

**Vom Labor in die Klinik:
Molekulare Charakterisierung
zirkulierender Tumorzellen zur
Selektion zielgerichteter Therapien am
Beispiel des metastasierten
Mammakarzinoms**

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2 Einleitung

2.1 Krebs

2.1.1 Die Charakteristika von Krebs

Der Begriff Krebs bezeichnet die Fähigkeit von Zellen, abnormal zu wachsen und schließt das Potential ein, in andere Teile des Körpers einwandern oder streuen zu können. Hierin unterscheiden sich maligne von benignen Tumoren, die diese Fähigkeit nicht aufweisen. Beim Menschen können über 200 verschiedene Arten von Krebserkrankungen auftreten. Dabei wird je nach Art des betroffenen Gewebes zwischen Karzinomen, die von epithelialen Zellen abstammen, Sarkomen, die aus Bindegewebe entstehen, Lymphomen und Leukämien, die aus hämatopoetischen Zellen hervorgehen, sowie Keimzelltumoren und Blastomen unterschieden (1).

Im Jahr 2018 gab es weltweit 18,1 Millionen Krebs-Neuerkrankungen. Im selben Jahr sind weltweit ungefähr 9,6 Millionen Menschen an Krebs gestorben. Derzeit erkranken eine von sechs Frauen bzw. einer von fünf Männern im Laufe ihres Lebens an Krebs und eine von elf Frauen bzw. einer von acht Männern stirbt an Krebs (2).

Im Zuge der Krebsentstehung durchlaufen gesunde Zellen den Prozess der malignen Transformation und erwerben bestimmte Eigenschaften, Die von Hanahan und Weinberg als die „*Hallmarks of Cancer*“ postuliert wurden (3):

Tumorzellen müssen unabhängig von Wachstumssignalen sein, sodass sie auch in Abwesenheit von Wachstumsstimuli in Form diffusibler Wachstumsfaktoren, Komponenten der extrazellulären Matrix oder Zell-Zell-Adhäsions bzw. Interaktionsmolekülen proliferieren können. Dies wird zum Beispiel durch die Aktivierung von Onkogenen gewährleistet (3,4).

Tumorzellen müssen unabhängig von wachstumsinhibierenden Signalen sein. In normalen Zellen steuern diffusible oder immobilisierte Wachstumsinhibitoren die zelluläre Quieszenz und die Homöostase des Gewebes. Eine derartige Wachstumsinhibition kann zum Beispiel durch den Verlust von Tumorsuppressorgenen umgangen werden (3,5).

Tumorzellen müssen Apoptose verhindern. Normalerweise führt Apoptose in Folge eines physiologischen Signals zum programmierten Tod abnormaler Zellen und stellt damit eine Barriere zur Entstehung von Krebs dar. Apoptose kann zum Beispiel durch die Überexpression von Überlebensfaktoren verhindert werden (3,6).

Zusätzlich zu den vorherigen Charakteristika, die zum Entkoppeln des Zellwachstums von der Umgebung sorgen, wird ein unlimitedes replikatives Potential benötigt. Ein limitiertes replikatives Potential, das sogenannte Hayflick-Limit (7), kann unter anderem durch eine angeschaltete Telomerase-Aktivität umgangen werden (3,8).

Trotz unbeschränkter Proliferationsfähigkeit von Tumorzellen wird das Tumorwachstum durch die Versorgung mit Nährstoffen aus vorhandenen Blutgefäßen limitiert. Als fünftes Kennzeichen müssen Krebszellen demnach in der Lage sein, Angiogenese zu induzieren, was unter anderem durch die Hochregulation von VEGF¹ gewährleistet wird (3,9).

Das sechste erworbene Kennzeichen ist die Fähigkeit in das umliegende Gewebe einzuwandern und metastasieren zu können. Durch die Inaktivierung des Adhäsionsproteins E-Cadherin kann es Krebszellen beispielsweise gelingen, sich vom Tumor zu lösen und an entfernte Stellen des Körpers zu streuen (3,10).

Hinzu kommen die Deregulation des zellulären Energiestoffwechsels, was zum Beispiel in Form „aerober Glykolyse“ ein kontinuierliches Zellwachstum und eine kontinuierliche Proliferation ermöglicht (11), und die Fähigkeit, den Angriff von und die Eliminierung durch Immunzellen zu verhindern (12).

Diesen Charakteristika von Krebs liegen zwei „*Enabling Characteristics*“ zu Grunde: das Auftreten von genomischer Instabilität oder von Mutationen und tumorfördernde Entzündungsreaktionen. Mutationen und Aberrationen entstehen durch Mutagene, den Zusammenbruch der genomischen Erhaltungsmaschinerie oder durch das Umgehen des Überwachungssystems, das normalerweise die genomische Integrität kontrolliert und dafür sorgt, dass genetisch geschädigte Zellen Seneszenz oder Apoptose durchlaufen (12–14). Entzündungsreaktionen tragen dazu bei, dass Faktoren zum Tumor gelangen, die Wachstum und Angiogenese begünstigen, den Zelltod verhindern und die Epithelial-Mesenchymale-Transition (EMT) induzieren (12,15).

2.1.2 Der Prozess der Metastasierung

Trotz Verbesserungen in der Krebsdiagnose und -therapie entwickeln viele Krebspatienten im Laufe der Zeit eine fortgeschrittene metastasierte Erkrankung, die weitestgehend nicht heilbar

¹ Der Übersichtlichkeit halber wurden zur Bezeichnung von Proteinen die gängigen Abkürzungen verwendet. Der vollständige Name der erwähnten Proteine ist im Abkürzungsverzeichnis aufgeführt.

und für die Mehrheit (ca. 90%) der Krebs-assoziierten Todesfälle verantwortlich ist (16). Bei der Metastasierung handelt es sich um einen mehrstufigen Prozess, der auch als Metastasierungskaskade bezeichnet wird. Dabei kommt es zunächst zur lokalen Invasion von Zellen des Tumors in das umgebende Gewebe. Anschließend intravadiert diese Zellen in das zirkulatorische System, um schließlich an einer entfernten Stelle durch die Wand der Blutgefäße zu extravadiert und in das Parenchym einzudringen. Dort können sie nun Kolonien und schließlich klinisch detektierbare Metastasen ausbilden (16–18).

Eine wichtige Rolle beim Metastasierungsprozess spielt die EMT. Dieser Begriff bezeichnet den teilweisen Verlust epithelialer Eigenschaften und den Erwerb bestimmter mesenchymaler Fähigkeiten, sodass die Zellen ein Spektrum intermediärer Stadien zwischen epitheliale und mesenchymalem Pol aufweisen (19–21). EMT kann durch parakrine Signale in Form von TGF- β , WNT, *Platelet-derived Growth Factor* oder Interleukin-6 sowie durch Nikotin, Alkohol und ultraviolettes Licht ausgelöst werden (22–24). Damit gehen eine gesteigerte Motilität, eine gesteigerte Invasivität und die Fähigkeit einher, Komponenten der extrazellulären Matrix zu degradieren (25–27). Gleichzeitig wird dadurch die Fähigkeit zur Tumorinitiation, zur Tumorprogression und zur Metastasierung gesteigert (28–30). Karzinomzellen eher mesenchymalen Charakters zeigen außerdem eine gesteigerte Resistenz gegenüber Radio- und Chemotherapien (31,32). Eine Invasion kann aber auch EMT-unabhängig wie zum Beispiel durch die Amplifikation der Zentrosomen induziert werden (33,34).

Die Einwanderung von Krebszellen in das tumorumgebende Gewebe kann entweder in Form einzelner Zellen oder in Form einer großen zusammenhängenden Kohorte von Zellen als kollektive Migration erfolgen (35). Diese Tumorzellen können anschließend entweder direkt oder über lymphatische Gefäße ins Blutgefäßsystem intravadiert (36) und als zirkulierende Tumorzellen (CTCs), die oft eine Kombination epithelialer und mesenchymaler Marker aufweisen (37), entweder als einzelne Zellen oder als multizelluläre Cluster (38,39) entfernte Stellen des Körpers erreichen. Die meisten dieser CTCs überleben nur wenige Sekunden oder Minuten im Blutgefäßsystem, sodass die Wahrscheinlichkeit für eine einzelne CTC, eine Metastase zu gründen, extrem gering ist (40). Als CTCs haben die Tumorzellen mit diversen Herausforderungen wie dem Verlust der Zell-Matrix-Interaktion, was normalerweise Apoptose auslösen würde und als Anoikis bezeichnet wird, dem hydrodynamischen Fluss und Scherkräften zu kämpfen und sind außerdem Angriffen des Immunsystems ausgeliefert (41).

Unter anderem um diesen Herausforderungen zu entgehen, interagieren CTCs mit anderen Zellen des Zirkulationssystems wie Thrombozyten, Neutrophilen, Monozyten oder endothelialen Zellen. So können anhaftende Thrombozyten Tumorzellen vor der Erkennung und Lyse durch NK-Zellen schützen (42–44). Auch die Interaktion mit Neutrophilen, die die zytotoxische CD8⁺ T-Zell-Antwort und die Beseitigung durch NK-Zellen inhibieren können, hat eine immunsuppressive Funktion (45,46). Außerdem können Thrombozyten, Neutrophile und Monozyten die Fähigkeit zur Metastasierung positiv beeinflussen: Thrombozyten induzieren oder stabilisieren das EMT-Programm (47); Neutrophile tragen zum Überleben der CTCs im Gefäßsystem, zur Adhärenz an Endothelzellen und zur Extravasation bei (48); und auch Monozyten, die zu metastasenassoziierten Makrophagen differenzieren können, haben auf die Extravasation und das anschließende Wachstum der Metastase einen positiven Effekt (49,50). Die Extravasation bezeichnet dabei den Prozess, bei dem die Tumorzellen die Endothelwand durchdringen, was als transendotheliale Migration bezeichnet wird (51),

Der Ort der metastatischen Kolonialisierung wird zu einem gewissen Teil von der Anordnung des Blutgefäßsystems bestimmt. Der Großteil des organspezifischen Tropismus geht aber auf gewebespezifische Adaption zurück (52). So lässt sich zum Beispiel der Knochen-Tropismus von Brustkrebszellen auf eine Reihe von Molekülen wie PTHrP, IL-11, und MMPs zurückführen, die die Stimulation der osteoklastischen Aktivität über RANKL begünstigen. Dies führt zur Freisetzung von Wachstumsfaktoren der Knochenmatrix und steigert dadurch die Tumorzellproliferation (53). Auch nach der Extravasation werden die meisten Zellen entweder vom Parenchym des Gewebes eliminiert oder erlangen zunächst einen dormanten Zustand, in dem sie als einzelne disseminierte Tumorzelle oder als Mikrometastase bis zu mehrere Jahren verweilen können (54). Die Gründung einer metastatischen Kolonie geht dabei vermutlich von einer sogenannten Tumorstammzelle aus, die tumor- bzw. metastaseninitiierende Eigenschaften besitzt (29,30,40).

2.2 Brustkrebs

2.2.1 Allgemeines über das Mammakarzinom

Brustkrebs, auch als Mammakarzinom (MK) bezeichnet, entwickelt sich aus Gewebe der Brust und ist die zweithäufigste Krebserkrankung und die fünfhäufigste Krebstodesursache. Bei Frauen ist Brustkrebs mit 24,2% sowohl die am häufigsten diagnostizierte Krebserkrankung als auch mit 15,0% die häufigste Krebstodesursache, obwohl durch eine

frühzeitige Erkennung und eine bessere Behandlung die Sterberate bei Brustkrebs in den vergangenen Jahren kontinuierlich gesunken ist (2).

Die weibliche Brust besteht aus Drüsenläppchen, den *Lobuli glandulae mammae*, die ein Lumen aufweisen, um das Milchbildungszellen und Myoepithelzellen angeordnet sind. Dieses Lumen mündet in den *Ductus lactifer*. Mehrere Lobuli bilden jeweils einen *Lobus glandulae mammae*. Jede Brustdrüse besteht aus zehn bis 20 Lobi, deren Milchgänge, die *Ducti lactifer colligens*, in die Brustwarze münden. Hinzu kommen umgebendes Fett- und Bindegewebe sowie Blut- und lymphatische Gefäße (55).

Ein MK geht dabei vor allem aus Zellen des Glandular-Gewebes hervor. Dabei wird zwischen lobulären, ductalen, medullären, muzinösen, adenoid zystischen, inflammatorischen und tubulären Karzinomen (56) unterschieden. MKs, die aus Zellen der Ducti hervorgehen und auch als nicht-spezifischer Typ bezeichnet werden, stellen mit 75% die häufigste MK-Form dar; gefolgt von lobulären MKs mit 10%, die aus Zellen der Lobuli entstehen (57).

Weiterhin können MKs anhand des Vorhandenseins verschiedener Rezeptoren in unterschiedliche molekulare Subtypen unterteilt werden. Es wird zwischen Luminal A, Luminal B, HER2/neu (HER2)-positiv und basalartig unterschieden (58). Karzinome vom luminalen Subtyp exprimieren den Östrogen- und/oder den Progesteronrezeptor (ER bzw. PR). Dabei zeigen Karzinome vom Subtyp Luminal A einen geringen Anteil Ki67-positiver Zellen, während bei Karzinomen vom Subtyp Luminal B ein hoher Anteil der Zellen Ki67-positiv ist. Hormonrezeptor-positive HER2-negative Karzinome werden mit einer endokrinen Therapie (ET) behandelt. Bei hohem Risiko wird zuvor eine Chemotherapie durchgeführt. Zum Subtyp der HER2-positiven MKs werden solche gezählt, die eine starke HER2-Überexpression oder bei moderater Überexpression eine Amplifikation des *ERBB2*-Gens aufweisen. HER2-positive Karzinome werden mit gegen HER2 gerichteten Antikörpern und – außer bei sehr kleinen Tumoren – mit einer Chemotherapie behandelt. Karzinome des basalartigen Typs exprimieren weder Hormonrezeptoren noch HER2 und werden deshalb auch als triple-negativ bezeichnet. Sie werden in der Regel mit einer Chemotherapie behandelt. Weitere Therapiemöglichkeiten ergeben sich beim MK durch neuartige Wirkstoffe wie zum Beispiel Checkpoint-, Angiogenese- oder PARP-Inhibitoren (59,60).

