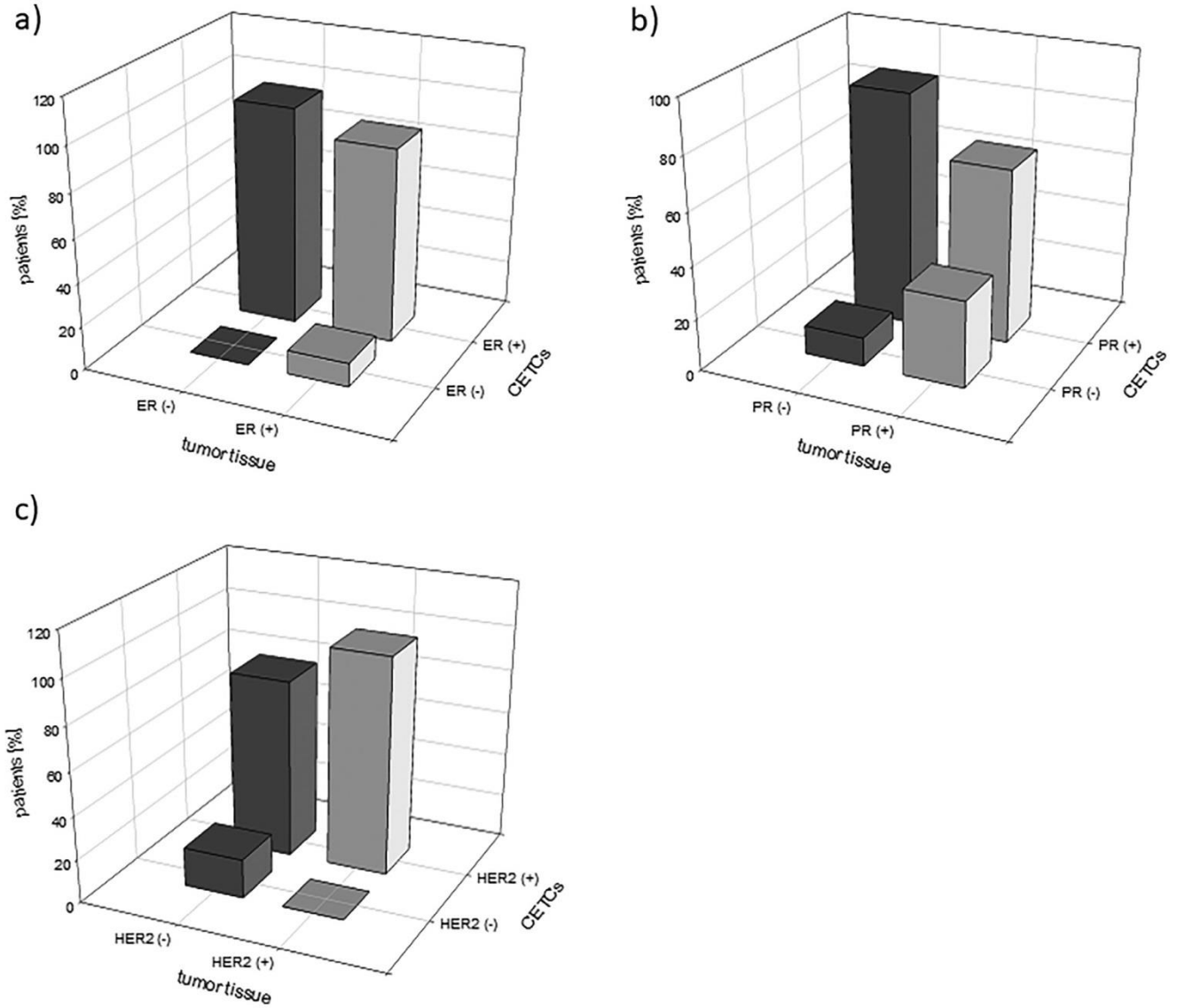

Fig. 3. Percentage of patients comparing (a) ER, (b) PR and (c) HER2 status in CETCs and tumor tissue.

patients with negative hormone receptor status and/or negative HER2 status in the primary tumor tissue had at least 1% of CETCs with expression of these receptors (Fig. 3).

We compared the ER and PR status in CETCs prior to RT with the receptor status at the end of RT. There was no influence of the RT on the hormone receptor status in CETCs when comparing the values prior to and at the end of RT, which means that patients who were positive or negative for ER or PR prior to RT remain positive or negative for the respective marker at the end of RT.

In addition, we examined the number of CETCs with regard to the different molecular subtypes of breast cancer before initiation of RT. According to international definition, Luminal A cancers were defined as ER and/or PR positive, HER2-negative, low level Ki67 (n=23; 44%), Luminal B ER and/or PR positive, HER2-negative, high level Ki67 (n=11; 21%), basal-like tumors were defined as ER, PR and HER2 negative (triple negative) (n=7; 14%), and HER2-like tumors were defined as HER2-positive (n=11; 21%). By comparing the molecular subtypes of the primary tumors with respect to the number of CETCs detected prior to RT, triple negative breast cancer patients had significantly more CETCs as compared to patients with luminal A or luminal B subtypes (median 16 vs. 5 CETCs/100µl of cell suspension, $p<0.05$) (Fig. 4a). Patients with HER2 positive/hormone receptor negative tumors had the highest median CETC numbers but due to the low number of patients with this subtype the difference was not significant.

In addition, lymph node involvement had an influence on the number of CETCs prior to RT. Patients with positive lymph nodes, who had not yet received chemotherapy, had statistically significantly more CETCs before start of RT as compared to patients without lymph node involvement (median 10 vs. 5 CETCs/100µl of cell suspension; $p<0.05$) (Fig. 4b). Patients with lymph node involvement, who previously had received chemotherapy (neoadjuvant or adjuvant) did not have higher CETC numbers as compared to patients without lymph node involvement prior to RT.

Follow-up of CETC counts during RT

During our study and the analysis we were surprised that RT does not have the same impact on CETCs in all patients. We identified 2 patient groups with antidromic behavior of CETC numbers during RT. Both groups were equally distributed with respect to T- and N-status, molec-

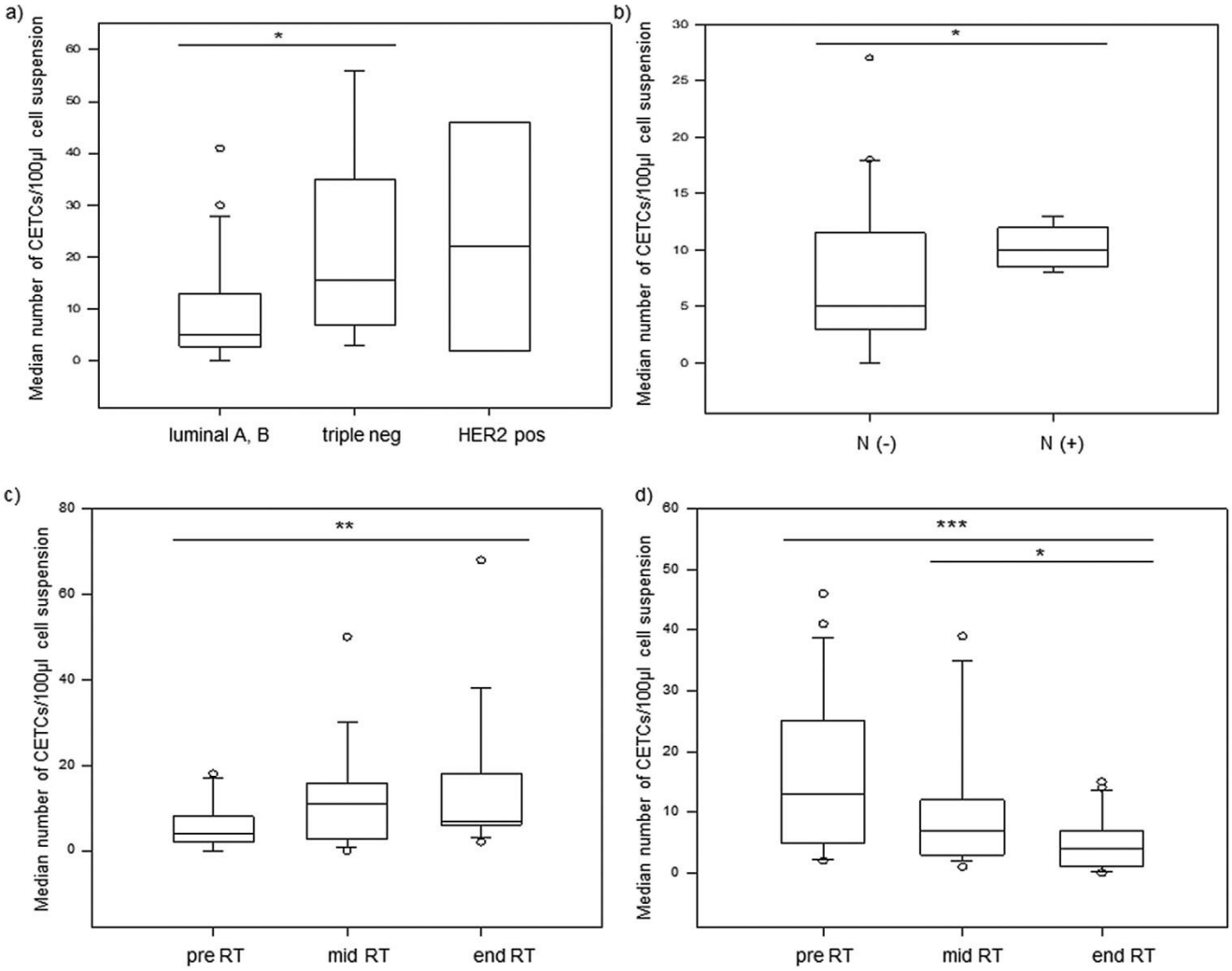

Fig. 4. Box plots of the median numbers of CETCs depending on (a) different molecular subtypes of breast cancer and (b) lymph node metastases. (c) box plots for the median number of CETCs in the patient group with increasing CETC numbers during RT and (d) in the patient group with decreasing CETC numbers.

ular subtype, histology, grading and administration of hormone therapy (Table 1). The only difference between the two groups was the observation that more patients with increasing cell numbers have had adjuvant chemotherapy whereas in the patient group with decreasing CETC numbers more patients had had neoadjuvant chemotherapy. 25 patients had increasing CETC numbers during RT whereas in 27 patients we observed decreasing CETC numbers. Pre RT CETC numbers differed significantly between the two groups (median 4 vs. 13 CETCs/100µl cell suspension; $p<0.01$), respectively (Fig. 4c and 4d).

In the patient group with increasing CETCs, cell numbers peaked at midterm of RT in 53% of patients. At the end of RT, the CETC numbers decreased but the median CETC numbers of all patients were still significantly higher as compared to the median value prior to RT (4 vs. 7 CETCs/100µl cell suspension; $p<0.01$) (Fig. 4c). The mean increase in CETC numbers from pre RT to end of RT was 2.91 ± 2.09 fold.

In the patient group with decreasing CETC numbers the mean decrease was 4.46 ± 3.68 fold. A continuous decrease in the CETC numbers was observed in this patient group. The difference in the CETC numbers was statistically significant between start of RT and end of RT (median 13 vs. 4 CETCs/100µl of cell suspension; $p<0.001$), as well as between midterm of RT and end of RT (median 7 vs. 4 CETCs/100µl of cell suspension, $p<0.05$) (Fig. 4d).

Number of CETCs in the context of prior chemotherapy

As cytostatic chemotherapy might have an influence on the number of CETCs, we investigated the CETC count dependent on chemotherapy before initiation of RT. 25 patients were treated with chemotherapy, 13 of which received neoadjuvant chemotherapy. 27 patients had not received any cytostatic chemotherapy. In the patient group treated with neoadjuvant chemotherapy, treatment led to pathological complete response (pCR) in 3 patients (23%). Patients who had received neoadjuvant chemotherapy had statistically significantly higher CETC numbers prior to RT as compared to patients who had received adjuvant chemotherapy or who had not received any chemotherapy (median 21, 4 and 6 CETCs/100µl cell suspension, respectively, $p<0.05$) (Fig. 5a). Patients who received adjuvant chemotherapy had slightly lower CETC numbers prior to RT as compared to patients who did not receive any cytotoxic chemotherapy before initiation of radiotherapy. In patients who received neoadjuvant chemotherapy, the number of CETCs decreased significantly at the end of RT from 21 (prior to RT) to 5 (at the end of RT) CETCs/100µl of cell suspension (Fig. 5b). Patients who received adjuvant chemotherapy had a trend to increasing median CETC numbers during RT (median 4, 11 and 11 CETCs/100µl cell suspension, prior to RT, midterm and end of RT, respectively) (Fig. 5c). In patients who did not receive chemotherapy there was only a marginal, nonsignificant

Table 1
Patient characteristics and CETC examination results.

Clinicopathological characteristics	Number of patients	Median number of CETCs	Number of patients with decreasing CETCs during RT	Number of patients with increasing CETCs during RT
Age				
≤50 years	13	**18**	**9**	**3**
>50 years	39	**6**	**13**	**18**
Tumor size				
pT1	30	**7**	**15**	**15**
pT2	19	**8**	**9**	**9**
pT3	2	**17**	**1**	**1**
pT4	1	**2**	**1**	
Lymph nodes				
pN0	31	**6**	**15**	**16**
pN1	17	**9**	**9**	**7**
pN2	3	**11**	**1**	**2**
pN+	1	**0**	**0**	**1**
pN3				
Stage				
I	24	**6**	**12**	**12**
II	20	**9**	**10**	**9**
III	8	**8**	**4**	**4**
ER status				
Positive (>1%)	42	**6**	**19**	**22**
Negative (≤1%)	10	**22**	**7**	**3**
PR status				
Positive (>1%)	34	**5**	**15**	**18**
Negative (≤1%)	18	**16**	**11**	**7**
HER2 status				
Positive (3+)	11	**10**	**5**	**6**
Negative (<3+)	41	**7**	**21**	**19**
Grading				
G1	10	**6**	**5**	**4**
G2	29	**10**	**15**	**14**
G3	13	**6**	**6**	**7**
Ki-67 index				
< 13%	30	**7**	**13**	**15**
> 13%	22	**10**	**12**	**9**
Molecular subtype				
Luminal A,B	34	**5**	**15**	**12**
Basal like	7	**21**	**5**	**2**
HER2 enriched	11	**10**	**5**	**6**
Histology				
invasive ductal	44	**9**	**23**	**21**
invasive lobular	6	**16**	**2**	**4**
tubular	1	**0**	**0**	**1**
mucinous	1	**2**	**1**	**0**
Chemotherapy				
No	27	**6**	**13**	**13**
adjuvant	12	**4**	**3**	**9**
neoadjuvant	13	**21**	**10**	**3**
Endocrine therapy				
Yes	23	**6**	**9**	**13**
No	29	**8**	**17**	**11**

change in the CETC numbers during RT. In contrast to chemotherapy, endocrine therapy had no influence on the median numbers of CETCs (data not shown).

Characterization of circulating cancer stem cells (cCSCs)

Cell culture for the identification of cCSCs was performed for each patient at each time of measurement. The formation of tumorspheres from cCSCs *in vitro* was controlled every 7 days under the light microscope. After 18-21 days of culture tumorspheres were collected, processed as described above and counted. As shown in Fig. 6a tumorspheres cultured from cCSCs were very compact. They were positive for EpCAM and reached a diameter of 50-90 µm. The EpCAM staining was very heterogeneous in the individual cells within individual tumorspheres 6b.

Tumorspheres had typical stem cell characteristics being positive for ALDH1 as shown in Fig. 6c. ALDH1 positivity varied strongly between individual tumorspheres from one patient and ranged between 60%-90%. In addition, they were positive for EpCAM, positive for CD44 (Fig. 6d) and negative or marginally positive for CD24 (Fig. 6e). CD44 staining varied strongly between individual cells in one individual tumorsphere.

Follow-up of cCSCs counts during RT

Irrespective of the molecular tumor subtype, tumorsphere formation *in vitro* was observed in 29/52 patients at least at one point of measurement. Prior to RT tumorspheres were detected in 31% of patients, midterm of RT in 25% of patients and at the end of RT in 41% of patients. In 44% of patients no tumorsphere formation was observed at any measurement point.

There were 15 patients with increasing and 14 patients with decreasing numbers of tumorspheres during RT. The mean increase in the patient group with increasing tumorsphere numbers was 6 ± 5 fold. There were significant differences between the number of tumorspheres prior to and at the end of RT (mean 1 vs. 7 tumorspheres/100µl of cell suspension, $p<0.001$) as well as between midterm of RT and end of RT (mean 4 vs. 7 tumorspheres/100µl of cell suspension, $p<0.05$) (Fig. 7a). The mean decrease in the patient group with decreasing tumorsphere numbers was 5 ± 7 fold. Differences in tumorsphere numbers between start of RT and midterm of RT (mean 7 vs. 4 tumorspheres/100µl of cell suspension, $p<0.05$) as well as between start of RT and end of RT (mean 7 vs. 1 tumorspheres/100µl of cell suspension, $p<0.01$) were statistically significant (Fig. 7b).

Chemotherapy had an effect on the number of tumorspheres grown *in vitro* prior to RT, yet this was not statistically significant. Comparable to the number of CETCs prior to RT, the number of tumorspheres *in vitro* was higher in the patient group after neoadjuvant chemotherapy as compared to patients who received adjuvant chemotherapy (mean 7 vs 4 tumorspheres/100µl of cell suspension (Fig. 7c). The highest tumorsphere number was observed in patients who did not receive chemotherapy prior to RT (mean 10 tumorspheres/100µl of cell suspension).

Tumorsphere formation *in vitro* was observed more frequently in patients after neoadjuvant chemotherapy (61%) as compared to patients with adjuvant chemotherapy (42%) or without chemotherapy (48%).

In 22 patients out of the 29 patients (76%), CETC and tumorsphere numbers developed in parallel: in 14 patients, both the number of CETC and the number of tumorspheres decreased during the course of RT, whereas in 8 patients, the number of tumor cells and the number of tumorspheres increased. However, in the patient group after neoadjuvant chemotherapy as well as in the patient group who did not receive chemotherapy an inverse development in tumorsphere and CETC numbers was observed: most patients in this patient group had increasing tumorsphere numbers but decreasing CETC numbers during RT.

Discussion

Although nowadays breast cancer is often diagnosed at an early stage it is still a leading cause of cancer mortality [3]. Due to intra- and intertumoral heterogeneity but especially due to potential changes in tumor cell properties during the course of disease, which can be caused by treatment effects, longitudinal analysis of circulating tumor cells promises a more complete understanding of the complex processes underlying treatment response or relapse[3]. Biomarkers from the primary tumor may help to identify low- and high risk patients but the analysis of the actually disseminated tumor cells would be preferable [3]. Especially in early stage where treatment is expected to be curative, monitoring circulating tumor cells could predict early disease recurrence and survival [20,36].

It has been shown that the characteristics of the cells released from the primary tumor may change during disease progression leading to