2.2.2 Die endokrine Therapie

Ungefähr 70% aller MKs exprimieren den ER (61). Bei einem ER-positiven metastasierten MK ist die ET die empfohlene Erstlinientherapie, sofern keine viszerale Krise vorliegt. Die ET kann dabei zum einen mit sogenannten selektiven ER-Modulatoren (SERMs) oder -Degradierern (SERDs) erfolgen, die auf den ER-Signalweg zielen. Zum anderen kann eine östrogendeprivierende Therapie (EDT) in Form von Aromataseinhibitoren (AIs) oder *Gonadotropin Releasing-Hormon* Analoga (GnRH-As) verwendet werden. AIs inhibieren das Enzym Aromatase, das den Aromatisierungsschritt bei der Synthese von Östrogen katalysiert; GnRH-As reduzieren den Östrogenspiegel im Blut (59,60).

Obwohl nahezu alle Patientinnen mit einem ER-positiven metastasierten MK zunächst gut auf eine gegen den ER-Signalweg gerichtete Therapie ansprechen, entwickeln letztendlich ebenso fast alle eine Resistenz, was schließlich zum Fortschreiten der Erkrankung führt. Mögliche Resistenzmechanismen können eine veränderte ER-Expression, Amplifikationen oder Translokationen des *ESR1*-Gens, eine veränderte Expression von Wachstumsfaktoren, die Aktivierung des PI3K/AKT1/mTOR Signalwegs, eine veränderte Expression von Zellzyklusregulierern, die Überexpression von ER-Koaktivatoren, hochregulierte Autophagie, EMT und die Heterogenität des Tumors sein (62). Außerdem kann es zu Mutationen im *ESR1*-Gen kommen, die insbesondere in dem Bereich zu beobachten sind, der für die Liganden-Bindedomäne kodiert (63,64).

Das *ESR1*-Gen kodiert für den Östrogenrezeptor α (ER α), einen Liganden-abhängigen Transkriptionsfaktor für Gene, die mit dem Zell-Überleben, der Proliferation und dem Tumorwachstum assoziiert sind (65). Das ER α -Protein besteht aus einer N-terminalen Domäne (NTD), einer DNA-Bindedomäne und einer Liganden-Bindedomäne (LBD). Hinzu kommen zwei Aktivierungsdomänen, AF1 und AF2, die Teil der NTD bzw. der LBD sind. Die Aktivierungsdomänen sind an der Rekrutierung von Koaktivatoren und Korepressoren für bestimmte Zielgene beteiligt (66). Die Aktivierungsdomäne AF2 befindet sich am Ende der LBD und besteht aus vier flexiblen Helices, deren Konformation vom Liganden abhängt. Entscheidend für die östrogenabhängige Aktivität von AF2 ist dabei die Helix 12. Sobald die LBD Östrogen gebunden hat, wird die Bindestelle für ER α -Koaktivatoren frei gegeben (67). Die Substitution der Aminosäuren L536, Y537 und D538 führen zu einer Konformationsänderung, wodurch die Helix 12 in die Agonistenposition gelangt. Dies hat eine konstitutive Aktivität trotz Abwesenheit des Liganden zur Folge (68,69). Auch die Aminosäure E380 befindet sich in räumlicher Nähe des Carboxy-Endes von Helix 12 und

induziert die Abstoßung von Helix 5 und Helix 12. Wird diese Aminosäure gegen ein Glutamin getauscht, entfällt diese Abstoßung und Helix 12 kann in die Agonistenposition gelangen, was wiederum zur konstitutiven Aktivität des Proteins führt (70).

ESR1 Mutationen treten in lediglich 0,5% der noch unbehandelten primären MKs auf, wohingegen 14% bis 54% der MK-Metastasen bei vorheriger Behandlung mit einer ET, insbesondere mit AIs, derartige Mutationen aufweisen. Dies deutet darauf hin, dass diese Mutationen durch klonale Selektion sehr seltener resistenter Klone entstehen oder unter dem Selektionsdruck einer ET im Laufe der Erkrankung erworben werden (71,72). In diversen Studien wurde untersucht, ob sich *ESR1*-Mutationen als prognostische und prädiktive Biomarker eignen. In der BOLERO-2-Studie (NCT00863655) wurden die beiden *ESR1* Mutationen Y537S und D538G in der zirkulierenden Tumor-DNA (ctDNA) analysiert. Die Detektion dieser Mutationen war dabei mit einem kürzeren Gesamtüberleben assoziiert (73). Basierend auf Proben der SoFEA (NCT00253422)- und EFECT (NCT00065325)-Studien wurde retrospektiv untersucht, ob sich die Detektion von *ESR1*-Mutationen als Kriterium für eine Therapieentscheidung eignen könnte: Die Detektion von *ESR1*-Mutationen war dabei mit einem kürzeren progressionsfreien Überleben bei Behandlung mit dem AI Exemestan verglichen zum SERD Fulvestrant assoziiert. Bei Patientinnen ohne *ESR1*-Mutationen war kein signifikanter Unterschied zwischen dem Exemestan- und dem Fulvestrant-Arm zu beobachten (74,75). Patientinnen mit Mutationen in der LBD des *ESR1*-Gens könnten von höheren Dosen oder potenteren SERMs und SERDs profitieren (71). Außerdem könnten *Small Molecules* wie AZD9496, ein potenter und selektiver Antagonist von ER α , der D538G und Y537C/N/S mutiertes ER α *in vitro* und im *Xenograft*-Modell herunterreguliert (76), oder mTOR-, PI3K- und HSP90-Inhibitoren (77) zur Therapie genutzt werden. Weiterhin könnten ER-Koaktivatoren als mögliche Ziele einer Therapie gegen Tumorzellen in Frage kommen, die von mutiertem ER α abhängig sind (78).

2.3 Der Begriff *Liquid Biopsy*

Im Laufe der letzten Jahre und Jahrzehnte hat sich die zielgerichtete Therapie zu dem bevorzugten Weg in der Krebstherapie entwickelt. Allerdings sind die dazu benötigten Biopsien der Metastasen invasiv, teuer, mit möglichen Komplikationen verbunden, teilweise schlecht wiederholbar und häufig nicht durchführbar, weil sich der klinische Allgemeinzustand des Patienten verschlechtert hat oder der Tumor nicht zugänglich ist (79).

Außerdem spiegelt das aus einer Biopsie gewonnene genomische Profil häufig nur ein limitiertes Bild des Tumors wieder, das auf einen bestimmten Zeitpunkt beschränkt ist und nicht die Heterogenität der verschiedenen Subklone des Tumors darstellen kann (80). Solche Einschränkungen des prädiktiven Nutzens sind besonders relevant, wenn sich das genetische Profil des Tumors in Folge einer erworbenen Resistenz durch den von unterschiedlichen Therapien ausgeübten Selektionsdruck dynamisch ändert, der das Wachstum bestimmter Subklone begünstigt (81).

Eine Möglichkeit, die mit einer herkömmlichen Biopsie verbundenen Schwierigkeiten zu umgehen, liegt in der Durchführung sogenannter *Liquid Biopsies*, die als dynamisches diagnostisches Werkzeug genutzt werden könnten (82,83). Als mögliche Analyten kommen dabei CTCs, zellfreie DNA (cfDNA), die in Krebspatienten ctDNA enthält, zirkulierende zellfreie RNA, zirkulierende extrazelluläre Vesikel, Exosomen, *Tumor-Educated Platelets*, Proteine und Metabolite in Frage (84). Derartige Tumor-Biomarker-Tests, die in der Klinik Verwendung finden, sollten drei Kriterien erfüllen: Die Bestimmung von Analyten sollte akkurat, zuverlässig und reproduzierbar sein, was als analytische Validität bezeichnet wird; ein solcher Test sollte in der Lage sein, eine Population in zwei Gruppen mit signifikant unterschiedlichem klinischem Resultat zu teilen, was als klinische Validität bezeichnet wird; und Patienten, die den Test erhalten, sollten ein besseres Behandlungsergebnis erreichen, was als klinischer Nutzen bezeichnet wird (85). Dabei ist allerdings zu beachten, dass auch Biomarker-Tests, die von der *U. S. Food and Drug Administration* (FDA) anerkannt wurden, nicht notwendigerweise einen klinischen Nutzen aufweisen müssen, während auf der anderen Seite Biomarker-Tests existieren, die nicht bei der FDA eingereicht wurden und als *Laboratory-Developed Test* vermarktet werden (84). Im Bereich *Liquid Biopsy* sind das CellSearch-System zur Detektion von CTCs bei Patienten mit metastasiertem Mamma-, Prostata- und Kolorektalkarzinom (86), der Cobas EGFR Mutations Test v2 zur Detektion von *EGFR*-Mutationen in der cfDNA von Patienten mit Lungenkarzinom (87), der Epi proColon Test zur Bestimmung des Methylierungsstatus des *SEPT9*-Promotors in der cfDNA beim *Screening* für das kolorektale Karzinom (88) und das Therascreen PIK3CA RGQ PCR Kit zur Detektion von *PIK3CA*-Mutationen in Gewebe und *Liquid Biopsies* von Patientinnen mit fortgeschrittenem oder metastasiertem MK (89) von der FDA anerkannt. Für die drei letzteren konnte der klinische Nutzen bereits gezeigt werden (84).

2.4 Zirkulierende Tumorzellen

2.4.1 Die Anreicherung zirkulierender Tumorzellen

CTCs wurden zuerst von Thomas Ashworth im Jahre 1869 beschrieben, der während einer Autopsie dem Tumor ähnliche Zellen im Blut an entfernten Stellen im Körper gefunden hat (90). CTCs sind extrem selten, so dass meist selbst in metastasierten Patienten nur wenige CTCs vor einem Hintergrund von Milliarden Blutzellen bzw. Millionen von mononukleären Zellen des Blutes (MNCs) (91) vorkommen. Daher wird die technisch extrem anspruchsvolle Detektion von CTCs häufig mit dem Bild der Suche nach der Nadel im Heuhaufen verglichen. In Patienten ohne detektierbare Metastasen sind CTCs sogar noch deutlich seltener (92,93).

Die Detektion von CTCs ist ein zweistufiger Prozess, der aus Anreicherung und Identifikation besteht.

Bei der Anreicherung von CTCs können grundsätzlich drei Strategien verfolgt werden: CTCs können anhand der Expression bestimmter Proteine auf der Zelloberfläche, anhand physikalischer Eigenschaften oder anhand ihrer Funktionalität angereichert werden. Dabei ist die erste Strategie oberflächenmarkerabhängig, während die anderen beiden als oberflächenmarkerunabhängig bezeichnet werden (94).

Die Anreicherung anhand der Expression bestimmter Proteine kann durch positive oder negative Selektion erfolgen. Bei der negativen Selektion werden bevorzugt CD45-positive Leukozyten depletiert. Die meisten Technologien, die auf der positiven Selektion beruhen, verwenden Antikörper, die gegen das epitheliale Zelladhäsionsmolekül (EpCAM) gerichtet sind. Am häufigsten wird dabei das CellSearch-System verwendet, das die einzige durch die FDA anerkannte Technologie zur Anreicherung von CTCs darstellt und als Goldstandard bezeichnet werden kann. Das CellSearch-System verwendet gegen EpCAM gerichtete Antikörper, die an Ferrofluid-Partikel gekoppelt sind und an epitheliale Zellen in einem Standardvolumen von 7,5 ml Blut binden. Anschließend werden die gebundenen Zellen mit

Tabelle 1: Technologien zur Anreicherung von CTCs

Die Tabelle zeigt eine Übersicht über die wichtigsten kommerziell erhältlichen Technologien zur Anreicherung von CTCs.