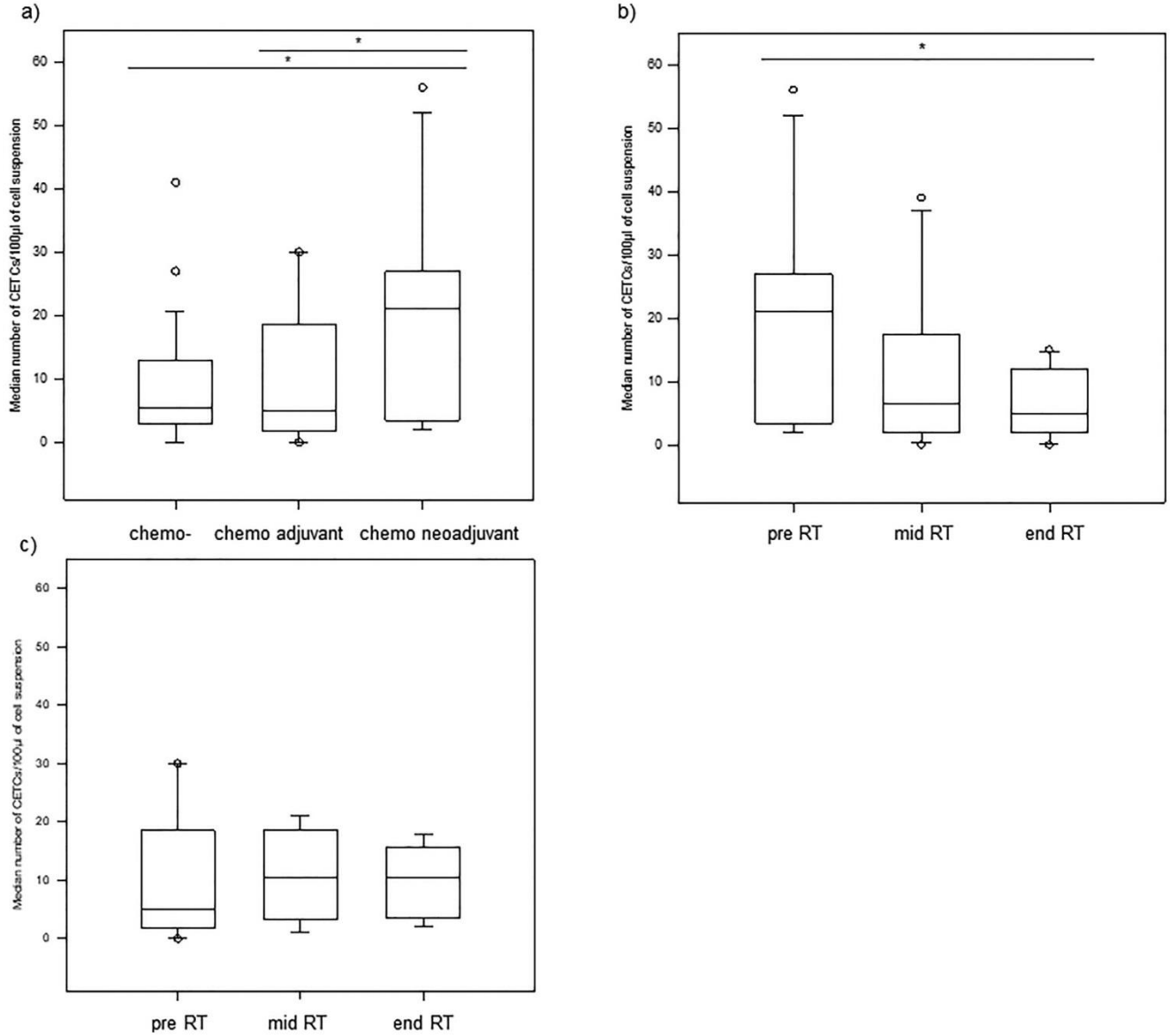

Fig. 5. (a) Median number of CETCs prior to RT with respect to chemotherapy. (b) median number of CETCs during RT in patients who received neoadjuvant chemotherapy and (c) median number of CETCs during RT in patients who received adjuvant chemotherapy.

ineffective treatment in the absence of the respective marker as indicated by the discrepancy in hormone receptor and HER2 status between primary tumor and metastatic lesions in one-third of metastatic breast cancer patients [37]. Our study shows that the expression of hormone receptors and amplification of HER2 in CETCs initially reflect hormone receptor and HER2 status of the primary tumors and was concordant in 70% for ER, 46% for PR and 55% for HER2 expression indicating that these cells are highly probably tumor cells disseminated from the primary tumor. These results are in agreement with the study of Kalinsky et al, who observed a concordance of 68% in ER/PR status between primary tumor and circulating tumor cells in early stage breast cancer [38] and Zhang et al reporting a concordance between HER2 status in primary tumor and circulating tumor cells in 38% of patients [39]. During subsequent RT almost no changes in hormone receptor status were observed suggesting that RT does not have any selective effect on marker expression and RT does not selectively eliminate cell populations.

In agreement with Xu et al. we observed higher CETC numbers in triple negative and HER2 positive patients as compared to luminal A/B subtypes before initiation of RT, indicating that patients with highly aggressive disease have increased CETC numbers and higher risk for metastasis formation [40]. Currently, the treatment of patients with triple negative breast cancer is the biggest challenge in breast cancer. In early stages, the use of neoadjuvant chemotherapy is the standard of care and achieving a pCR after neoadjuvant chemotherapy correlates with improved survival outcomes [41]. In our short follow up of 7 months we observed one relapse in a triple negative breast cancer patient. The patient suffered from a stage II (T2N1M0) breast cancer and had been treated with neoadjuvant chemotherapy. As she did not achieve a pCR she was additionally treated with adjuvant chemotherapy (Fig. 7d). Her numbers in CETCs and tumorspheres, grown *in vitro* from cCSCs, increased during RT followed by a relapse 7 months after end of RT. If this observation is confirmed in a higher number of patients monitoring CETCs during the course of RT could help in decision making for fur-

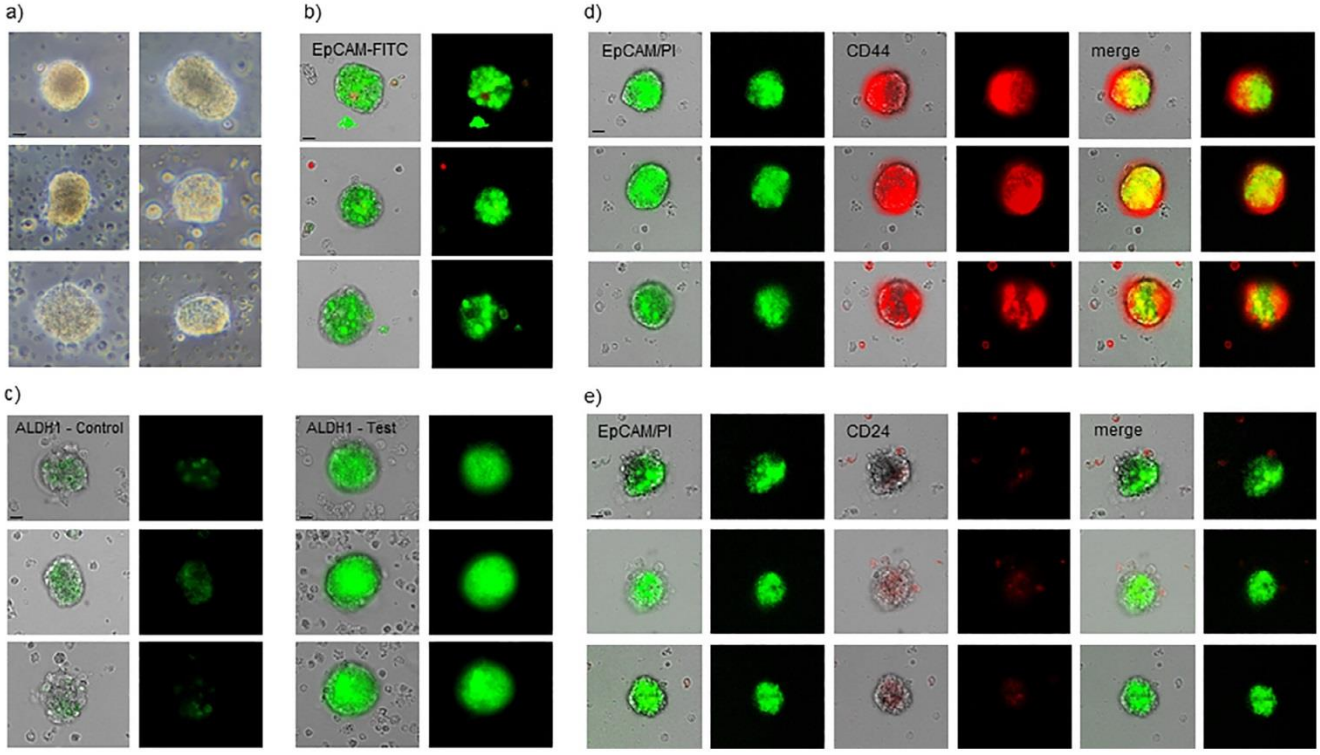

Fig. 6. Typical images of tumorspheres grown *in vitro* from cCSCs from one breast cancer patient (a) from culture flask and (b) fluorescence scanning microscope. (c) ALDH1 activity of tumorspheres. (d) Images of tumorspheres being positive for EpCAM and CD44 and (e) Images of tumorspheres being negative or marginally positive for CD24. Scale bar: 10μm.

ther diagnostic and therapeutic interventions to early detect recurrence, especially in this aggressive subgroup of breast cancer.

Furthermore, in accordance with other studies [12,36] the number of CETCs was associated with patients‘ nodal stage. Positive axillary lymph nodes are a predictor of increased risk for local and distant recurrence and directly correlated with overall survival [42].

Goodman et al showed that patients in whom circulating tumor cells can be detected (24% of all patients with at least 1 cell/20 ml of blood) fare better with RT after BCS than without RT. RT seems to reduce the risk of recurrence and death from breast cancer suggesting elimination of microscopic tumor foci [33] which might seed cells into the circulation. In the present study population, breast conserving procedure was performed in 90% of patients. Increasing CETC numbers were observed in 40% of patients and decreasing CETC numbers in 44% of patients from the start of RT to end of RT. Regarding clinicopathological features, age and endocrine therapy there was no difference in the groups with increasing and decreasing cell numbers. The only difference is that in the patient group with decreasing cell numbers almost all patients (80%) were treated with neoadjuvant chemotherapy and that these patients after neoadjuvant chemotherapy had statistically significantly more CETCs before RT than patients with adjuvant chemotherapy or without chemotherapy. While neoadjuvant therapy enables tumor downsizing for BCS by disintegration of tumor tissue, assessing *in vivo* response to therapy and provide prognostic information based on pathological response [2] the reduction in tumor size and the destruction of tumor tissue may lead to the mobilization and release of tumor cells into the bloodstream. Monitoring CETCs during neoadjuvant chemotherapy in early breast cancer patients [22] showed that the initial decrease in the number of CETCs correlated with the reduction in tumor size. Comparable observations during RT have been made in NSCLC patients where increased numbers of circulating tumor cells during the course of neoadjuvant RT were detected due to mobilization of viable tumor cells from intact tumors into the circulation [43].

The group of patients with increasing CETC numbers during RT mainly comprised patients who had not received chemotherapy or who had received adjuvant chemotherapy. In this subpopulation we observed in half of the patients a peak in CETC numbers at midterm of RT followed by a decrease.

It is not easy to distinguish dormant circulating tumor cells from those with proliferating capacity initiating new tumors at distant sites [3]. It is assumed that only a small subpopulation of circulating tumor cells, the so called circulating cancer stem cells, can grow into metastases. They have properties of stemness and represent the most aggressive fraction of cells [44]. Different approaches for the identification of cancer stem cells from the primary tumor are available, one of them a functional assay, the sphere-formation assay [45] is commonly used to study stem cell properties of CSCs. In the present study we used a tumorsphere-formation assay developed by us to grow circulating cancer stem cells from the peripheral blood. To our knowledge, this is the first study to monitor the number of circulating cancer stem cells during RT in breast cancer patients. In about one half of patients tumorsphere formation *in vitro* could be observed at least at one point of measurement expressing typical stem cell markers for breast cancer. Again, there were two patient groups, one with increasing and one with decreasing tumorsphere numbers *in vitro* during RT. The group with increasing tumorsphere numbers *in vitro* comprised mainly patients with an unfavorable hormone receptor status and higher Ki-67 index. In addition, in this patient group, many patients had not received any chemotherapy. Thus, as reported previously [31], the number of tumorspheres correlated with unfavorable characteristics of the primary tumor. In patients who had received neoadjuvant chemotherapy, tumorsphere formation *in vitro* was more frequently observed as compared to patients who received adjuvant chemotherapy or no chemotherapy. After neoadjuvant chemotherapy an increase in tumorsphere numbers during RT was found in 63% of patients and in 69% of patients who had not received any chemotherapy. Like CETCs cells with cancer stem cell properties may be-

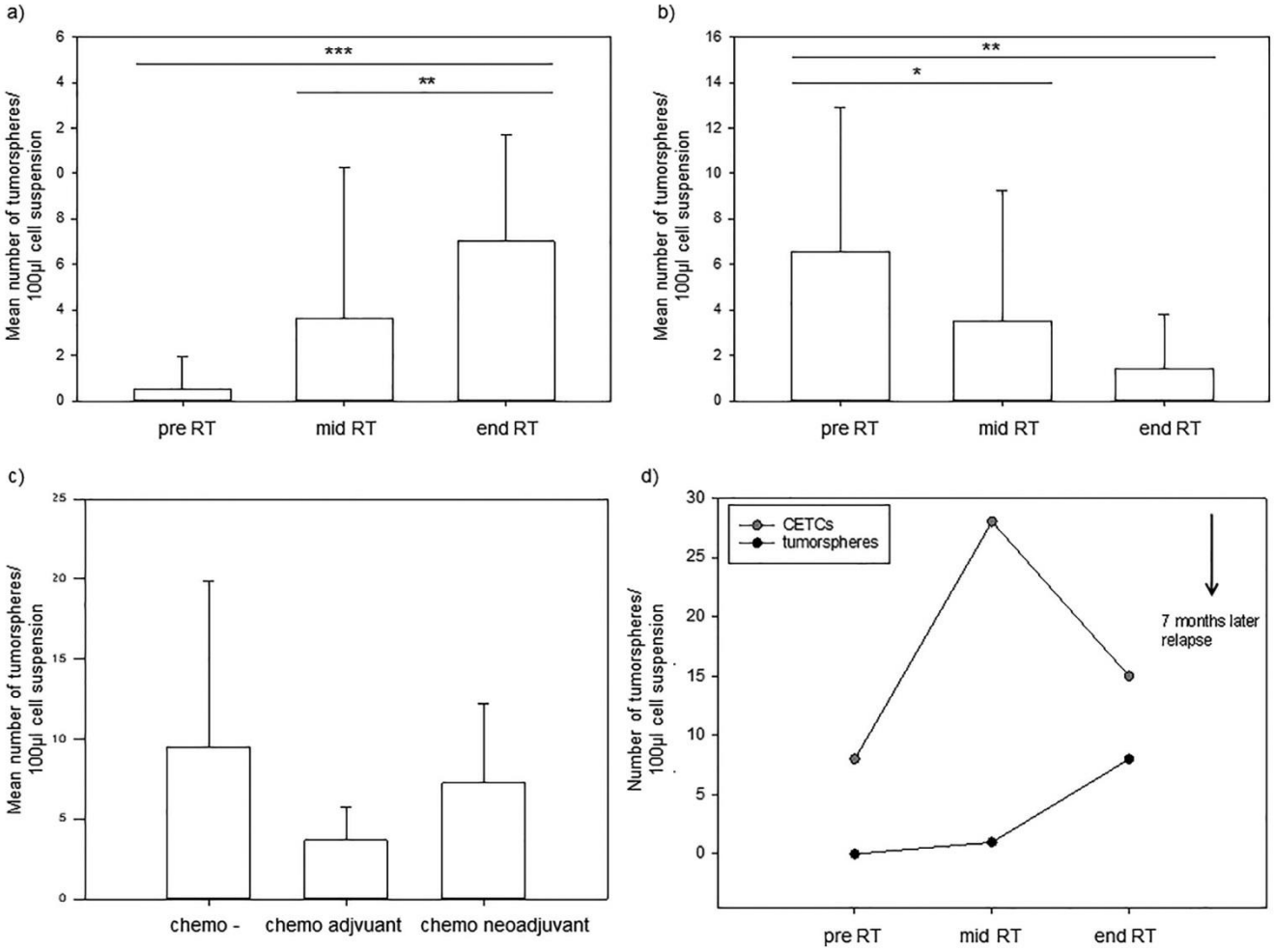

Fig. 7. Mean variation in the number of tumorspheres during RT in the patient group with (a) increasing tumorsphere numbers and (b) decreasing tumorspheres numbers. (c) Mean numbers of tumorspheres prior to RT with respect to chemotherapy. (d) Course of CETC and tumorsphere numbers during RT in one breast cancer patient after neoadjuvant and adjuvant chemotherapy.

come released from the primary tumor during neoadjuvant chemotherapy. In the patient group after neoadjuvant chemotherapy we observed more frequently a decrease in the number of CETCs antidromic to an increase in the number of tumorspheres grown from cCSCs *in vitro* than in the other patients, indicating that cancer stem cells might be radioresistant.

Our results of decreasing single CETC numbers during RT but increasing numbers of tumorspheres after neoadjuvant treatment might also support the hypothesis, that under conditions of radiation-induced stress, cancer cells may undergo dedifferentiation into stem cell-like cells. These cells might subsequently obtain phenotypes and functions of CSCs, including radioresistance, which indicates that radiation may directly result in the generation of novel CSCs from non-stem cancer cells [46]. Changes in the number of CETCs, but especially changes in the number of tumorspheres cultured from cCSCs *in vitro*, could be relevant for recurrence of cancer disease.

Conclusion

We could show that CETCs before RT have the same molecular properties as the cells in the primary tumor, which in turn indicates that the detected cells are tumor cells. Furthermore, we were able to identify a subpopulation of CETCs which were able to grow clonally *in vitro* into so called tumorspheres and possess stem cell properties. Our preliminary results shows that changes in the number of CETCs, but especially changes in the number of tumorspheres cultured *in vitro* from cCSCs during adjuvant radiotherapy could reflect the activity of residual tumor burden after breast conserving surgery. This could help to identify patients with increased risk of local recurrence.

Declaration of Competing Interest

All authors declare that they have no competing interests.

CRediT authorship contribution statement

Dorothea Sonja Schott: Conceptualization, Methodology, Validation, Investigation, Writing - original draft, Visualization, Supervision, Project administration. **Monika Pizon:** Methodology, Validation, Writing - original draft, Visualization. **Ulrich Pachmann:** Writing - review & editing. **Katharina Pachmann:** Conceptualization, Methodology, Writing - review & editing, Project administration. **Rainer Schobert:** Writing - review & editing, Project administration. **Andrea Wittig:** Writing - review & editing, Project administration. **Matthias Mäurer:** Conceptualization, Writing - original draft, Supervision, Project administration.

Ethics approval and consent to participate

All subjects have provided written informed consent before participation in the study which has been approved by the Ethics Committee of Jena.

Consent for publication

All authors have agreed to the submission of the manuscript.

Availability of data and material

All data are fully available without restriction.

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5.8 Publikation VI

Influence of endocrine therapy on the ratio of androgen receptor (AR) to estrogen receptor (ER) positive circulating epithelial tumor cells (CETCs) in breast cancer

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Influence of endocrine therapy on the ratio of androgen receptor (AR) to estrogen receptor (ER) positive circulating epithelial tumor cells (CETCs) in breast cancer

Monika Pizon*, Daniel Lux, Ulrich Pachmann, Katharina Pachmann and Dorothea Schott

Abstract

Background: The androgen receptor (AR) is expressed in the majority of breast cancers and across the main breast cancer subtypes. Despite the high frequency of AR expression in breast cancer its appraisal remains controversial because its role is complex, dependent on the hormonal milieu. The aim of the current study was to investigate the frequency of AR and ER positive CETCs in breast cancer patients.

Methods: The number of vital CETCs was determined from blood of 66 patients suffering from breast cancer and the expression of AR and ER on these cells was investigated using the maintrac method.

Results: Numbers of CETCs/mL blood were significantly higher in patients with advanced disease as compared to patients with early stage disease. The fraction of AR positive CETCs was significantly higher than the fraction of ER positive CETCs (90% vs. 50%; $P < 0.001$). Patients with positive lymph nodes had less AR positive CETCs as compared to patients with negative lymph node status. The AR:ER ratio was higher in patients receiving tamoxifen therapy as compared to patients without tamoxifen therapy whereas treatment with aromatase inhibitor had no influence on AR:ER ratio.

Conclusions: The ratio of AR to ER positive CETCs, obviously, is influenced by endocrine therapy, more specifically therapy with tamoxifen. Since AR expression seems to be one of the possible mechanism of resistance to endocrine therapy this may provide a new biomarker to select patients who might benefit from combination treatment of ER and AR inhibitors.

Keywords: Breast cancer, Circulating epithelial tumor cells, Androgen receptor, Androgen to estrogen ratio

Background

The biology of the androgen receptor (AR) and its therapeutic importance have been investigated extensively in prostate cancer [1]. However, AR is also widely expressed in other human tissues, including testis, ovary and breast [2]. In recent years AR has attracted a great deal of attention in the management of breast cancer, because it turned out that AR is expressed in about 80% of breast cancers depending on the subtype, often at a higher level than ER [2–4]. Like ER and PgR, AR is a nuclear transcription factor. The binding of steroid hormone androgens activates AR [5]. According to reports [1], over 70% of ER-positive breast cancers, approximately 60% of HER2-positive breast cancers, and 30% to 45% of TNBC (triple negative breast cancers) express AR. It has a multifunctional role and there is still a conundrum as to whether it acts as a tumor suppressor or an oncogene. Similar to ER and PR, AR expression is an important prognostic factor for disease free and overall survival [6, 7]. In addition, it is an independent prognostic marker associated with favorable clinico-pathological features, and a predictor of response to chemotherapeutic agents [1, 8]. AR expression is associated with a

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well-differentiated state and with more indolent breast cancers [6, 7]. In contrast, some studies showed that AR promotes the growth of ER+ breast cancer and TNBC via distinct mechanisms [9]. Furthermore, AR expression is associated with resistance to anti-estrogen therapies [1]. Resistance to established endocrine therapies is a well-documented phenomenon occurring de novo in 30% to 50% of all ER+ tumors and ultimately all metastatic ER+ breast cancers. It could result from tumor cell adaption from estrogen dependence to androgen dependence [6]. Recent studies suggest that not the expression level of AR alone predicts benefit from adjuvant endocrine therapy with tamoxifen but its relation to ER expression levels in primary tumors is of importance [5]. Thus, suboptimal response to ER-directed endocrine therapy may be due to the AR:ER ratio. A high AR:ER protein ratio has been reported to be indicative of a shorter time to relapse in patients treated with tamoxifen [6]. However, despite these emerging data, the role of AR in breast cancer is still not fully elucidated and the biology of AR in breast cancer remains incompletely understood [1]. No biomarkers are currently available to track changes in AR expression in blood over time in response to AR-targeting treatment. Circulating epithelial tumor cells (CETCs) used as "liquid biopsy" may become a tumor-specific biomarker of response to therapy. Circulating tumor cells are rare tumor cells that escape from solid tumors, travel into peripheral blood and can seed distant metastases. They have been reported to be a surrogate marker for tumor treatment response in primary breast cancer [10], and their presence has been linked to shorter survival in patients with metastatic breast, prostate, colorectal and lung cancer [1]. The purpose of our study was to better characterize AR and ER expression on CETCs in breast cancer contributing a new biomarker for targeted AR therapy, especially in patients with tamoxifen resistance.