Strategie	Prinzip	Technologie	Hersteller	Art der Anreicherung	inkludierte Identifikation
Oberflächen-marker	Negative Depletion	CD45-Beads	Thermo Fisher	Immunomagnetische Depletion von CD45-positiven Zellen (95)	ICF
		RosetteSep	Stemcell Technologies	Vernetzung von Erythrozyten mit ungewünschten Zellen (40)	
		AdnaTest	Qiagen	Immunomagnetische Anreicherung von z.B. Muc1-, HER2- und EpCAM-positiven Zellen (96)	
Positive Selektion		MACS	Miltenyi	Immunomagnetische Anreicherung (97)	Immunzyto-fluoreszenz (ICF)
		CellSearch	Menarini	Immunomagnetische Anreicherung (98)	
		Isoflux	Fluxion Biosciences	Immunomagnetische Anreicherung von z.B. EpCAM und EGFR positive Zellen (99)	
		CellCollector	Gilupi	In vivo Anreicherung mittels eines Antikörper-beschichteten Drahtes (100)	

physikalische Eigenschaften	Größe und Steifigkeit	ScreenCell	ScreenCell	Filter (Porengröße: $7,5 \pm 0,36 \mu\text{m}$ für fixierte Zellen; $6,5 \pm 0,33 \mu\text{m}$ für lebende Zellen) (101)
		ISET	Rarecells Diagnostics	Filter (Porengröße: $8 \mu\text{m}$) (102)
		MetaCell	MetaCell	Filter (Porengröße: $8 \mu\text{m}$) (103)
		Parsortix	Angle	dreidimensionaler Mikrofilter (Abstand für Anreicherung: $6,5 \mu\text{m}$) (104)
Hydro-dynamik		ClearCell	Biolidics	hydrodynamische Trennung von Blutbestandteilen (105)
		Vita-Assay	Vitatex	Anreicherung mittels Matrixproteinen, die von invasiven CTCs gebunden werden (106)
Funktionalität				ICF

Hilfe eines Magneten zurück gehalten (98,107). Auch zahlreiche weitere Technologien wie zum Beispiel der AdnaTest oder der IsoFlux arbeiten mit EpCAM-spezifischen Antikörpern, die in diesen beiden Fällen gebunden an magnetische *Beads* verwendet werden (96,99). Dabei kann eine Kombination verschiedener Antikörper gegen unterschiedliche Epitope die Wiederfindungsrate gegebenenfalls erhöhen (108). Alternativ zu magnetischen Partikeln oder magnetischen *Beads* können EpCAM-spezifische Antikörper auch an die Oberflächen mikrofluidischer Chips (CTC- oder Herringbone-Chip) oder an einen *in vivo* angewendeten Draht (CellCollector) gekoppelt werden (100,109,110). Eine Schwierigkeit bei der oberflächenmarkerabhängigen Anreicherung von CTCs anhand von Antikörpern, die gegen epitheliale Marker gerichtet sind, stellt allerdings die EMT dar, da während dieses Prozesses die Expression dieser Proteine einschließlich derer von EpCAM herunter reguliert wird (111–113). Außerdem gibt es einige Krebstypen, die EpCAM nur niedrig oder gar nicht exprimieren (114).

Die Anreicherung anhand physikalischer Eigenschaften basiert auf dem größeren Durchmesser und der geringeren Deformierbarkeit von Tumorzellen im Vergleich zu den umgebenden Blutzellen. Erythrozyten haben eine Größe von 7,0 bis 8,5 μm , Granulozyten eine Größe von 7,3 bis 13,2 μm , Lymphozyten eine Größe von 5,2 bis 10,1 μm und Monozyten sind 9,7 bis 10,5 μm groß (115). Die durchschnittliche Größe von MK-CTCs liegt bei 13,1 μm , die von Prostatakarzinom-CTCs bei 10,7 μm und die von CTCs des kolorektalen Karzinoms (CRC) bei 11,0 μm (116). Unterschiede in der Deformierbarkeit können die größenbasierte Anreicherung beeinflussen, da Zellen sich verformen können und somit auch durch Poren passen, die deutlich kleiner sind als sie selbst. Bei Neutrophilen wurde mittels Rasterkraftmikroskopie eine Steifigkeit von unter 200 Pa bestimmt, während die Steifigkeit von Krebszellen im Bereich von 200 bis 2000 Pa liegt (115). Einfache Systeme zur CTC-Anreicherung anhand physikalischer Eigenschaften basieren lediglich auf einer Membran (ISET- oder ScreenCell-System) (101,102). Bei komplexeren Systemen wie dem Parsortix-System handelt es sich um mikrofluidische Chips oder Kassetten (104,117). Zusätzlich zur filtrationsbasierten Anreicherung ist es möglich, größere CTCs von kleineren Blutzellen mittels hydrodynamischer Isolation zu trennen (ClearCell) (105). Oberflächenmarkerbasierte und auf physikalischen Eigenschaften beruhende Anreicherungen können auch kombiniert werden: Beim CTC-iChip werden zunächst hydrodynamisch Erythrozyten von MNCs getrennt und anschließend die CTCs antikörperbasiert mittels positiver und negativer Selektion angereichert (118).

Die dritte Strategie zur Anreicherung von CTCs basiert auf deren Funktionalität. Bei der CAM (*Cell Adhesion Matrix*)-Methode (Vita Assay) werden Blutproben zum Beispiel auf eine mit einer Matrix beschichtete Oberfläche gegeben. Die im Blut enthaltenen CTCs binden nun an die Matrixproteine, wobei die gleichen Mechanismen genutzt werden, die es Tumorzellen ermöglichen, in umgebendes Gewebe einzuwandern (106).

2.4.2 Die Identifikation zirkulierender Tumorzellen

Die Identifikation von CTCs aus der Gesamtheit der angereicherten Zellen erfolgt in der Regel anhand einer morphologischen Untersuchung und einer Immunzytofluoreszenzfärbung. Im CellSearch werden Zellen als CTCs klassifiziert, die mindestens 4 µm groß sind, positiv für Zytokeratin sind (gefärbt wird für die Zytokeratine 4, 5, 6, 8, 10, 13, 18 und 19) und einen Nukleus aufweisen, der mittels 4',6-Diamidin-2-phenylindole-Färbung detektiert wird und mindestens zu 50% von Zytokeratin umschlossen sein muss. Außerdem dürfen die Zellen nicht positiv für die als Ausschlusskriterium verwendete CD45-Färbung sein (98,107).

Alternativ kann die Identifikation auf mRNA-Ebene mit Hilfe tumorassoziierter Transkripte wie *EPCAM*, *MUC1*, *ERBB2* oder *KRT19*, sowie EMT-assoziiierter Marker oder durch Stammzellmarker erfolgen (113,119). Problematisch ist allerdings, dass dabei zum einen nur eingeschränkt quantitative Aussagen getroffen werden können und es zum andern essentiell ist, präzise *Cut-Off*-Level zu definieren, da tumorassoziierte Transkripte wie *KRT-19* teilweise auch in gesunden Individuen gefunden werden können (120).

Eine dritte Möglichkeit der Identifikation von CTCs besteht darin, sekretierte Proteine zu analysieren. Beim Epispot-Assay werden angereicherte CTCs für kurze Zeit auf einer Membran kultiviert, die mittels Antikörpern sekretierte CTC-spezifische Proteine detektiert, die anschließend durch weitere fluorophormarkierte Antikörper sichtbar gemacht werden können (121).

2.4.3 Die diagnostische Leukapherese

Trotz hoch entwickelter Anreicherungs- und Identifikationsverfahren stellt die Detektion von CTCs auf Grund ihrer Seltenheit eine große Herausforderung dar – insbesondere dann, wenn das analysierte Blutvolumen zu gering ist. Tibbe et al. haben berechnet, dass bei einer Detektionsrate von 85% auf Grund der Poisson-Statistik nur zu einer Wahrscheinlichkeit von

34% wenigstens eine CTC in dem standardmäßig analysierten Blutvolumen von 7,5 ml zu finden ist, falls 1000 Tumorzellen, die die gesuchten Marker aufweisen, in einem Gesamtblutvolumen von 5 l vorhanden sind (122). Geht man nun davon aus, dass in der nicht-metastasierten Situation die CTC Zahl nur 9 ± 6 pro l beträgt, bedeutet dies, dass in der Mehrzahl der Patienten ein Blutvolumen von mindestens 0,75 bis 7,5 l analysiert werden müsste, um mindestens zehn CTCs zu detektieren (123).

Eine mögliche Lösung für diese Einschränkung stellt die diagnostische Leukapherese (DLA) dar. Bei einer Leukapherese werden PBMCs mit einer Dichte von 1,055 bis 1,08 g/ml aus Vollblut getrennt und angereichert – und mit ihnen auch epitheliale Zellen, deren Dichte der von PBMCs ähnelt (124). In zahlreichen Studien, bei denen LAs zur Ernte von Stammzellen der Hämatopoese zur autologen Transplantation vor einer hoch dosierten Chemotherapie durchgeführt wurden, konnten Krebszellen im Leukapheresat nachgewiesen werden (125). Dabei war der Nachweis von Krebszellen im Leukapheresat mit einer höheren jährlichen Rezidivrate assoziiert (126), während die Anreicherung von CD34-positiven hämatopoetischen Stammzellen vor der Transplantation zu einem signifikant verlängerten Überleben führte (127). Dies deutet darauf hin, dass die nachgewiesenen Krebszellen nach der Re-Infusion beim Fortschreiten der Erkrankung beteiligt waren (126).

Bei der DLA wird im Vergleich zu einer LA ein verkürztes Protokoll angewandt, sodass innerhalb von 60 bis 70 Minuten MNCs aus ungefähr zwei bis drei Litern Blut angereichert werden. Außerdem wird zuvor keine Stammzellmobilisierung durchgeführt (128). Während der DLA wird das Blut über einen peripheren Venenzugang entnommen, mit Antikoagulantien versetzt und zentrifugiert. Dabei werden die MNCs von Erythrozyten und dem Plasma getrennt und gesammelt. Die übrigen Blutbestandteile werden über einen Venenzugang im zweiten Arm wieder zurückgeführt (129). Am Ende einer DLA werden ungefähr 40 ml DLA-Produkt gewonnen, in dem ca. 20×10^8 MNCs enthalten sind. Aliquots von 2×10^8 Zellen können anschließend für die CTC Analyse mittels CellSearch® verwendet werden (128). Mittels DLA lässt sich die Anzahl der erhaltenen CTCs bei metastasierten MK-Patientinnen um das über 200-fache im Vergleich zur Analyse von 7,5 ml Blut steigern. In der primären Situation konnte außerdem die Anzahl der CTC-positiven Patienten von 15% bei der Analyse von 7,5 ml Blut auf 55% bei der Analyse von 2×10^8 MNCs des DLA-Produktes gesteigert werden (130).

2.4.4 Der prognostische Nutzen von CTCs

Der prognostische Wert der CTC-Bestimmung und Zählung ist durch zahlreiche Studien belegt. Ein hoher Wert an detektierten CTCs ist mit einem reduzierten progressionsfreien Überleben und einem reduzierten Gesamtüberleben beim metastasiertem MK (131), metastasiertem CRC (132), metastasiertem kastrationsresistentem Prostatakarzinom (CRPC) (133), metastasiertem nichtkleinzelligen Lungenkarzinom (NSCLC) (134) und metastasiertem kleinzelligen Lungenkarzinom (SCLC) (135) assoziiert. Beim MK liegt der *Cutoff* bei fünf CTCs pro 7,5 ml analysiertem Blut, die mittels CellSearch Systems detektiert wurden. Die Bestimmung der CTCs wurde jeweils vor Beginn einer neuen Therapie durchgeführt (131).

Auch beim nicht-metastasiertem MK (136,137), lokal-fortgeschrittenem Pankreaskarzinom (138), nicht metastasiertem CRC (139) und nicht metastasiertem Lungenkarzinom ist die CTC-Zahl prognostisch relevant. In der adjuvanten Situation war bei MK-Patientinnen (Patienten mit kleinen nodal negativen Tumoren und geringem Risiko ausgenommen) bereits schon die Detektion einer einzelnen CTC mittels CellSearch-Analyse mit einer schlechteren Prognose verbunden und korrelierte mit einem reduzierten krankheitsfreien Überleben, einem reduzierten metastasenfreien Überleben, einem reduzierten Brustkrebs-spezifischen Überleben und einem reduzierten Gesamtüberleben (136). Auch bei MK-Patientinnen in der neoadjuvanten Situation haben bereits Patientinnen mit einer mittels CellSearch-System detektierten CTC ein verringertes Gesamtüberleben, ein reduziertes metastasenfreies Überleben und ein reduziertes lokalrezidivfreies Überleben. Die Detektion jeder weiteren CTC führte zu einer weiteren Verschlechterung der Prognose. Es konnte allerdings keine Korrelation zwischen der Detektion von CTCs und der pathologischen Komplettremission nachgewiesen werden (137).

Die Bestimmung der CTC-Zahl könnte außerdem zum Überwachen des Ansprechens auf eine Behandlung verwendet werden. Patientinnen mit hoher Ausgangs-CTC-Zahl, aber geringer CTC-Zahl nach einem Chemotherapie-Zyklus, haben eine signifikant bessere Prognose als solche Patientinnen, bei denen die Zahl der gefundenen CTCs konstant hoch bleibt, und haben sogar eine fast vergleichbare Prognose wie Patientinnen mit geringer Ausgangs-CTC Zahl (86). Auch beim metastasierten CRPC, metastasierten CRC und metastasierten SCLC konnte eine Korrelation zwischen der beobachteten Dynamik der CTC-Zahl und dem Ansprechen auf eine Therapie beobachtet werden (135,140–143).

2.4.5 Der prädiktive Nutzen von CTCs

CTCs wurden in zahlreichen Studien als diagnostische, prognostische und prädiktive Biomarker beim MK in Betracht gezogen. Dabei wurden neben der Bestimmung der CTC-Zahl zum Beispiel Analysen von Punktmutationen (144), *ERBB2*-Amplifikationen (145), *Copy Number Variations* (146), DNA-Methylierungen (147), sowie Untersuchungen auf Ebene des Transkriptoms (148) und des Proteoms (149) durchgeführt.

Derzeit wird in diversen klinischen Studien untersucht, inwieweit CTCs einen prädiktiven Nutzen aufweisen. Dabei können CTCs als Surrogat-Tumormaterial dienen – zum Beispiel zur Detektion der Androgenrezeptor (AR)-*Splicing*-Variante AR-V7, deren Detektion eine Resistenz gegenüber androgendeprivierender Therapie beim Prostatakarzinom voraus sagen könnte (150) –, es können CTC-Anzahl oder die Veränderung der CTC-Zahl während der Therapie betrachtet werden oder es können biologische Eigenschaften in Betracht gezogen werden, die spezifisch für CTCs oder die Metastasierung sind (151). Die Verwendung von CTCs als prädiktiver Biomarker ist insbesondere dann interessant, wenn bezüglich einer Eigenschaft eine Diskordanz zwischen Primärtumor und CTCs zu beobachten ist (152–155). Auf diesen Beobachtungen basierend wurde bzw. wird in einigen klinischen Studien die gegen HER2 gerichtete Therapie mit Lapatinib oder Trastuzumab bei Patientinnen mit HER2-negativem Primärtumor, aber HER2-positiven CTCs (156–158) bzw. CTCs mit Amplifikationen des *ERBB2*-Gens (159), getestet.