Methods

Blood collection and processing

Peripheral blood (7.5 mL) from 66 patients with breast cancer in different stages of disease was drawn into normal blood count tubes with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant and processed within 48 h of collection. The sampling of peripheral blood was carried-out 6–12 weeks after end of standard therapy (tumor resection, adjuvant chemotherapy, adjuvant radiotherapy). In patients with local or distant recurrence the blood was collected prior to treatment of recurrent disease. In parallel, control blood samples were collected from 15 healthy female and male donors aged 20–40 years. The study was conducted in accordance with Good Clinical Practice Guidelines and the declaration of Helsinki.

Maintrac®

For CETC enumeration and further characterization the maintrac® approach was used as reported previously [11]. Briefly, 1 mL blood was subjected to red blood cell lysis using 15 mL of erythrocyte lysis solution (Qiagen, Hilden, Germany) for 15 min in the cold, spun down at 700 g and re-diluted in 500 µL of PBS-EDTA. 5 µL of fluorescein-isothiocyanate (FITC)-conjugated anti-human epithelial cell adhesion molecule antibody (EpCAM) (clone HEA-125, Miltenyi Biotec GmbH, Germany) at a final concentration of up to 10^7 cells/100 µL cell suspension were added and incubated for 15 min in cold. The corresponding isotype control for EpCAM (Mouse IgG1 K FITC, Miltenyi Biotec GmbH, Germany) was used at the same final concentration. The samples were subsequently diluted with 430 µL PBS-EDTA. A defined volume of the cell suspension and propidium iodide (PI) (Sigma-Aldrich, USA) was transferred to wells of ELISA plates (Greiner Bio-one, USA). Analysis of red and green fluorescence of the cells was performed using a Fluorescence Scanning Microscope, ScanR, (Olympus, Hamburg, Germany), enabling detection and relocation of cells for visual examination of EpCAM positive cells. For data analysis we used the ScanR Analysis software (Olympus, Hamburg, Germany). Vital CETCs were defined as EpCAM-positive cells, with intact morphology lacking in nuclear PI staining and, only these cells were counted. We used fluorospheres (Flow-Check 770, Beckman Coulter) for daily verification of optical components and detectors of the microscope, which are required to ensure the consistent analysis of samples.

Cell lines

For the AR staining Sk-Br-3 (data not shown) and MCF-7 cell lines were used as positive controls and the SW-620 cell line as a negative control (Fig. 1). For the ER staining MCF-7 cell line was used as a positive control and SW-620 cell line as a negative control. We obtained all cell lines from the CLS cell lines service (Eppenheim, Germany). MCF-7 cells were grown in Minimum Essential Medium Eagle ready-to-use medium (CLS cell lines service (Eppenheim, Germany). Sk-Br-3 and SW-620 cells were grown in Dulbecco's modified Eagle's medium with 4,5 g/L glucose, 2 mM L-glutamine (Gibco, Thermo Fisher Scientific, Waltham, USA) and 10% FBS. Cells were maintained at 37 °C in 5% CO_2. For immunofluorescence analysis cells were detached from cell culture flasks using StemPro® Accutase® Cell Dissociation Reagent (Gibco, Thermo Fisher Scientific, Waltham, USA), washed and stained for AR or ER with the same protocol as patients' samples.

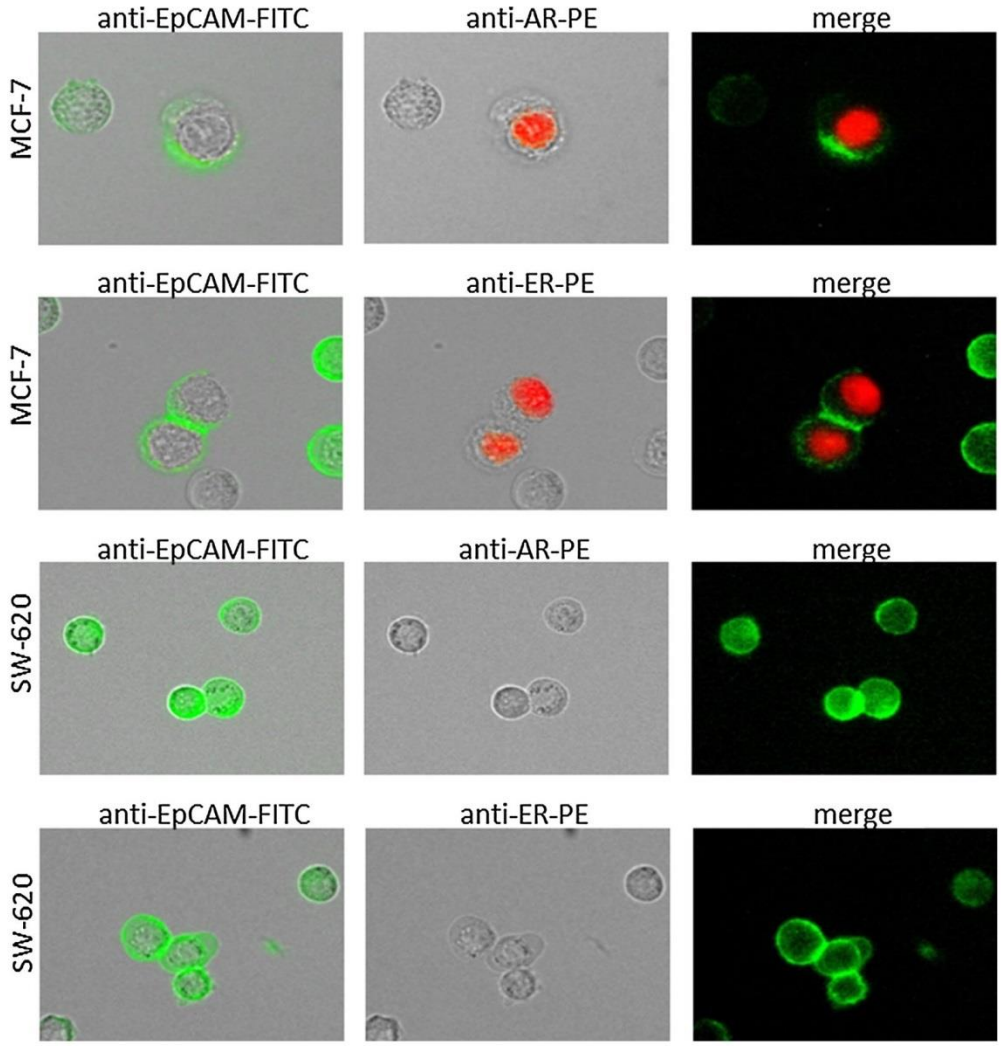

Fig. 1 Positive and negative control for AR and ER staining. As positive control we use MCF-7 cell line. MCF-7 cells are positive for EpCAM (green) and AR (red) or ER (red). SW-620 cell line is used as a negative control. SW-620 cells are positive for EpCAM and strictly negative for AR or ER

AR/ER-analysis

The analyses of AR (Fig. 2a) and ER (Fig. 2b) expression on the CETCs were performed with an extended maintrac® approach. For AR expression analysis we used an anti-human AR phycoerythrin (PE)-conjugated antibody (clone 523339, R&D Systems, Minneapolis, Canada) at a final concentration of 0.12 µg/mL and for ER we used an anti-human ER-PE conjugated antibody (clone E115, abcam, Cambridge, USA) at a final concentration of up to 10^7 cells/100 µL cell suspension. The corresponding isotype controls for AR (Mouse IgG2B PE-conjugated Antibody, R&D Systems, Minneapolis, Canada) and ER (Rabbit IgG PE, abcam, Cambridge, USA) were used at the same final concentration. Finally, cells were visually inspected looking for a green and red surface staining, but also a well-preserved nucleus. For excluding expression of AR and ER on hematopoietic cells we additionally performed staining with EpCAM–FITC, AR–PE/ER–PE and CD45-Pacific blue antibodies (data not shown). The results for AR and ER were calculated as percentage of total number of CETCs.

Statistical analysis

Statistical analysis was performed using the software programs SigmaPlot version 13.0 (Systat Software Inc., Chicago, USA) for Windows. Comparisons between the variables were performed with the Student t test (dichotomous variables) or ANOVA (variables with more

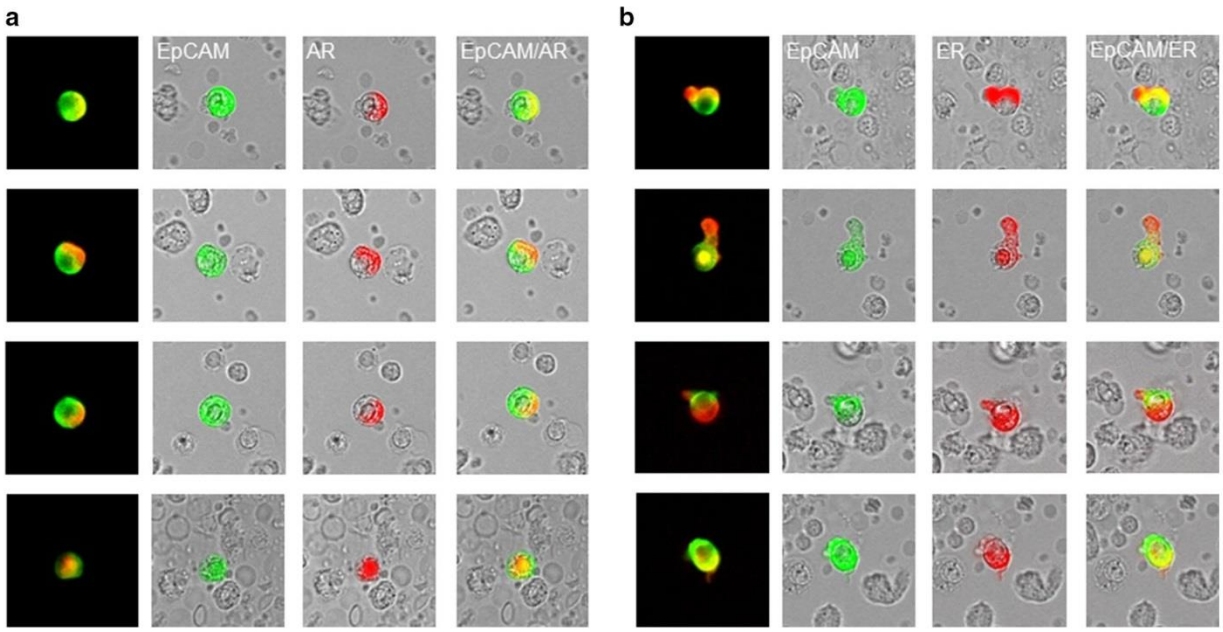

Fig. 2 Androgen and estrogen expression on CETCs. **a** Representative images of EpCAM (green) and AR staining (red) on CETCs in one patient. **b** Representative images of EpCAM (green) and ER staining (red) on CETCs in the same patient. There is a substantial heterogeneity in EpCAM, AR and ER expression level across the CETCs from the same patient

than two categories), taking into account the possibility of using nonparametric tests. The correlation was calculated with the Pearson or Spearman rank correlation coefficient. Receiver operating characteristic (ROC) curve was used to find the optimal cut-off point of CETC counts. The optimal cut-off point was determined by choosing the CETC count with the highest sum of sensitivity and specificity regarding to the stage of disease. Area under curve (AUC) analysis was applied to demonstrate the performance for better distinguishing patients in early and advanced stages of disease. The significance level was $P < 0.05$.