Inwieweit CTCs einen prädiktiven Nutzen aufweisen, kann derzeit noch nicht abschließend beurteilt werden. Einige klinische Studien zeigten allerdings nicht die erwarteten Ergebnisse und konnten somit aus unterschiedlichen Gründen einen solchen Nutzen nicht nachweisen (156,159–161).

Auch in der Phase III-Studie SWOG S0500 (NCT00382018) konnte kein prädiktiver Nutzen von CTCs gezeigt werden (162). In dieser klinischen Studie wurden in der Erstlinientherapie des metastasierten MKs Patientinnen mit mehr als fünf CTCs pro 7,5 ml Blut nach einem Chemotherapie-Zyklus, also solche mit schlechter Prognose, randomisiert und gegebenenfalls mit einer alternativen Chemotherapie behandelt. Allerdings profitierten die Patientinnen von diesem Therapiewechsel nicht. Möglicherweise ist das Scheitern der SWOG S0500-Studie darauf zurück zu führen, dass durch den Studienaufbau Patienten selektiert wurden, die generell resistent gegenüber einer weiteren Chemotherapie waren (162,163).

Tabelle 2: Auf CTCs basierende Phase III-Studien beim Mammakarzinom

Diese Tabelle zeigt eine Übersicht über abgeschlossene sowie derzeit laufende CTC-basierte Phase III-Studien beim Mammakarzinom (MK). Bezogen auf das gesamte CTC-Feld kommt die hier nicht dargestellte VISNU-1-Studie (NCT01640405) hinzu, die beim kolorektalen Karzinom ebenfalls auf der Anzahl der detektierten CTCs basiert.

Studie	basiert auf	Primary Objective	Ergebnis
SWOG500 (NCT00382018) (162)	CTC-Zahl (≥ 5), bestimmt mit CellSearch	Die Studie untersucht, ob metastasierte MK-Patientinnen von einem Wechsel der Chemotherapie bei persistierenden CTCs profitieren.	Negativ: kein signifikanter Vorteil im Arm mit CTC basierter Therapie
STIC-CTC (NCT01710605) (164)	CTC-Zahl (≥ 5), bestimmt mit CellSearch	Die Studie untersucht, ob eine auf der CTC-Zahl vor Beginn der Erstlinien-Therapie basierende Therapieentscheidung (endokrine Therapie oder Chemotherapie) bei Östrogenrezeptor positiven metastasierten MK-Patientinnen der Arztentscheidung überlegen ist	Positiv: signifikant verlängertes PFS nach CTC-basiertem Wechsel auf Chemotherapie und keine signifikante Verschlechterung nach CTC-basiertem Wechsel auf endokrine Therapie
CirCé01 (NCT01349842) (165)	CTC-Zahl (≥ 5), bestimmt mit CellSearch	Die Studie untersucht, ob metastasierte MK-Patientinnen bei persistierenden CTCs von einem Wechsel der Chemotherapie nach einem Zyklus der Drittlinien- oder späteren Chemotherapie profitieren	Rekrutierung abgeschlossen; Ergebnis ausstehend
DETECT-III (NCT01619111) (166)	HER2-Positivität von CTCs, bestimmt mit CellSearch	Die Studie untersucht bei MK-Patientinnen in der metastasierten Situation mit HER2-negativem Primärtumor die Wirksamkeit einer anti-HER2 Therapie, falls HER2-positiven CTCs nachgewiesen werden können	noch nicht abgeschlossen

Die Ergebnisse anderer klinischer Studien deuten dagegen darauf hin, dass CTCs einen klinischen Nutzen aufweisen: In der STIC-CTC-Studie (NCT01710605) wurde der klinische Nutzen der Ausgangs-CTC-Zahl in der Erstlinientherapie von Patientinnen mit ER- positivem metastasiertem MK untersucht. Die Art der Therapie – entweder eine endokrine Therapie oder eine Chemotherapie – wurde in dem einen Arm durch den Arzt und in dem anderen Arm durch die Anzahl der CTCs bestimmt. Bei geringer CTC-Zahl wurden die Patientinnen mit einer endokrinen Therapie behandelt, bei hoher CTC-Zahl mit einer Chemotherapie. Dabei zeigten Patientinnen, die auf Grund der CTC-Bestimmung eine Chemotherapie an Stelle einer endokrinen Therapie erhielten, ein signifikant längeres progressionsfreies Überleben und einen Trend zu einem längeren Gesamtüberleben. Gleichzeitig hatten Patientinnen, bei denen die Therapie basierend auf der CTC-Bestimmung von einer Chemotherapie zu einer endokrinen Therapie indiziert wurde, kein signifikant kürzeres progressionsfreies oder Gesamtüberleben im Vergleich zu denen, die basierend auf der Arztentscheidung eine Chemotherapie trotz geringer CTC-Zahl erhalten hatten (164).

In der CirCé01-Studie (NCT01349842) wird derzeit in der Drittlinien- oder späteren Chemotherapie beim metastasiertem MK getestet, ob sich persistierende CTCs als Indikator für einen Therapiewechsel eignen (165).

Die derzeit laufende DETECT-III-Studie (NCT01619111) testet die klinische Verwendbarkeit von CTCs basierend auf deren Biomarker-Expression. In dieser zweiarmigen Studie erhalten Patientinnen mit HER2-negativem Primärtumor, auf deren CTCs mittels CellSearch-Analyse die Expression von HER2 nachgewiesen werden kann, im Studienarm zusätzlich zur Standardtherapie Lapatinib (166,167). In einer vorherigen randomisierten Phase II-Studie mit Patientinnen nach adjuvanter Chemo- und Radiotherapie konnte bereits gezeigt werden, dass ein Großteil der noch vorhandenen CTCs HER2-positiv war und eine folgende anti-HER2-Therapie das Risiko eines Rezidivs reduzierte und das krankheitsfreie Überleben verlängerte (157).

2.4.6 Die Kultivierung von CTCs

Eine weitere zukünftige Anwendung von CTCs könnte die Entwicklung von CTC-basierten Xenograft-Modellen und Zellkulturen sein. Lebensfähige CTCs könnten dadurch einerseits zum Testen von Therapeutika in *in vitro*-Experimenten verwendet und andererseits könnten

weitere Einblicke in deren funktionale Eigenschaften und die Mechanismen des Prozesses der Metastasierung gewonnen werden (168).

Auf Grund der extrem geringen Anzahl von CTCs stellt die Kultivierung von CTCs eine sehr große Herausforderung dar. Voraussetzungen für eine erfolgreiche Kultivierung von CTCs sind: eine Blutabnahme in Röhrchen ohne Fixativ, ein möglichst schneller Transport des abgenommenen Blutes ins Labor, die zügige Anreicherung der CTCs, ohne diese dabei zu fixieren oder zu permeabilisieren und die Identifikation geeigneter Kulturbedingungen (169). Hinzu kommt, dass teilweise ein Großteil der in Patienten vorkommenden CTCs bereits apoptotisch und somit nicht für die Kultivierung geeignet ist (170–172).

Die Kurzzeitkultivierung über wenige Tage, die häufig auf einer Membran erfolgt, mit deren Hilfe die Anreicherung der CTCs durchgeführt wurde, wurde bereits für diverse Tumortypen dokumentiert (103,121,173–176).

Inzwischen konnten in einigen Fällen auch CTCs über längere Zeiträume kultiviert werden. Dies beschränkt sich jedoch in der Regel auf CTCs von einzelnen Patienten im fortgeschrittenen Stadium mit außergewöhnlich vielen CTCs (169).

Die erfolgreiche Kultur von CTCs wurde zuerst von Zhang et al. beschrieben. Zhang et al. konnten drei CTC-Linien auf EpCAM-negativen, ALDH1-positiven, HPSE-positiven CTCs mit EGFR-Amplifikation aufbauen, die im CellSearch nicht nachweisbar waren. Die kultivierten CTCs waren hoch invasiv und bildeten nach der Injektion in Nacktmäuse Hirn- und Lungenmetastasen (177). Wenig später konnten Yu et al. CTCs von sechs Patientinnen mit lumbalem MK *in vitro* expandieren (77). Auch beim CRC wurde eine auf CTCs basierte Zelllinie beschrieben. Die CTCs wiesen eine hohe Ähnlichkeit zum Primärtumor und zu Lymphknotenmetastasen des Patienten auf und waren nach einer Injektion in immundefiziente Mäuse tumorigen (178). Weiterhin konnten Prostatakarzinom-CTCs mittels eines 3D-Organoid-Systems kultiviert werden (179). CTCs von Patienten mit Lungenkarzinom im frühen Stadium konnten mit Hilfe eines 3D-Co-Kultur-Modells expandiert werden, das die Tumor-Mikroumgebung simulieren soll (180).

Eine Alternative zur *in vitro* Expansion stellt die *in vivo* Kultur dar. Mit Hilfe eines solchen *Xenograft*-Assays, bei dem die angereicherten CTCs in die Tibia von immundefizienten Mäusen injiziert wurden, konnte gezeigt werden, dass insbesondere die Fraktion der EpCAM-positiven, CD44-positiven, CD47-positiven und MET-positiven CTCs eine metastaseninitiierende Aktivität aufweist (40). Ebenfalls im *in vivo* Experiment – allerdings

nach subkutaner Injektion von CTCs von MK- und Prostatakarzinompatienten – konnten Rossi et al. CTCs im Blut und im Knochenmark von NOD/SCID-Mäusen detektieren (181). Die besten Voraussetzungen für funktionale Analysen stellen CTCs des kleinzelligen Lungenkarzinoms dar, da diese Patienten die höchsten CTC-Zahlen aufweisen (135). Hodgkinson et al. konnten zeigen, dass sich CDX-Modelle mit Hilfe solcher CTCs in immungeschwächten Mäusen generieren lassen, die das Ansprechen auf eine Platin- oder Etoposid-basierte Chemotherapie widerspiegeln (182).

Kommentar zu Tabelle 3:

Die Auswahl der hier aufgeführten CTC-Kultivierungen erfolgte anhand ihrer Relevanz in der Literatur (168,169,183). Die Anreicherung, aber auch der Nachweis der erfolgreichen Kultivierung bzw. Expansion wurde dabei auf höchst unterschiedliche Art und Weise durchgeführt. So erfolgte in einigen Fällen der Nachweis lediglich durch eine Immunfluoreszenz-Analyse für einen neoplastischen Marker, während in anderen Studien mittels genetischer Analysen die Abstammung vom Tumor sichergestellt werden konnte. Idealerweise sollte zusätzlich eine *Short Tandem Repeat*-Analyse durchgeführt werden. Inwieweit alle der beschriebenen Beobachtungen also tatsächlich eine erfolgreiche Kultivierung oder Expansion darstellen, kann nicht immer abschließend sichergestellt werden.

Tabelle 3: Übersicht über die CTC-Kultivierung

Diese Tabelle zeigt eine Übersicht ausgewählter in der Literatur dokumentierter Erfolge bei der Kultivierung von CTCs (siehe Kommentar zu Fehler! Verweisquelle konnte nicht gefunden werden., Seite 24).

Autor	Tumortyp	initiale Kultur	Anreicherung	Ausgangsmaterial	Anzahl CTCs mit CellSearch bestimmt	Erfolgsrate bei Kultivierung
Zhang et al. (177)	metastasiertes Mammakarzinom	<i>in vitro</i>	Fluoreszenz aktivierte Zellsortierung (FACS) (ALDH1+/CD45-)	20 bis 45 ml Blut	0	7,90%
Yu et al. (77)	metastasiertes Mammakarzinom	<i>in vitro</i>	CTC-iChip (Depletion mittels anti-CD45 und anti-CD66 Antikörpern)	20 ml Blut	3 bis 3000 CTCs/6 ml Blut	16,70%
Gao et al. (179)	metastasiertes Prostatakarzinom	<i>in vitro</i>	RosetteSep	Blut (Volumen nicht angegeben)	nicht angegeben (es wurden nur Proben von Patienten mit wenigstens 100 CTCs/7,5 ml Blut analysiert)	5,90%
Cayrefourq et al. (178)	metastasiertes kolorektales Karzinom	<i>in vitro</i>	RosetteSep	10 ml Blut	≥302 CTCs/7,5 ml Blut	2,80%

Zhang et al. (180)	Lungenkarzinom (SCLC), frühes Stadium	<i>in vitro</i>	CTC-Chip	≤ 4,5 ml Blut	1 bis 11 CTCs/1 - 1,5 ml Blut (bestimmt mittels ICF nach Anreicherung mit dem CTC- Chip)	73,70%
Baccelli et al. (40)	metastasiertes Mammakarzinom	<i>in vivo</i>	RosetteSep oder FACS	Blut (Volumen nicht angegeben)	≥ 1109 CTCs in äquivalentem Volumen	3,60%
Rossi et al. (181)	metastasiertes Mamma- und Prostatakarzinom	<i>in vivo</i>	CellSearch Profile Kit	7,5 ml Blut	51 bis 207 CTCs pro 7,5 ml Blut	100%
Hodgkinson et al. (182)	metastasiertes Lungenkarzinom (SCLC)	<i>in vivo</i>	RosetteSep	10 ml Blut	≥ 458 CTCs pro 7,5 ml Blut	66,70%

2.5 Zielsetzung

Die Idee der *Liquid Biopsy* ist es, durch die Analyse von Tumor-Bestandteilen im Blut die Probleme, Limitierungen und mögliche Komplikationen zu umgehen, die mit Biopsien von Tumorgeweben verbunden sind. Als ein möglicher Analyt kommen dabei CTCs in Frage, deren klinischer Nutzen derzeit in zahlreichen Studien untersucht wird.

Ziele dieser Dissertation waren:

- a) Die Entwicklung von Methoden und Arbeitsschritten zur Charakterisierung von CTCs
- b) Die Identifikation von Zielstrukturen für zielgerichtete, CTC-basierte Therapien
- c) Das Verstehen der Biologie von CTCs unter Therapie von MK-Patientinnen
- d) Die Übertragung dieser Erkenntnisse in die klinische Anwendung

Diese Ziele sollten in vier Arbeitspaketen realisiert werden:

Ad a und b) Die Mutationsanalyse des *ESR1*-Gens in einzelnen CTCs zur Erweiterung des Verständnisses von Tumorzell-Heterogenität in Antwort auf eine ET

Im Rahmen der DETECT-III-Studie wird der klinische Nutzen der HER2-Analyse auf CTCs von metastasierten MK-Patientinnen mit HER2-negativem Primärtumor und die anschließende Gabe einer HER2-gerichteten Therapie untersucht. An diese klinische Studie angeschlossen werden in translationalen Projekten CTCs isoliert und charakterisiert. Dies soll dazu beitragen, Resistenzen zu verstehen und potenzielle Ansätze für die personalisierte Therapie auf CTC-Ebene zu identifizieren. Dabei könnten insbesondere Mutationen im *ESR1*-Gen eine vielversprechende Zielstruktur für *Liquid Biopsies* darstellen. Daher sollte eine Methode zur NGS-basierten Analyse von Mutationen im gesamten *ESR1*-Gen nach der *Whole Genome Amplification* (WGA) des Genoms einzelner CTCs entwickelt werden. Anschließend sollten CTCs von metastasierten MK-Patientinnen analysiert, die Heterogenität der CTCs betrachtet und die identifizierten Mutationen zur zuvor erhaltenen Therapie korreliert werden.

Ad a und b) Die Entwicklung einer Methode zur Kultivierung von CTCs aus DLA-Produkten als Grundlage für zukünftige Studien zur Biologie von CTCs und für individuelle CTC-basierte *Drug Screening*-Versuche

Insbesondere die Analyse lebensfähiger CTCs könnte in der Zukunft eine vielversprechende Möglichkeit des Nutzens von *Liquid Biopsies* darstellen. Zum einen bestünde die Möglichkeit, *in vitro* Therapeutika an Tumorzellen der Patienten zu testen, und zum anderen könnten durch die Verwendung CTC-basierter Zellkulturen weitere Einblicke in den Prozess der Metastasierung gewonnen werden. Die Kultivierung von CTCs aus dem Blut stellt auf

Grund ihrer Seltenheit jedoch eine große Herausforderung dar und ist dadurch auf eine sehr geringe Anzahl an Patienten limitiert. Die DLA erlaubt es, die Positivitätsrate und die Menge der erhaltenen CTCs zu steigern. Ziel dieser Studie war es, herauszufinden, ob die DLA eine Möglichkeit darstellt, CTCs für die Kultivierung zu erhalten. Zunächst sollte ein Weg gefunden werden, CTCs aus DLA-Produkt anzureichern, ohne dabei deren Viabilität zu beeinträchtigen.

Ad c und d) Eine vergleichende multiparametrische Verlaufsstudie als *Proof-of-Concept* zur Untersuchung des klinischen Nutzens einer CTC-basierten Mutationsanalyse zur Therapiefindung im Vergleich zur klassischen Tumoranalyse

Im dritten Teil dieser Dissertation sollte unter Zuhilfenahme der in den ersten beiden Teilen entwickelten Methoden der klinische Nutzen von CTCs – insbesondere der Analyse von Mutationen – in einer *Proof of Concept*-Studie betrachtet werden. Dazu wurden CTCs von fünf metastasierten MK-Patientinnen mittels *Whole Exome Sequencing* (WES) analysiert, um klinisch relevante Mutationen zu detektieren. Weiterhin wurden *Liquid Biopsies* einer Index-Patientin über einen Zeitraum von drei Jahren entnommen und multiparametrisch analysiert. Schließlich sollten mit Hilfe der im zweiten Teil der Dissertation entwickelten Methode *in vitro* in Frage kommende Therapeutika getestet werden.

Ad d) Die Entwicklung eines *Next Generation Sequencing* (NGS)-Panels zur Identifikation therapierelevanter Mutationen in CTCs für die Realisierung zielgerichteter, CTC-basierter Therapien mit derzeit zugelassenen Therapeutika

Die Mutationsanalyse von CTCs mittels WES stellt eine zeitaufwendige und kostenintensive Methode dar, wodurch ihr Einsatz in der Klinik erschwert wird. Daher sollte im vierten Teil dieser Dissertation ein PCR-basiertes NGS-*Panel* entwickelt werden, das die Identifikation therapierelevanter Mutationen in den Genen *PIK3CA*, *ESR1*, *AKT1* und *ERBB2* auf CTC-Ebene ermöglicht. Diese Gene wurden ausgewählt, da im Falle einer detektierten Mutation zugelassene zielgerichtete Therapien zur Verfügung stehen.

3 Manuskripte

3.1 Manuskript 1:

„Detection of *ESR1* Mutations in Single Circulating Tumor Cells on Estrogen Deprivation Therapy but not in Primary Tumors from Metastatic Luminal Breast Cancer Patients.”

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Detection of *ESR1* Mutations in Single Circulating Tumor Cells on Estrogen Deprivation Therapy but Not in Primary Tumors from Metastatic Luminal Breast Cancer Patients

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Mutations in the ligand-binding domain (LBD) of the *ESR1* gene result in resistance to estrogen deprivation therapy (EDT) in breast cancer. Their detection might enable optimization of therapy strategies. However, the predictive utility of the primary tumor (PT) is limited, and obtaining serial biopsies of metastatic lesions is challenging. To underline their application as a liquid biopsy, single circulating tumor cells (CTCs) were analyzed with a next-generation sequencing approach for the *ESR1* coding region. CTCs from 46 metastatic luminal breast cancer patients were enriched using CellSearch system and isolated by micromanipulation. Their genomic DNA was amplified and the *ESR1* gene was sequenced. Furthermore, tissue samples from corresponding PTs and/or metastatic lesions were investigated. *ESR1* mutations were detected in 12 patients—exclusively in patients treated with EDT ($P = 0.048$). In seven cases mutations were located in the hotspot regions in the LBD. Six novel mutations were identified. *ESR1* mutations were absent in PT tissue samples and were detected only in metastases obtained after CTC characterization. Single-cell CTC analysis for *ESR1* mutations could be of clinical value to identify patients who progress under EDT and therefore benefit from an early switch to an alternative endocrine therapy or other treatment regimens. Furthermore, our data indicate that mutations outside the LBD's hotspot regions might also contribute to resistance to EDT. (*J Mol Diagn* 2020, 22: 111–121; <https://doi.org/10.1016/j.jmoldx.2019.09.004>)

Over the past few decades, targeted therapy has become the preferred approach to treat biomarker-positive breast

cancers. Most breast cancers express the estrogen receptor (ER) and are therefore predicted to be endocrine

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responsive.¹ Endocrine therapy (ET) is the recommended first-line treatment for ER-positive metastatic breast cancer (MBC) and may either target the ER pathway with selective ER modulators or degraders or by drugs that cause estrogen deprivation, such as aromatase inhibitors (AIs). Although nearly all patients with ER-positive MBCs initially respond to ER pathway targeting therapy, almost all of them will ultimately develop secondary resistance that results in a relapse. Potential resistance mechanisms include altered ER expression, amplifications or translocations of the *ESR1* gene, altered expression of growth factor receptors, activated phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling, altered expression of cell-cycle regulators, overexpression of ER coactivators, up-regulated autophagy, epithelial-to-mesenchymal transition, and tumor heterogeneity.² Furthermore, *ESR1* mutations in the ligand binding domain (LBD) coding sequence are frequently observed after ET in MBC patients. These mutations result in an estrogen-independent constitutive activation of the ER α .^{3,4} *ESR1* mutations are found in approximately 0.5% of primary breast cancers compared with 14% to 54% in BC metastases, which suggests that these mutations arise through clonal selection of rare resistant tumor cells or are acquired under the selection pressure of ET during the course of disease.⁵ Studies have investigated the role of *ESR1* mutations as potential predictive and prognostic biomarkers and have revealed that *ESR1* mutations are likely to be associated with a more aggressive disease.⁶ Therefore, ET that targets *ESR1*-mutated MBC might be an appealing concept.⁷ However, tissue availability for *ESR1* or other mutation analyses is a major challenge. Biopsies of recurrent or metastatic lesions are invasive or cannot be performed because of an inaccessible location or due to other clinical reasons. To overcome this challenge, the idea of deriving information about the tumor or the metastatic lesions from liquid biopsies, for example, circulating tumor cells (CTCs) or circulating tumor DNA, became popular.^{8,9} CTCs are shed into the blood by tumor tissue and are commonly considered as precursor cells for metastasis formation.^{10,11} CTCs are an independent prognostic indicator of progression-free and overall survival in the adjuvant,¹² neoadjuvant,¹³ and metastatic^{14,15} setting. However, to date, the predictive utility of CTCs is unclear.¹⁶

In this study, we analyzed *ESR1* mutations in CTCs isolated from peripheral blood from MBC patients who harbor an ER-positive primary tumor (PT) in single cell resolution. The presence of *ESR1* mutations was compared with the *ESR1* mutational status of the PT. Although *ESR1* mutations predominantly occur in two hotspots in the LBD, additional mutations outside these hotspots have been reported.^{5,17} Thus, we developed a next-generation sequencing approach to sequence the whole *ESR1* coding region.

Materials and Methods

Cell Lines

SUM44 cells, with an acquired *ESR1* mutation after long-term estrogen deprivation, were cultured in phenol red-free RPMI supplemented with 10% dextran charcoal stripped fetal bovine serum (both from Thermo Fisher Scientific, Waltham, MA) as previously described.¹⁸

Patients

CTCs were analyzed from 46 patients with MBC, who had a PT of luminal subtype. A tumor was defined as ER positive if at least 1% of the tumor cells were determined as ER α positive with the use of immunohistochemistry. Patients were selected from the DETECT III (NCT01619111) and DETECT IV (NCT02035813) study cohorts (available from <https://clinicaltrials.gov/ct2/home>; last accessed December 21, 2018), in which patients with Her2/neu-negative MBC being CTC positive are included. All patients gave their informed consent for the use of their blood samples for CTC analysis and for translational research projects. Patients' characteristics were anonymized by using sample identifiers (Ethical approvals MC-531 and MC-LKP-668). Clinical patient data are shown in Table 1.

Enrichment and Enumeration of CTCs

Enrichment and enumeration of CTCs was performed with the CellSearch Circulating Epithelial Cell Kit (Menarini, Florence, Italy) according to the manufacturer's instructions. Briefly, 10 mL of peripheral blood was collected into CellSave Preservative Tubes (Menarini) and was processed within 96 hours; 7.5 mL of blood was used for enumeration of CTCs. Enrichment is based on immunomagnetic ferrofluid conjugated with an epithelial cell adhesion molecule-directed antibody. Subsequent characterization of CTCs was performed with immunofluorescent staining directed against cytokeratins (CKs) to identify CTCs, CD45 to exclude leukocytes, and DAPI to confirm nucleo-morphologic integrity.¹¹

Enrichment of CTCs positive for epithelial cell adhesion molecule without automated subsequent immunofluorescent characterization was performed with the CellSearch Epithelial Cell Profile Kit (Menarini) according to the manufacturer's instructions. CTCs were enriched in a total volume of approximately 900 μ L and spun onto glass slides (Hettich, Tuttlingen, Germany) for subsequent characterization.

Table 1 Clinical Patient Data ($n = 46$)

Characteristic	Value
Age, years	
Mean	62.9
Median	63.5
Range	43–81
Tumor size, n (%)	
T1	11 (23.9)
T2–4	31 (67.4)
NA	4 (8.7)
Nodal status, n (%)	
Negative	13 (28.3)
Positive	30 (65.2)
NA	3 (6.5)
M status at time of diagnosis, n (%)	
0	35 (76.1)
1	9 (19.6)
NA	2 (4.3)
Tumor staging, n (%)	
0	1 (2.2)
I	5 (10.9)
II	9 (19.6)
III	11 (23.9)
IV	9 (19.6)
NA	11 (23.9)
Histology, n (%)	
Lobular	18 (39.1)
NST	20 (43.5)
DCIS	4 (8.7)
Others	4 (8.7)
Grading, n (%)	
2	35 (76.1)
3	10 (21.7)
NA	1 (2.2)
Menopause status, n (%)	
Postmenopausal	41 (89.1)
Premenopausal	2 (4.3)
NA	3 (6.5)
Received lines of therapy in metastatic setting, n (%)	
0	12 (26.1)
1	16 (34.8)
2	10 (21.7)
3	8 (17.4)

The eighth edition of UICC TNM classification was used.
DCIS, ductal carcinoma *in situ*; NA, no available information; NST, invasive breast cancer of no special type.

Isolation of Single Cells by Semiautomatic Micromanipulation

Single CTCs were isolated from the CellSearch cartridges by micromanipulation with the CellCelector (ALS, Jena, Germany), a semiautomated micromanipulator that consists of an inverted fluorescent microscope (CKX41; Olympus, Tokyo, Japan) equipped with a charge-coupled device camera system (XM10-IR; Olympus) and a robotic arm with a vertical glass capillary of 30 μm in diameter as described previously.¹⁹

Isolated cells were deposited in PCR tubes for whole genome amplification (WGA).