Results

The patient characteristics according to AR and ER expression are shown in Table 1. 21 patients were classified as stage I (32%), 28 in stage II (42%), 9 in stage III (14%) and 7 in stage IV (11%). Patients with advanced stage of disease (stage III/IV) had statistically significantly more CETCs as compared to patients with early stages (stage I/II) of disease ($P < 0.05$) (Fig. 3a). The median number of CETCs in early stage of disease was 30 CETCs/100 µL of blood (ranging from 10 to 290), and in advanced stage of disease the median number of CETCs was 95 CETCs/100 µL of blood (ranging from 15 to 565). As negative control we tested blood samples from 15 healthy controls and confirmed that none of the samples were positive for CETCs. The receiver operating characteristic (ROC) curve shows that the number of CETCs was a predictor of advanced stage of disease. The AUC for number of CETCs was 0.74 ($P = 0.01$) (Fig. 3b). The cut-off number of CETCs predictive for advanced stage of disease for breast cancer was 80/100 µL blood with a sensitivity of 85% and a specificity of 60%. AR positive CETCs were observed in all examined patients. Median percentage of AR positive CETCs was 90% and ranged from 25 to 100%. The percentage of AR positive CETCs correlated with lymph node and hormone receptor status. Patients with a negative lymph node status had a statistically significantly higher fraction of AR positive CETCs as compared to patients with lymph node involvement (93% vs 86%, $P < 0.05$) (Fig. 4a). In addition, the percentage of AR positive CETCs was significantly higher in TNBC as compared to hormone receptor positive patients (median 91 vs 83, $P < 0.05$) (Fig. 4b). ER positive CETCs could be observed in 94% of patients. Median percentage of ER positive CETCs was 50% and ranged from 11 to 100%. Interestingly, the fraction of AR positive CETCs was significantly higher than the fraction of ER positive CETCs (90% vs. 50%; $P < 0.001$) (Fig. 5).

Table 1 Patient characteristics and CETC examination results

Clinico-pathological characteristics	Number of patients (%) with CETCs	Median of CETC counts	*P*-value	Median (%) of AR positive CETCs	*P*-value	Median (%) of ER positive CETCs	*P*-value
Age (years)							
≤ 50	26 (39)	85	*0.001*	90	0.98	50	0.91
> 50	40 (61)	30		89		50	
Tumor size							
T1	29 (44)	35	0.34	83	0.42	50	0.86
T2	34 (51)	35		80		50	
T3	2 (3)	120		73		62	
n.a	1 (2)						
Lymph node metastasis							
Positive	25 (38)	40	0.63	86	*0.04*	50	0.63
Negative	40 (61)	35		93		50	
n.a	1 (1)						
Distant metastasis							
Positive	7 (11)	85	0.11	88	0.99	49	0.97
Negative	58 (88)	35		89		50	
n.a	1 (1)						
Stage							
I/II	49 (74)	30	*0.009*	90	0.93	51	0.16
III/IV	17 (26)	95		89		62	
HER2 status							
Positive	21 (32)	35	0.58	89	0.93	56	0.37
Negative	45 (68)	35		90		52	
ER/PR status							
Positive	51 (77)	35	0.97	87	0.74	52	0.82
Negative	15 (23)	35		88		46	
Radiation							
Yes	24 (36)	40	0.93	92	0.19	48	0.27
No	34 (52)	40		88		52	
n.a	8 (12)						
Hormone receptor status							
TNBC	10 (16)	55	0.31	91	*0.02*	56	0.55
ER/PR pos.	51 (84)	35		83		46	
Hormone therapy							
Yes	33 (50)	35	0.77	89	0.57	47	0.07
No	32 (49)	35		91		60	
n.a	1 (1)						
Chemotherapy							
Yes	30 (45)	50	0.51	90	0.47	52	0.96
No	36 (55)	35		88		48	
Tamoxifen therapy							
Yes	22 (33)	35	0.64	90	0.75	45	*0.042*
No	43 (66)	35		91		58	
n.a	1 (1)						
Aromatase inhibitor							
Yes	19 (29)	50	0.29	85	0.22	50	0.35
No	46 (70)	35		91		39	
n.a	1 (1)						

Statistically significant values are in italics

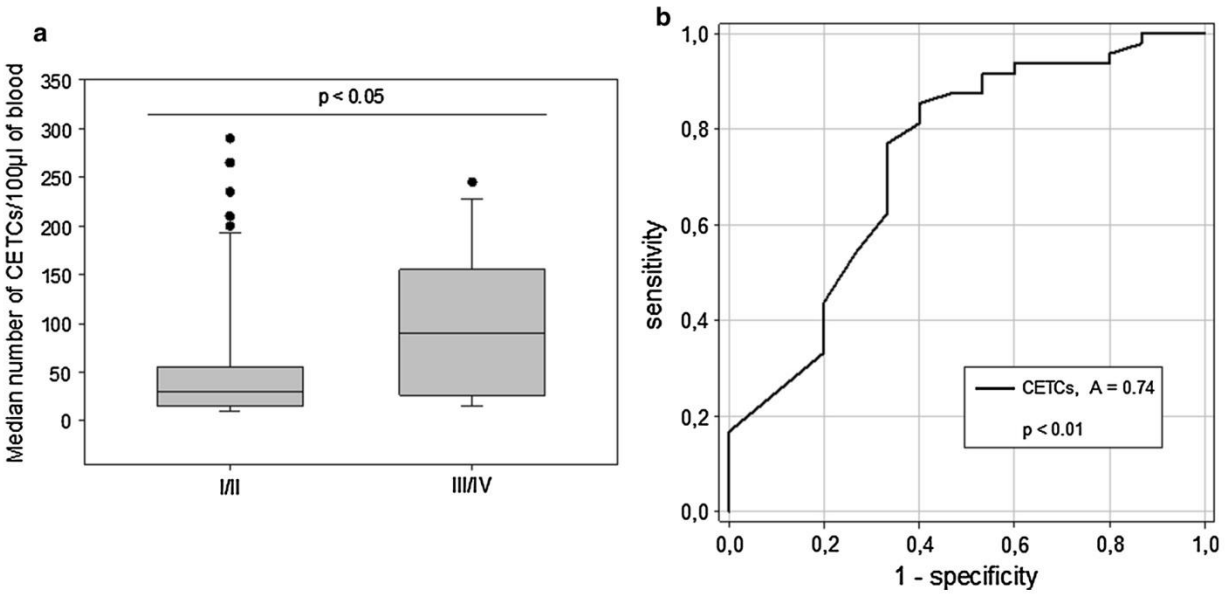

Fig. 3 Number of CETCs in early (I/II) and advanced stage of disease (III/IV). **a** Box plot shows the number of CETCs in early and advanced stage of disease. Patients in advanced stage had significantly more CETCs as compared to patients in early stage of disease, *P < 0.05. **b** Determination of the cut-off point of CETC numbers by ROC curve analysis. ROC curve analysis was performed to determine the cut-off point of CETC numbers in terms of accuracy for discrimination of localized and advanced stage of disease. The value with the highest sum of sensitivity and specificity was chosen as a cut-off point: CETC number > 80 was determined as high CETC level and < 80 was determined as low CETC level. The AUC was 0.74, **P < 0.01

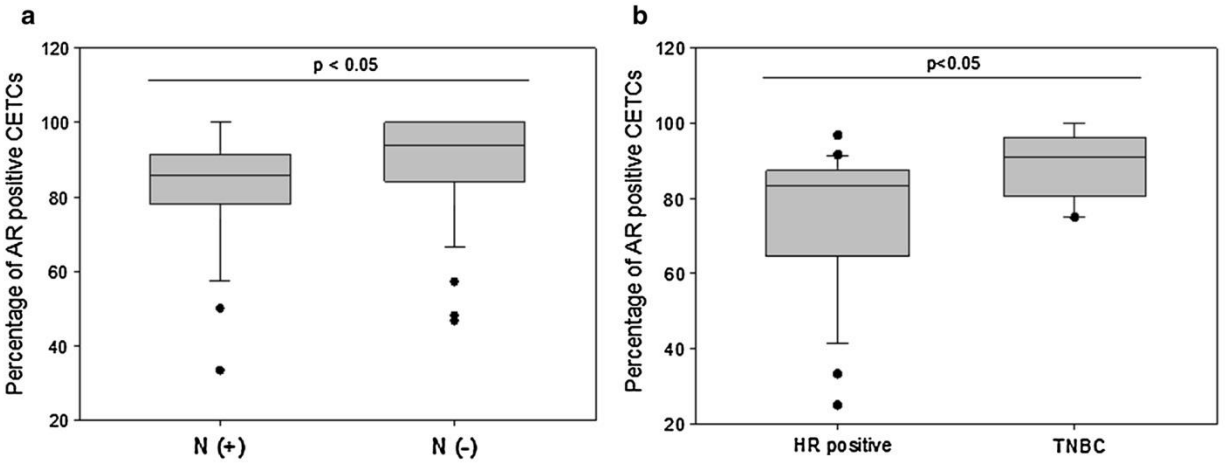

Fig. 4 Box plot analysis of AR expression on CETCs in breast cancer patients with respect to lymph node and hormone receptor status. **a** We identified significant enhanced number of AR positive CETCs in patients without lymph node metastasis (N−) as compared to patients with lymph node involvement (N+), *P < 0.05. **b** In TNBC patients we observed elevated numbers of AR positive CETCs as compared to patients with HR positive primary tumors, *P < 0.05

Women who received tamoxifen therapy had a higher ratio of AR-positive:ER-positive cells as compared to women not treated with tamoxifen (2.2 vs 1.3, P < 0.05) (Fig. 6) whereas there was no such association between the AR:ER ratio and treatment with aromatase inhibitors (AIs).