Whole Genome Amplification

For genomic analysis chromosomal DNA of isolated single cells was amplified with the Ampli1 WGA Kit (Menarini) according to the manufacturer's protocol.

The DNA integrity of the WGA products was determined with genome integrity index analysis using the Ampli1 QC Kit (Menarini). WGA products with at least two amplified PCR products were further processed for DNA sequencing.

Spike in Experiment

For validation of the workflow 200 cells of the SUM44 cell line with acquired *ESR1* Y537S mutation were spiked into 7.5 mL of healthy donor blood. For detachment from the culture flask an enzyme-free cell dissociation buffer (Thermo Fisher Scientific) was used. The tumor cells were enriched with the CellSearch, isolated with the CellCelector and the genome was amplified with the Ampli1 WGA Kit similarly to CTCs.

DNA Extraction from Blood

For analysis of germline *ESR1* variants DNA from white blood cells was extracted with the QIAamp DNA Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol.

To determine *ESR1* mutation status in PTs and metastatic tissues DNA was purified from formalin-fixed, paraffin-embedded (FFPE) tissue with the GeneRead DNA FFPE Kit (Qiagen) according to the manufacturer's protocol. Tumor content was determined by experienced pathologists (I.E., S.J.) inspecting a sequential tissue section (5 μm) stained with hematoxylin and eosin and immunohistochemistry against CKs (clone AE1/AE3; catalog number 42-9003-82; Thermo Fisher Scientific) as described previously.²⁰

Sequencing

ESR1 DNA fragments were amplified and barcoded in two steps. In the first step, *ESR1* exons were amplified with gene-specific primers (Table 2) that contained primer binding sites for universal Multiplicom MID Dx primers (Agilent Technologies, Santa Clara, CA). In the second step, Multiplicom primers were used in a multiplex PCR to attach unique barcodes for sequencing to the PCR products. KAPA2G Fast Multiplex Mix (F. Hoffmann-La Roche, Basel, Switzerland) was used. One microliter of WGA product and 1 $\mu\text{mol/L}$ gene-specific primers were applied in the first PCR. In the second PCR, 2.5 $\mu\text{mol/L}$ of Multiplicom primers and 0.5 μL of first-round PCR products were used.

Table 2 Primer Overview

Target	Forward primer	Reverse primer
Exon 1 A	5'-AAGACTCGGCAGCATCTCCAACGGTC -TGCACCCTGCCCCGCG-3'	5'-GCGATCGTCACTGTTCTCCAGCCGGTCTGACCGTAGACCTG-3'
Exon 1 B	5'-AAGACTCGGCAGCATCTCCAAAGCCC -GCCGTGTACAACACTAC-3'	5'-GCGATCGTCACTGTTCTCCACACCCCGACGGGCGGCGCGG-3'
Exon 2	5'-AAGACTCGGCAGCATCTCCATTAAT -GGATTTACTGTTTTTTTC-3'	5'-GCGATCGTCACTGTTCTCCAAAAAATAGAAGTCGTTTTCAAC-3'
Exon 3	5'-AAGACTCGGCAGCATCTCCATAGTT -AATAGGACATAACGACTA-3'	5'-GCGATCGTCACTGTTCTCCACCCAAGGGCCCTGGGAGAG-3'
Exon 4 A	5'-AAGACTCGGCAGCATCTCCATAAACT -AATTTTTTTTTTCCACC-3'	5'-GCGATCGTCACTGTTCTCCAGCATCCAACAAGGCACTGAC-3'
Exon 4 B	5'-AAGACTCGGCAGCATCTCCAGCTCTAA -GAAGAACAGCCTG-3'	5'-GCGATCGTCACTGTTCTCCATAGTTAAAGCTGCGCTTCG-3'
Exon 5	5'-AAGACTCGGCAGCATCTCCATTTGAGT -CAGCAGGGTTTTTC-3'	5'-GCGATCGTCACTGTTCTCCAGCTACTCCTAAGCTACAGCC-3'
Exon 6	5'-AAGACTCGGCAGCATCTCCATATTTA -TTTATTTTTCGTATG-3'	5'-GCGATCGTCACTGTTCTCCATAGTTAAGCAAAATAATAGA-3'
Exon 7	5'-AAGACTCGGCAGCATCTCCAAGACCTC -ATCCTCTTTGAGC-3'	5'-GCGATCGTCACTGTTCTCCATCTCCTGTAGGAAGCCCACA-3'
Exon 8	5'-AAGACTCGGCAGCATCTCCAAGTAGTC -CTTCTGTGTCTTC-3'	5'-GCGATCGTCACTGTTCTCCATATCTGAACCGTGTGGGAGC-3'

Final PCR products were purified with Agencourt AMPure XP beads (Beckmann Coulter, Brea, CA) and quantified with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific) with the use of the Broad Range Quant-iT dsDNA Assay Kit (Thermo Fisher Scientific).

Equimolar amounts of DNA libraries were pooled to 2 nmol/L and sequenced on a MiSeq system (Illumina, San Diego, CA) with 151 bp of paired end reads.

FASTQ files were analyzed with the Galaxy web platform, using the public server at usegalaxy.org (last accessed June 28, 2019).²¹ Reads were aligned to the human reference genome (hg19) with Bowtie2 version 2.3.4.2. Aligned sequences were screened for mutations with the IG Viewer version 2.3.25.²² Identified mutations were covered by >100× and had a variant allele frequency > 12.5% for WGA products and >1% for FFPE tissue analysis.

Immunofluorescence Analysis

For immunofluorescence analysis cells were fixed with CellSave reagent, permeabilized with 0.1% Triton X-100 (Merck, Darmstadt, Germany), and stained for nucleic acid (DAPI; F. Hoffmann-La Roche), CKs (clone C11, Alexa Fluor 488 conjugated, catalog number GTX11212; Gene-Tex, Irvine, TX), CD45 (clone 35-ZS, Alexa Fluor 647 conjugated, catalog number sc-1178 AF647; Santa Cruz Biotechnology, Dallas, TX), and ERα (clone SP1, catalog number RM-9101-S; Thermo Fisher Scientific). As secondary antibody, a goat anti-rabbit IgG antibody (catalog number A-11012; Thermo Fisher Scientific) was used for ERα analysis.

Results

Patient Characteristics

In this study, *ESR1* mutation status of CellSearch enriched and micromanipulated single CTCs obtained from the blood of 46 MBC patients was analyzed. PTs of all patients enrolled in this study were ER positive.

In the primary setting 54.3% of the patients received EDT—either AI or gonadotropin-releasing hormone analogues (GnRH-As); 19.6% of the patients were treated with other ETs (eg, tamoxifen) in the primary setting. In the metastasized setting 50.0% of patients were treated with an EDT; 6.5% received other ETs. In total 80.4% of the patients received EDT during primary and/or metastatic treatment regimens and 8.7% received other kinds of ETs. A portion (10.9%) of the patients were ET naive before *ESR1* analysis; 23.9% received EDT therapy during both primary and metastatic treatment regimens.

CellSearch analysis of blood samples revealed CTC counts between two and approximately 40,000 CTCs per 7.5 mL of blood. The median CTC count per 7.5 mL of blood was 46.

Identification of *ESR1* Mutations in CTCs

For sequencing of the *ESR1* coding region a primer panel that covered all eight exons was designed. Sequencing primers were adapted to the location of MseI restriction sites of the Ampli1 WGA. Because the MseI restriction site at the 3'-end of exon 6, the last 22 nucleotides of this exon were not covered with our amplification-based next-generation

Table 3 ESR1 Mutations in Circulating Tumor Cells

Patient	WT	G160C	K252R	N348S	E380Q	D426E	L507F	Y537C	Y537N	D538G	D541Q
1	—	—	—	—	—	—	—	1*	—	—	—
2	—	—	—	—	—	—	—	1	—	—	—
3	1	—	—	—	—	—	—	—	—	7	—
4	1	—	—	—	—	—	—	—	—	—	—
5	5	—	—	—	—	—	—	—	—	—	—
6	2	—	—	—	—	—	—	—	—	—	—
7	2	—	—	—	—	—	—	—	—	—	—
8	1	—	—	—	—	—	—	—	—	—	—
9	3	—	—	—	—	—	—	—	—	—	—
10	4	—	—	—	—	—	—	—	—	—	—
11	3	—	—	—	—	—	—	—	—	5	—
12	3	—	—	—	—	—	—	—	—	—	—
13	4	—	—	—	—	—	—	—	—	—	—
14	3	—	—	—	—	—	—	—	—	—	—
15	2	—	—	—	—	—	—	—	—	—	—
16	3	—	—	—	—	—	—	—	—	—	—
17	1	—	—	—	—	—	—	—	—	—	—
18	4	—	—	—	—	—	—	—	—	—	—
19	2	—	—	—	—	—	—	—	—	—	—
20	6	—	—	—	—	—	—	—	—	—	—
21	4	—	1	—	—	—	—	—	—	—	—
22	1	—	—	—	—	—	—	—	—	—	—
23	2	—	—	—	—	—	—	—	—	—	—
24	1	—	—	—	—	—	—	—	—	—	—
25	—	—	—	—	—	—	1	—	—	—	—
26	1	—	—	—	—	—	—	—	—	—	—
27	2	—	—	—	5	—	—	—	—	—	—
28	3	—	—	—	—	1	—	—	—	—	—
29	1	—	—	—	—	—	—	—	—	—	—
30	1	—	—	—	—	—	—	—	—	—	—
31	3	—	—	—	—	—	—	—	—	—	—
32	1	—	—	1	—	—	—	—	—	—	—
33	4	—	—	—	—	—	—	—	—	—	—
34	3	—	—	—	—	—	—	—	—	—	—
35	1	—	—	—	—	—	—	—	—	—	—
36	3	—	—	—	—	—	—	—	—	—	—
37	—	—	—	—	1	—	—	—	—	—	—
38	3	—	—	—	—	—	—	—	—	—	—
39	3	—	—	—	—	—	—	—	1	—	—
40	2	—	—	—	—	—	—	—	—	—	—
41	3	—	—	—	—	—	—	—	—	—	—
42	2	—	—	—	—	—	—	—	—	—	—
43	3	—	—	—	—	—	—	—	—	—	—
44	1	2	—	—	—	—	—	—	—	—	—
45	5	—	—	—	—	—	—	—	—	—	—
46	1	—	—	—	—	—	—	—	—	—	1

One to eight CTCs of a total of 46 patients were analyzed. Numbers of CTCs with observed mutations exceeding a variant allele frequency of 12.5% and covered by at least 100 reads are displayed. Only missense mutations were considered. A detailed overview is shown in [Supplemental Table S1](#).

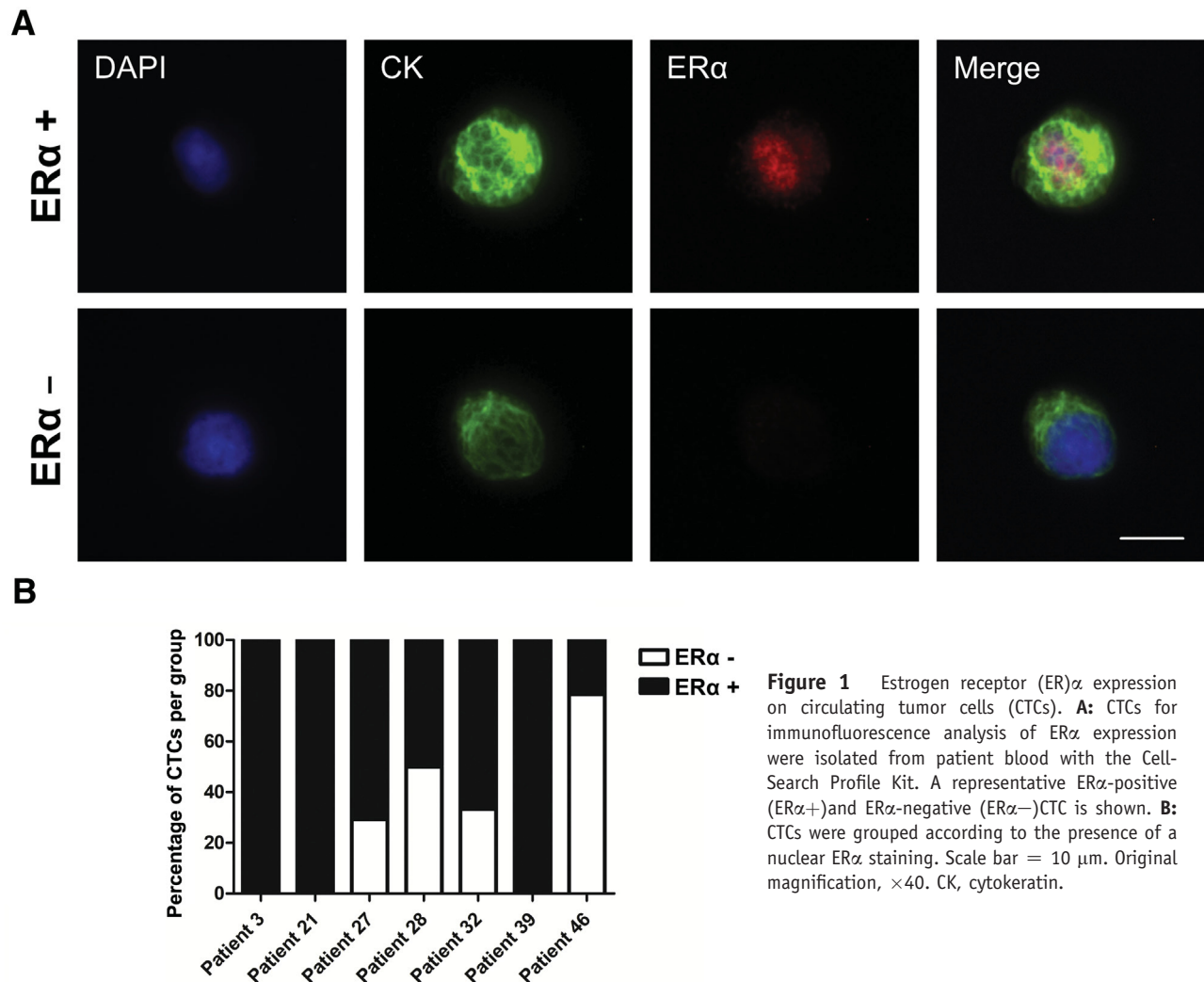
*This cell harbored the mutation Y537C and the mutation L408S that is not shown in this table.

—, no cell with the respective mutation was found; CTC, circulating tumor cell; WT, wild-type.

sequencing approach. Furthermore, it was observed that exon 1 of *ESR1* was not amplified during Ampli1 WGA, resulting in no sequencing data for exon 1.

For *ESR1* mutation analysis, DNA from single CTCs was amplified by WGA. The sequencing approach was validated with cells from the SUM44 cell line that had been spiked

into healthy donor's blood and enriched with CellSearch. These cells have acquired an *ESR1* Y537S mutation as resistance to long-term estrogen deprivation. The *ESR1* Y537S mutation was detected in 25 of 32 analyzed cells, resulting in an assay sensitivity of 78.1% ([Supplemental Figure S1](#)).



Subsequently, one to eight CTCs of each patient with sufficient DNA quality determined by genome integrity analysis were used for *ESR1* sequencing. In CTCs from 13 of 46 patients missense mutations in the *ESR1* gene were detected (Table 3 and Supplemental Table S1). Mutations E380Q, Y537C, Y537N, or D538G located in the LBD hotspots were found in seven patients. In cases with more than one CTC analyzed, a heterogeneous *ESR1* mutational status was determined; 25.0% to 72.0% of the CTCs harbored a mutant *ESR1* gene, whereas the other CTCs displayed *ESR1* wild-type sequences. In one case two different *ESR1* mutations were identified in one single CTC: the mutation Y537C and the not yet described mutation L408S (patient 1). In most cases heterozygous mutations were observed with a variant allele frequency of approximately 50%. Only CTCs from patient 27 were found to harbor exclusively homo- or hemizygous mutations. In one case (patient 11) CTCs with heterozygous, homo-, or hemizygous as well as CTCs without an *ESR1* mutation were detected. Two of the three CTCs of patient 44 harbored the *ESR1* variant G160C. This variant was also

detected in this patient's white blood cells with a similar variant allele frequency of 51% (Supplemental Table S2). In all other cases no mutations were found in DNA extracted from white blood cells, suggesting that germline mutations could be excluded in these cases. Furthermore, mutations K252R, N348S, D426E, L507F, and D541Q located outside the LBD's hotspot regions of the *ESR1* gene were detected in CTCs from five patients. The latter mutations were only detected in single WGA products.

In seven cases with CTCs that carried *ESR1* mutations additional CTC samples were available. Immunofluorescence analysis revealed that 21.4% to 100% of the CTCs were positive for a nuclear ER α staining (Figure 1).

Occurrence of *ESR1* Mutations Is Associated with Prior Endocrine Deprivation Therapy

Next, the presence of *ESR1* mutations was compared with patients' treatment during primary and metastatic therapy. Patients were classified into two groups: the first group received either AI or GnRH-A therapy (37 patients).

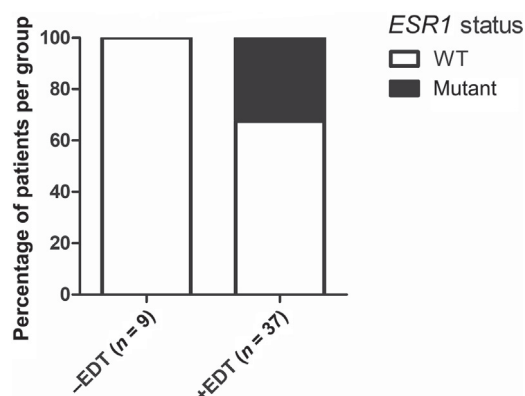


Figure 2 Presence of *ESR1* mutations was associated with obtained therapy. Patients were classified into two groups according to their type of treatment during primary and metastatic setting. One group contained patients treated with estrogen deprivation therapy (+EDT)—either aromatase inhibitors (AIs) or gonadotropin releasing hormone-analogues (GnRH-As). The other group contained patients without EDT treatment (−EDT). Patient 44—harboring the variant G160C in her circulating tumor cells (CTCs) and the germline DNA—was classified as *ESR1* mutation negative. $P = 0.0476$, determined by one-tailed Fishers exact test.

Because both AI and GnRH-A suppress estrogen synthesis, they were classified as EDT. The second group comprised patients who were not treated with EDT and received no (five patients) or other kinds of ET (four patients), such as tamoxifen or fulvestrant. *ESR1* mutations were solely observed in patients treated with EDT; 32.4% of these patients harbored CTCs with *ESR1* mutations. One of the patients with *ESR1*-mutated CTCs was treated with GnRH-A. No *ESR1*-mutated CTCs were found in two patients who had also received GnRH-A. Twelve of 34 patients treated with AI showed *ESR1* mutations in CTCs. Thus, a significant association of *ESR1* mutation status in CTCs to a received EDT therapy was observed (Figure 2). An association between the presence of *ESR1* mutations and whether the patients received EDT during adjuvant treatment or in the metastatic situation was not observed (Supplemental Figure S2).

Mutations Are Not Detected in Tumor Biopsies Sampled before CTC Analysis

Furthermore, it was investigated whether observed mutations were also found in FFPE tissue from the PTs or from metastatic lesions. PT tissue was available from 9 of 12 cases with *ESR1*-mutated CTCs (Figure 3). No *ESR1* mutations could be identified in DNA extracted from tumor specimen of all nine PTs.

ESR1 mutations from metastatic lesions were analyzed from six patients (Figure 3). Four samples were collected before CTC sampling—two from patients not treated with EDT before sample collection (patients 11 and 28), the other two from patients treated with EDT in adjuvant therapy (patients 27 and 46). All samples collected before CTC

sampling contained a wild-type *ESR1* gene. The other two samples were collected approximately 12 months (patient 3) and approximately 9 months (patient 39) after CTC sampling. In the sample from patient 3 the mutation D538G was detected, in the sample from patient 39 the mutation Y537N was observed (Table 4).

On CTC analysis and characterization, the patients were treated with various treatment schemes without regard to the herein reported analysis. Patients with *ESR1* mutations did not have a shorter overall survival than patients without *ESR1* mutations (Supplemental Figure S3).

Discussion

Developing *ESR1* mutations is a known resistance mechanism in tumor cells against AI and GnRH-A therapy. Thus, *ESR1* mutations are currently under investigation as potential predictive and prognostic biomarkers. In this context, liquid biopsies might become an important biomaterial, and in-depth characterization of CTCs obtained from patient blood might have the potential to provide information that could optimize patients' therapy.

Herein, we sequenced the *ESR1* gene of single CTCs' DNA amplified by WGA from MBC patients with PTs of luminal subtype. Point mutations that lead to amino acid changes were detected in CTCs from 12 of 49 patients. In seven cases, heterogeneity between analyzed CTCs was observed; *ESR1* mutations were only detected in a subset of the CTCs, whereas others were of wild-type *ESR1*. This might indicate that the analyzed CTCs originate from different metastases or from a different subclone of the same lesion. It is well known that CTCs differ in their phenotype and genotype due to tumor heterogeneity.²³ However, it cannot be excluded that the observed assumed heterogeneity may at least in part be caused by allelic dropout due to WGA. For example, in one CTC of patient 44 the expected *ESR1* variant G160C was not detected, most likely as a result of allelic dropout. In 7 of the 12 patients with mutated *ESR1* gene these mutations were localized to one of the hotspot regions located in the DNA sequence coding for the LBD at position E380, Y537, or D538. In addition, several other mutations were observed that have not been connected to breast cancer yet. However, these mutations were only detected in single WGA products. Because of prior WGA amplification, artifacts cannot be excluded.²⁴ For the final amplification, the Expand Long Template PCR System was used which contains a DNA polymerase exhibiting an error rate of 0.33×10^{-6} . Within the *ESR1* sequencing panel 1124 nucleotides were analyzed. Because of the cutoff of 12.5% to call a mutation, polymerase errors would have had occurred in the first round of the amplification. The likelihood of such a false-positive mutation in our analysis of a single CTC's *ESR1* gene is 0.148%. The likelihood of a mutation leading to a false-positive amino acid substitution

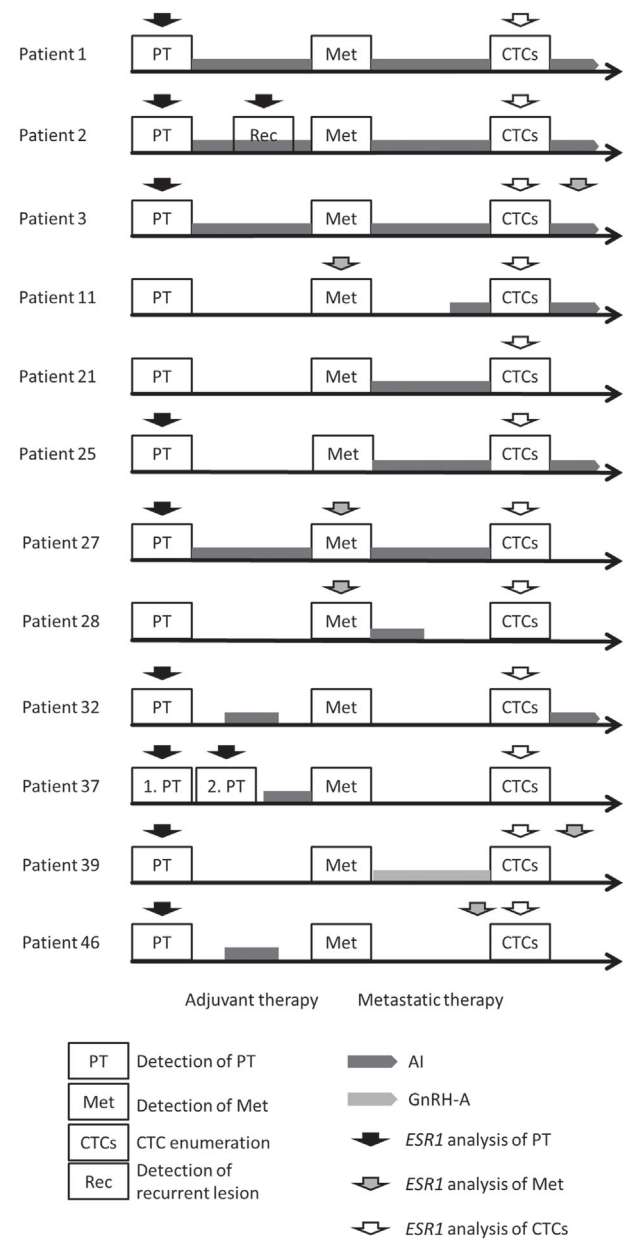


Figure 3 Sampling scheme. Time point of sample collection and period of estrogen deprivation therapy (EDT) of patients with observed *ESR1* mutations on circulating tumor cell (CTC) level are shown. **Arrows** indicate time point of sampling. Bars indicate period of EDT—either with aromatase inhibitors (AIs) or gonadotropin releasing hormone-analogues (GnRH-As). Only EDTs are shown. 1. PT, first primary tumor; 2. PT, second (contralateral) primary tumor; Met, metastatic lesion; Rec, recurrent lesion; PT, primary tumor.

is even lower. Thus, although it cannot be excluded, it is highly unlikely that the novel mutations detected here are false-positive events.

Furthermore, there are indications that the detected novel mutations are real mutations that might lead to resistance against EDT. First, the observed novel mutations only occurred in patients who had been treated with AI. Second, most mutations occurred in the LBD. Third, the mutations did not occur in regions that are particularly susceptible for

polymerase errors. Finally, all novel mutations—including those outside the LBD—affect amino acids that are highly conserved from mammals to zebrafish. Of interest, mutations N348S and L507F have already been identified in a screening approach for *ESR1* mutations that result in constitutively active ER α proteins.²⁵ These observed novel mutations need to be functionally tested in cell culture models to determine their effect on resistance against EDT or other ETs. Of note, applied prediction tools classify most of the observed novel mutations as deleterious or damaging (Supplemental Table S3).^{26–29}

Although all other mutations identified in CTCs were defined as somatic mutations, the *ESR1* variant G160C was also found in the germline DNA of one patient. This mutation has already been reported by another group in BC patients.³⁰ However, its frequency only slightly and not significantly exceeded the frequency in a control group. Thus, it is unlikely that it is connected with a higher risk of BC.