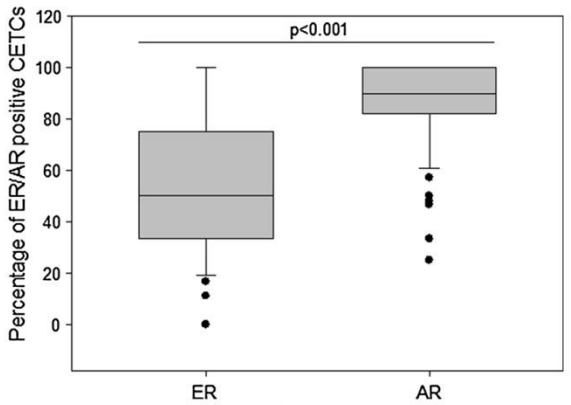

Fig. 5 Box plot of ER and AR positive CETCs detected in breast cancer patients. The box plot shows higher AR expression on CETCs compared to ER expression on CETCs, ***P < 0.001

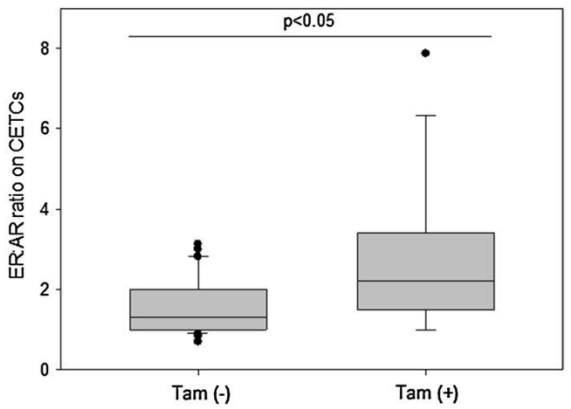

Fig. 6 Box plot analysis of ER:AR ratio on CETCs by patients with and without tamoxifen therapy. In patients who received tamoxifen therapy we identified a significant enhanced ER:AR ratio as compared to patients without tamoxifen therapy, *P < 0.05

Discussion

The detection of tumor cells circulating in the peripheral blood of metastatic cancer patients has been associated with both metastatic disease and a higher risk of progression [12]. With the maintrac® method, an approach designed to minimize cell loss during the labeling and analysis process we were one of the first who could detect circulating tumor cells also in non-metastatic patients. Cell numbers could be monitored during treatment and we could show that an increase in the number of CETCs during chemotherapy correlates with an unfavorable prognosis [10]. Similarly, an association between a poor prognosis and persisting numbers of circulating tumor cells following chemotherapy in early stage of disease has been reported by Rack et al. [13]. Using the maintrac® approach the number of CETCs from the peripheral blood of patients with solid tumors detected is higher than that detected with other methods in both, early and advanced stage of disease [14]. This is due to the omission of all enrichment steps during the sample preparation. In opposition, the fixation and magnetic enrichment in the CellSearch® method leads to a massive destruction of the tumor cells and also to a heavy cell loss [14]. However, preservative agent present in the CellSave tubes provide poor antigen preservation due to the cross-linking mechanism of fixation [15].

In primary disease the number of CETCs was significantly related to the stage of disease. Our results indicate that patients with higher CETC counts have a more advanced stage of disease than patients with lower CETC counts. The AUC to distinguish between early and advanced stage with the CETC count was 0.74 with a high sensitivity and specificity. Thus, the CETC count in the peripheral blood is predictive for the severity of cancer disease. Our results are in agreement with the results of Maestro et al. [16] who used the numbers of circulating tumor cell to determine the cut-off value for discrimination between localized and metastatic stages with an AUC for breast cancer cases of 0.76. This indicates that the number of CETCs can be used as an additional predictive marker for better distinguishing patients in early and advanced stages of disease. AR is expressed in about 70–90% of breast cancers and seems to play a major role in carcinogenesis. Currently, it is under investigation in the clinical setting as a therapeutic target [17]. In contrast to Fujii et al. [1] who found only in 18% of metastatic breast cancer patients circulating tumor cells positive for AR we were able to detect AR positive CETCs in all patients. Reyes et al. [18] observed AR positive circulating tumor cells in 100% of metastatic castration-resistant prostate cancers. Data regarding clinicopathologic parameters of tumor in relation to AR status are inconsistent. Some studies report the observation that there is no correlation between AR expression and lymph node involvement and other conventional parameters [19, 20]. Ogawa et al. [21] in contrast reported that AR expression is a favorable biomarker related to lymph node metastasis. Patients with negative lymph nodes had a higher rate of AR positive tumors. Our results show that a high rate of CETCs with AR expression is a sign of more well-differentiated state with a decreased aggressiveness. Patients without lymph node involvement had a higher percentage of AR positive CETCs as compared to patients with lymph node involvement.

Definition of triple negative breast cancers (TNBC) is the absence of expression for ER and PR and absence of overexpression for HER2 by immunohistochemistry

(IHC). It is well known that TNBC patients have more frequently axillary lymph node involvement and a shorter overall survival. Due to the lack of a receptor target chemo- and radiotherapy represent the only option for the treatment of TNBC [22]. In the literature the frequency of AR expression in TNBC is very variable ranging from 7 to 75% [22]. The prognostic value of AR expression in TNBC is not well understood and still controversial. On the one hand studies showed that patients with positive AR expression have poor overall survival, however, on the other hand other studies documented the opposite conclusion [23]. To the best of our knowledge we are the first who report about expression of AR on CETCs in TNBC. We found that TNBC patients had a higher percentage of AR positive CETCs as compared to patients with a hormone receptor positive primary tumor. Therefore, AR targeted therapy might become a new option in TNBC therapy. Furthermore, monitoring AR positive CETCs during androgen blockade could reflect the efficacy of therapy.

Our results in hormone receptor positive breast cancer are in agreement with the results from xenografts from cell lines reported by D'Amato et al. [24]. Statistically significantly more AR positive CETCs were detected in TNBC as compared to ER positive CETCs. Thus, AR overall is more frequently expressed in CETCs than ER or PR, however, the role of AR is complex, dependent on the hormonal milieu and remains controversial [24]. There are three types of resistance to SERM therapy as described by Fan and Jordan: metabolic resistance, de novo resistance and acquired resistance [25, 26]. More recently it has been demonstrated that another mechanism of resistance to anti-ER therapies may be the adaptation of the tumor from estrogen to androgen dependence [7, 24]. In hormone receptor positive breast cancer AR is frequently expressed in tumor tissue and related to prognosis. It is expected to be used as a predictive marker for hormone therapy and a potential therapeutic target for breast cancer [7]. Resistant cell populations seem to be in permanent evolution depending on selection pressure that enhances survival of new clones [27], this is corroborated by our results showing that patients with tamoxifen therapy had a higher AR:ER ratio as compared to patients without endocrine therapy whereas treatment with aromatase inhibitor had no influence on AR:ER ratio. In vitro studies have demonstrated that tamoxifen-therapy resistant tumors express higher level of AR and lower ER levels than tamoxifen-therapy sensitive tumors [28, 29]. Rangel et al. [30] reported a high AR:ER ratio to be associated with aggressive biological features and worse prognosis. Furthermore, Cochrane and coworkers demonstrated that AR:ER ≥ 2 was associated with an increased risk of tamoxifen failure in breast cancer [6]. If tumors with a high ratio of AR:ER expressing circulating tumor cells turn out to be resistant to tamoxifen therapy and this raises the question whether a blockade of AR may be an effective and new target to overcome tamoxifen resistance. Our study has some limitations. The study population is relative small and follow-up data for disease free and overall survival are missing. To address these limitations future studies need to include a larger cohort of patients and follow-up of patients.

Conclusion

In conclusion, with the maintrac platform we are able to identify CETCs in patients with breast cancer and to determine AR expression on those cells. Results from our study suggest that AR expression detected on CETC has capability to be a biomarker for identifying patients who might develop tamoxifen resistance. This needs to be clinically validated.

Authors' contributions

Conceptualization: KP, MP, DS and UP; Methodology: MP, DS and KP; Validation: MP, DS and DL; Investigation: DL; Writing-original draft preparation: MP and DS; Writing-review and editing, KP, UP; Visualization: MP and DS; Supervision: MP and DS; Project administration: MP and KP. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data are fully available without restriction.

Consent for publication

All authors have agreed to the submission of the manuscript.

Ethics approval and consent to participate

All subjects have provided written informed consent before participation in the study which has been approved by the Ethics Committee of the Bavarian Medical Association.

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6 Liste weiterer Publikationen im Rahmen der Dissertation

Annals of Oncology 27 (Supplement 6): vi526–vi544, 2016
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translational research

1545P The number of tumorspheres cultured from CETCs in breast cancer patients is directly related to stage of disease and administration of chemotherapy

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Background: Breast cancer is one of the leading causes of cancer death for women worldwide. Major hurdles for a successful treatment are cancer metastasis, resistance to therapy and disease recurrence. The presence of CETCs is closely related to metastasis formation, but the mechanisms through which CETCs promote recurrence of disease are still unclear. Therefore, the aim of this study was to determine the proliferative capacity of CETCs by analyzing the frequency of tumorsphere formation with subsequent phenotypic characterization of the spheres arising in breast cancer patients.

Methods: CETCs were cultured under conditions favoring growth of tumorspheres from 72 patients with breast cancer, including a subpopulation of 23 patients with metastatic disease. Cell viability, stem cell marker expression and ALDH 1 activity was evaluated by fluorescence scanning microscope (Olympus Scan^R).

Results: Sphere formation was observed in 79 % of patients with breast cancer. In the current study we found that the number of tumorspheres depended on stage of disease. Patients in stage IV had statistically significant more tumorspheres compared to patients in stage I (median 6 vs. 2; =0.002). The most important factor for growing of tumorspheres is obtaining chemotherapy. Patients with chemotherapy treatment had lower numbers of tumorspheres compared to patients without chemotherapy (median 2 vs. 5; =0.002). Interestingly, patients with HER2 positive primary tumor had higher number of tumorspheres with median 10. Analysis of surface marker expression profile of tumorspheres showed that spheres cultured from CETCs had typical phenotype of cancer stem cells with a high enzymatic activity for ALDH 1. There was no sphere formation in a control group with 50 healthy donors.

Conclusions: This study demonstrates that a small fraction of CETCs has proliferative activity. Identifying the CETC subset with cancer stem cell properties may provide more clinically useful prognostic information. Chemotherapy is the most important component in cancer therapy because it frequently reduces the number of tumorspheres.

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abstracts

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Frequency of PDL-1 expressed on circulating epithelial tumor cells (CETCs) compared to PDL-2 and its role in immune evasion in cancer progression

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Tagungsbeitrag: ASCO (*American Society of Clinical Oncology*) Annual Meeting 2016

Journal of Clinical Oncology 2016, 34, no. 15_suppl, 3071-3071;

DOI: 10.1200/JCO.2016.34.15_suppl.3071

Abstract

3071

Background: The current cancer research strongly focuses on the immune therapies, where the programmed death-1 receptor (PD-1), with its ligands PD-L1 and PD-L2 play an important role. It is known that PDL-1 is frequently upregulated in a number of different cancers and the relevance of this pathway has been extensively studied and therapeutic approaches targeting PD-1 and PD-L1 have been developed. However, PD-L2 has not received as much attention and its role in modulating tumor immunity is less clear. To identify potential patients who may benefit from PD-1/PD-L1/PD-L2 targeted immunotherapeutics, we used a non-invasive, real-time liquid biopsy for determining PD-L1 and PDL-2 expression in CETCs of breast cancer patients. **Methods:** CETCs were determined from blood of 66 patients suffering from breast cancer. The number of vital CETCs and the expression of PDL-1 and PDL-2 were investigated using the maintrac method. **Results:** PDL-1 expressing CETCs were detected in 95 % of breast cancer patients whereas only 75% patients had PDL-2 positive CETCs. There was no association between the frequency of PDL-1 positive CETCs and progression of cancer disease. We found that the fraction of PDL-1 positive CETCs is significantly higher than the fraction of PDL-2 positive CETCs (63.3% vs. 32.9%; p). Moreover, we observed a significant heterogeneity in PDL-1 and PDL-2 immunostaining intensity across CETCs from the same patients. **Conclusions:** Breast cancer patients have detectable CETCs with high frequency of PDL-1 regardless of stage of disease. PDL-1 seems to be a major factor in immune evasion and may be a promising target of anticancer therapies. Monitoring the frequency of PDL-1 positive CETCs could reflect individual patient's response for an anti-PD-1/PDL-1 therapy.

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1648P **B7-H3 (CD276) on circulating epithelial tumor cells (CETCs) correlates with proliferation marker Ki-67 and may be associated with aggressiveness of tumor in breast cancer patients**

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Background: CETCs in the peripheral blood are a prerequisite for the development of metastases. B7-H3 is an important immune checkpoint member of the B7 family and inhibits T-cell mediated anti-tumor immunity. It is highly overexpressed on a wide range of solid cancers and often correlates with both negative prognosis and poor clinical outcome in patients. Based on the clinical success of the inhibitory immune checkpoint blockade, mAbs against B7-H3 appear to be promising therapeutic strategy. In order to better understand the role of B7-H3 in cancer development we used a non-invasive, real-time biopsy for determining B7-H3 on CETCs in breast cancer patients.

Methods: Blood from 50 patients suffering from breast cancer were analyzed for CETCs. The number of vital CETCs and the expression of B7-H3 and Ki-67 were evaluated using the maintrac® method.

Results: CETCs were detected in all examined patients (ranged from 2-676 CETCs in 100 µl of blood). B7-H3 expression on the surface of CETCs was found in 82% of patients. Triple negative breast cancer patients had statistically significantly more B7-H3 positive CETCs than patients with hormone receptor positive tumor tissue (median 50 vs. 26.3; $p < 0.05$). The frequency of B7-H3 positive CETCs was significantly higher in patients who received radiation therapy compared to patients without irradiation (mean 42 vs. 29; $p < 0.05$). B7-H3 positive CETCs seem to be more aggressive because the percentage of B7-H3 positive CETCs correlated with the percentage of proliferation marker Ki-67 positive CETCs ($r = 0.689$ and $p < 0.001$). Interestingly, a significant relationship between Ki-67 expression level on the CETCs and nodal status was found.

Conclusions: Breast cancer patients have detectable CETCs with high frequency of B7-H3 expression regardless of stage of disease. B7-H3 seems to be an important factor in immune evasion and may be a promising target of anticancer therapies. Furthermore, radiation leads to an up-regulation of B7-H3 expression on CETCs, which could be a possible mechanism of acquired radio-resistance.

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Adjuvant radiation therapy to induce a transitory adaptive up-regulation of programmed death ligand 1 (PD-L1) on circulating epithelial tumor cells (CETCs) to affect the anti-tumor immune response in primary breast cancer patients

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Tagungsbeitrag: ASCO (*American Society of Clinical Oncology*) Annual Meeting 2017

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Abstract

11558

Background: Radiation therapy (RT) is an integral part of the treatment of breast carcinoma but unfortunately many patients experience local recurrence. During the inflammatory response that accompanies radiation tumor cells may develop multiple resistance mechanisms for example the up-regulation of PD-L1 on tumor cells which leads to immune evasion. Since CETCs arise from the tumor it is conceivable that under evolutionary pressure they might share some of the immune escape mechanism inherent to tumor cells. In this study we demonstrate that RT leads to a transitory adaptive up-regulation of PD-L1 expression on CETCs. **Methods:** CETCs and the expression of PD-L1 and Ki-67 were analyzed from 25 patients with primary non-metastatic breast cancer using the maintrac method. The fraction of PD-L1 and Ki-67 positive CETCs were assessed at baseline, 3 and 6 weeks after start of RT and 6 weeks after end of therapy. Additionally, copy number status of PD-L1 was determined using FISH. **Results:** Fractionated-dose RT leads to a significant increase in PD-L1 expression on CETCs with the highest expression level midterm of irradiation as compared to baseline (49% vs. 74%, $p < 0.01$). 6 weeks after end of RT the number of PD-L1 positive CETCs returned to baseline value. The up-regulation of PD-L1 was dose dependent. Patients who received higher total dose had significantly more PD-L1 positive CETCs as compared to patients treated with lower total dose midterm of RT (64% vs. 43, $p < 0.05$). Before start of therapy there was a correlation between the fraction of PD-L1 and Ki-67 positive CETCs ($r = 0.6$, $p < 0.01$). PD-L1 copy number gains were significantly associated with PD-L1 expression ($r = 0.6$, $P < 0.05$). **Conclusions:** RTleads to an up-regulation of PD-L1 expression on CETCs, which could be a possible mechanism of acquired radioresistance. Combining immunomodulatory agents with radiation might have the potential to overcome this resistance and could improve clinical outcome in breast cancer.

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Importance of androgen receptor (AR) to estrogen receptor ratio in breast cancer patients during endocrine therapy determined on circulating epithelial tumor cells (CETCs) in breast cancer patients

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Abstract

e24031

Background: The androgen receptor (AR) is expressed in the majority of breast cancers and across the three main breast cancer subtypes. Despite the high frequency of AR expression in breast cancer its role is complex, dependent on the hormonal milieu, and remains controversial. On one hand AR seems to be a good prognostic marker in ER^+tumors probably due to more differentiated state. On the other hand it is known that AR signaling and AR are involved in resistance to ER-directed endocrine therapies, in particular, the high AR:ER ratio. The aim of the current study was to investigate the frequency of AR and ER positive CETCs in breast cancer patients. **Methods:** The number of vital CETCs was determined from blood of 66 patients suffering from breast cancer and the expression of AR and ER on these cells were investigated using the maintrac method. **Results:** AR expressing CETCs were detected in 100% of breast cancer patients. Patients with advanced disease had statistically more CETCs as compared to patients in early stage of disease. The fraction of AR positive CETCs was significantly higher than the fraction of ER positive CETCs (90% vs. 50.0%; $p < 0.001$). Patients with lymph node metastasis had less AR positive CETCs as compared to patients with negative lymph node status. AR:ER ratio on CETCs was higher in patients during tamoxifen therapy compared to patients without endocrine therapy (2.2 vs 1.3, $p < 0.05$). Interestingly, treatment with aromatase inhibitor had no influence on AR:ER ratio. **Conclusions:** Breast cancer patients have detectable CETCs with a high frequency of AR regardless of stage of disease. AR to ER ratio may influence breast cancer response to endocrine therapy, especially with tamoxifen. It is crucial to identify biomarkers that would enable the selection of patients who might benefit from combination treatment with ER and AR inhibitors.

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Influence of radiotherapy on residual circulating epithelial tumor cells (CETCs) and their programmed cell death ligand 1 (PD-L1) status in breast cancer patients

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Abstract

Background: Preclinical studies have suggested that radiotherapy followed by immunotherapy could be a promising strategy for synergistic enhancement of treatment efficacy. Radiation induces immunogenic changes in cancer cells which can lead to adaptive up-regulation of PD-L1 expression. Moreover, radiotherapy reduces not only local recurrence but even more distant relapse indicating its influence also on the residual occult tumor such as circulating tumor cells. For this reason the aim of this study was to determine the PD-L1 status on CETCs before and during radiotherapy.

Methods: CETCs were determined in 54 primary breast cancer patients in early and locally advanced stages. The number of CETCs and their expression of PDL-L1 were investigated using the maintrac method. The fraction of PD-L1 positive CETCs was assessed prior to (baseline), 3 and 6 weeks after the start of radiotherapy.

Results: We found an association between the PD-L1 status on CETCs and aggressiveness of cancer disease before radiotherapy. Patients with positive lymph node status had more PD-L1 positive CETCs as compared to patients with negative lymph nodes (mean 73 vs. 50, $p<0.01$). HER2 expression of the primary tumor was also positively correlated with PD-L1 expression. Lower numbers of CETCs were detected in patients who had not received chemotherapy or adjuvant chemotherapy before radiation than in those who received neoadjuvant chemotherapy (median 20 vs 25 vs 120 CETC/100µl blood, $p<0.001$). Surprisingly, the fraction of PD-L1 positive CETC decreased during radiotherapy only in patients who had received neoadjuvant chemotherapy.

Conclusion: The number of CETCs and the fraction of PD-L1 positive CETCs were higher in patients who had received neoadjuvant chemotherapy and radiotherapy reduced both measured biomarkers. In the future PD-1/PD-L1 checkpoint inhibitors after radiotherapy might have additional benefit in breast cancer patients.

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8 Eidesstattliche Versicherungen und Erklärungen des Verfassers

(§ 8 Satz 2 Nr. 3 PromO Fakultät)

Hiermit versichere ich eidesstattlich, dass ich die Arbeit selbstständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe (vgl. Art. 64 Abs. 1 Satz 6 BayHSchG).

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Hiermit erkläre ich, dass ich die Dissertation nicht bereits zur Erlangung eines akademischen Grades eingereicht habe und dass ich nicht bereits diese oder eine gleichartige Doktorprüfung endgültig nicht bestanden habe.

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Ort und Datum Unterschrift (Dorothea Schott)