The presence of *ESR1* mutations was associated to the therapies that the patients had received before blood sampling for CTC analysis ($P = 0.0476$). *ESR1* mutations—both known hotspot mutations and novel mutations—were only detected in patients who received EDT, whereas no mutations were detected in patients who received no or other kinds of ET. Analysis of PT tissues revealed no detectable *ESR1* mutations. Furthermore, no *ESR1* mutations were detected in metastatic biopsies of four patients sampled before CTC analysis, although two of

Table 4 *ESR1* Mutations in Primary Tumor and Metastasis Tissue

Patient	PT	2. PT	Rec	Met (before CTC analysis)	CTCs	Met (after CTC analysis)
1	WT	—	—	—	L408S/Y537C	—
2	WT	—	WT	—	Y537C	—
3	WT	—	—	—	D538G	D538G (42.5%)
11	—	—	—	WT	D538G	—
21	—	—	—	—	K252R	—
25	WT	—	—	—	L507F	—
27	WT	—	—	WT	E380Q	—
28	—	—	—	WT	D426E	—
32	WT	—	—	—	N348S	—
37	WT	WT	—	—	E380Q	—
39	WT	—	—	—	Y537N	Y537N (65.0%)
46	WT	—	—	WT	L541Q	—

ESR1 mutation status of the primary tumor (PT), a recurrent lesion (Rec), and/or a metastatic lesion (Met) was analyzed from DNA extracted from formalin-fixed, paraffin-embedded tissue. WT indicates that a variant allele frequency below a cutoff of 1% was detected. Only missense mutations were considered. To determine the variant allele frequency of the observed mutation, tumor content was determined by hematoxylin and eosin staining and immunohistochemical analysis.

—, no tissue samples were available; CTC, circulating tumor cell; 2. PT, second (contralateral) primary was observed and a respective tissue sample was analyzed.

these patients had already received EDT in the adjuvant situation. This suggests that in these cases other resistance mechanisms might have driven the metastatic process and *ESR1* mutations occurred at a later time point. Only in two cases whereby the biopsies of liver metastases were taken approximately 12 and 9 months after blood sampling for CTC analysis, the same *ESR1* mutations as found in the CTCs were detected. Hence, our results match with the hypothesis that *ESR1*-mutant subclones are selected by EDT.

In line with the observations made for our patient cohort other research groups have already reported cases of patients with ER-positive breast cancer, whereby activating *ESR1* mutations have been detected in liquid biopsy samples on AI treatment but not in the PT.^{31–33}

ESR1 mutations have been shown to be clinically relevant and might act as a prognostic marker. In the BOLERO-2 trial the two *ESR1* mutations Y537S and D538G analyzed in circulating tumor DNA were associated with a shorter overall survival.⁶ In another study, the retrospective analysis of predictive and prognostic values of circulating *ESR1* mutations in advanced BC after progression on AI treatment revealed that overall survival was significantly shorter in patients with circulating *ESR1* mutations.³⁴ Furthermore, *ESR1* mutations are currently evaluated as a potential biomarker to guide therapeutic decisions. Especially AI-treated patients who harbor *ESR1* mutations have a poorer progression-free survival than patients without mutations.^{35,36} At least some activating *ESR1* mutations also seem to result in resistance to fulvestrant and tamoxifen.³⁷ However, patients with mutations within the LBD of ER α might benefit from higher doses or more potent selective ER modulators or degraders.⁵ Furthermore, small molecules such as AZD9496, a potent and selective antagonist and down-regulator of ER α that binds and down-regulates D538G and 537C/N/S ER α proteins *in vitro* and in a xenograft model,³⁸ targeting ER coactivators,³⁹ or mTOR, PI3K, and heat shock protein 90 inhibitors offer potential approaches to target tumor cells that depend on mutant ER α .³³

In the context of activating *ESR1* mutations as potential predictive biomarkers, the concept of a liquid biopsy could play an important role. For example, Clatot et al³⁴ showed that circulating *ESR1* mutations were detected before clinical progression in 75% of the cases. However, liquid biopsies and CTC analysis are also facing difficulties. Low CTC numbers and low numbers of CTC-positive patients are limiting the use of CTCs in the clinical routine. Furthermore, a high ratio of CTCs can be apoptotic, of low quality, and not suitable for DNA analysis. Polzer et al⁴⁰ have already shown that only 17.3% of WGA products of BC CTCs were of the highest quality as determined by a multiplex PCR quality control assay. In addition, specific alleles might get lost during WGA; thus, the absence of mutations does not necessarily mean that these mutations were not present in the initial cells. This effect of allelic

dropout is limiting all approaches for the analysis of DNA from single cells. Here, an assay sensitivity of 78.1% was observed. However, circulating tumor DNA analysis, the CTCs' counterpart in liquid biopsy, might also not fulfill the requirements for a clinical use, because it requires a priori knowledge of the target of interest in most cases. Here, it was shown that analysis of undetermined mutations might be promising and necessary.

If available, CTCs from a second blood sample of the patients with *ESR1*-mutated CTCs were analyzed for the expression of ER α . ER α -positive CTCs were observed in all samples, showing that detected mutations are likely to be present in the ER α protein of those CTCs. In many samples ER α -positive CTCs were accompanied by ER α -negative CTCs. This heterogeneity about the expression of ER α on CTCs matches previous findings by other groups.^{41,42}

Altogether, *ESR1* mutations were observed exclusively in CTCs from patients who had been treated with EDT. Furthermore, the mutations were not detectable in tissue biopsies from the PT or, if available, biopsies from a metastatic lesion taken before CTC analysis. Reasons might be the gain and selection of mutations during ET but also analytic limitations due to tumor heterogeneity that have to, at least in part, be considered. Therefore, these results underline the hypothesis that CTC analysis could be an important tool for guiding personalized therapy by identifying patients who might have a poorer outcome under EDT and might therefore benefit from an early switch to an alternative ET or other therapy.

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Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.jmoldx.2019.09.004>.

References

1. Huang B, Warner M, Gustafsson JÅ: Estrogen receptors in breast carcinogenesis and endocrine therapy. *Mol Cell Endocrinol* 2015, 418:240–244
2. Musgrove EA, Sutherland RL: Biological determinants of endocrine resistance in breast cancer. *Nat Rev Cancer* 2009, 9:631–643
3. Robinson DR, Wu YM, Vats P, Su F, Lonigro RJ, Cao X, Kalyana-Sundaram S, Wang R, Ning Y, Hodges L, Gursky A, Siddiqui J, Tomlins SA, Roychowdhury S, Pienta KJ, Kim SY, Roberts JS, Rae JM, Van Poznak CH, Hayes DF, Chugh R, Kunju LP, Talpaz M, Schott AF, Chinnaiyan AM: Activating *ESR1* mutations in hormone-resistant metastatic breast cancer. *Nat Genet* 2013, 45:1446–1451
4. Toy W, Shen Y, Won H, Green B, Sakr RA, Will M, Gala K, Fanning S, King TA, Hudis C, Chen D, Hortobagyi G, Greene G,

- Berger M, Baselga J: ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. *Nat Genet* 2013, 45:1439–1445
5. Jeselsohn R, Buchwalter G, De Angelis C, Brown M, Schiff R: ESR1 mutations—a mechanism for acquired endocrine resistance in breast cancer. *Nat Rev Clin Oncol* 2016, 12:573–583
6. Chandarlapaty S, Chen D, He W, Sung P, Samoil A, You D, Bhatt T, Patel P, Voi M, Gnant M, Hortobagyi G, Baselga J, Moynahan ME: Prevalence of ESR1 mutations in cell-free DNA and outcomes in metastatic breast cancer: a secondary analysis of the BOLERO-2 clinical trial. *JAMA Oncol* 2016, 2:1310–1315
7. Reinert T, Saad ED, Barrios CH, Bines J: Clinical implications of ESR1 mutations in hormone receptor-positive advanced breast cancer. *Front Oncol* 2017, 7:26
8. Pantel K, Alix-Panabières C: Circulating tumour cells in cancer patients: challenges and perspectives. *Trends Mol Med* 2010, 16:398–406
9. Barradas AMC, Terstappen LW: Towards the biological understanding of CTC: capture technologies, definitions and potential to create metastasis. *Cancers (Basel)* 2013, 5:1619–1642
10. Yu M, Stott S, Toner M, Maheswaran S, Haber DA: Circulating tumor cells: approaches to isolation and characterization. *J Cell Biol* 2011, 192:373–382
11. Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, Tibbe AGJ, Uhr JW, Terstappen LW: Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. *Clin Cancer Res* 2005, 10:6897–6904
12. Janni WJ, Rack B, Terstappen LW, Pierga J-Y, Taran F-A, Fehm T, Hall C, de Groot MR, Bidard F-C, Friedl TWP, Fasching PA, Brucker SY, Pantel K, Lucci A: Pooled analysis of the prognostic relevance of circulating tumor cells in primary breast cancer. *Clin Cancer Res* 2016, 22:2583–2593
13. Bidard F-C, Michiels S, Riethdorf S, Mueller V, Esserman LJ, Lucci A, et al: Circulating tumor cells in breast cancer patients treated by neoadjuvant chemotherapy: a meta-analysis. *J Natl Cancer Inst* 2018, 110:560–567
14. Bidard FC, Peeters DJ, Fehm T, Nolé F, Gisbert-Criado R, Mavroudis D, et al: Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. *Lancet Oncol* 2014, 15:406–414
15. Cristofanilli M, Hayes DF, Budd GT, Ellis MJ, Stopeck A, Reuben JM, Doyle GV, Matera J, Allard WJ, Miller MC, Fritsche HA, Hortobagyi GN, Terstappen LW: Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. *J Clin Oncol* 2005, 23:1420–1430
16. Banny-Paluchowski M, Krawczyk N, Fehm T: Potential role of circulating tumor cell detection and monitoring in breast cancer: a review of current evidence. *Front Oncol* 2016, 6:255
17. Toy W, Weir H, Razavi P, Lawson M, Goeppert AU, Mazzola AM, Smith A, Wilson J, Morrow C, Wong WL, De Stanchina E, Carlson KE, Martin TS, Uddin S, Li Z, Fanning S, Katzenellenbogen JA, Greene G, Baselga J, Chandarlapaty S: Activating ESR1 mutations differentially affect the efficacy of ER antagonists. *Cancer Discov* 2017, 7:277–287
18. Martin L-A, Ribas R, Simigdala N, Schuster E, Pancholi S, Tenev T, Gellert P, Buluwela L, Harrod A, Thornhill A, Nikitorowicz-Buniak J, Bhamra A, Turgeon M, Poulgiannis G, Gao Q, Martins V, Hills M, Garcia-murillas I, Fribbens C, Patani N, Li Z, Sikora MJ, Turner N, Zwart W, Oesterreich S, Carroll J, Ali S, Dowsett M: Discovery of naturally occurring ESR1 mutations in breast cancer cell lines modelling endocrine resistance. *Nat Commun* 2017, 8:1865
19. Neumann MH, Schneck H, Decker Y, Schömer S, Franken A, Endris V, Pfarr N, Weichert W, Niederacher D, Fehm T, Neubauer H: Isolation and characterization of circulating tumor cells using a novel workflow combining the CellSearch system and the CellSelector. *Biotechnol Prog* 2017, 33:125–132
20. Wurster M, Ruoff A, Meisner C, Seeger H, Vogel U, Juhasz-Böss I, Solomayer E, Wallwiener D, Fehm T, Neubauer H: Evaluation of ERalpha, PR and ERbeta isoforms in neoadjuvant treated breast cancer. *Oncol Rep* 2010, 24:653–659
21. Afgan E, Baker D, van den Beek M, Blankenberg D, Bouvier D, Čech M, Chilton J, Clements D, Coraor N, Eberhard C, Grüning B, Guerler A, Hillman-Jackson J, Von Kuster G, Rasche E, Soranzo N, Turaga N, Taylor J, Nekrutenko A, Goecks J: The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. *Nucleic Acids Res* 2016, 44:W3–W10
22. Thorvaldsdóttir H, Robinson JT, Mesirov JP: Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Brief Bioinform* 2013, 14:178–192
23. Reinhardt F, Franken A, Fehm T, Neubauer H: Navigation through inter- and intratumoral heterogeneity of endocrine resistance mechanisms in breast cancer: a potential role for Liquid Biopsies? *Tumor Biol* 2017, 39. 10.10428317731511
24. Klein CA, Schmidt-Kittler O, Schardt JA, Pantel K, Speicher MR, Riethmüller G: Comparative genomic hybridization, loss of heterozygosity, and DNA sequence analysis of single cells. *Proc Natl Acad Sci U S A* 1999, 96:4494–4499
25. Duraj-Thatte A: Fluorescent Gfp Chromophores as Potential Ligands for Various Nuclear Receptors; 2012. ProQuest Dissertations and Theses
26. Adzhubei I, Jordan DM, Sunyaev SR: Predicting functional effect of human missense mutations using PolyPhen-2. *Curr Protoc Hum Genet* 2013. Chap 7:Unit 7.20
27. Tavtigian SV, Deffenbaugh AM, Yin L, Judkins T, Scholl T, Samollow PB, de Silva D, Zharkikh A, Thomas A: Comprehensive statistical study of 452 BRCA1 missense substitutions with classification of eight recurrent substitutions as neutral. *J Med Genet* 2006, 43:295–305
28. Vaser R, Adusumalli S, Leng SN, Sikic M, Ng PC: SIFT missense predictions for genomes. *Nat Protoc* 2016, 11:1–9
29. Choi Y, Chan AP: PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. *Bioinformatics* 2015, 31:2745–2747
30. Andersen TI, Wooster R, Laake K, Collins N, Warren W, Skrede M, Eeles R, Tveit KM, Johnston SR, Dowsett M, Olsen AO, Møller P, Stratton MR, Børresen-Dale AL: Screening for ESR mutations in breast and ovarian cancer patients. *Hum Mutat* 1997, 9:531–536
31. Shaw JA, Guttery DS, Hills A, Fernandez-Garcia D, Page K, Rosales BM, Goddard KS, Hastings RK, Luo J, Ogle O, Woodley L, Ali S, Stebbing J, Coombes RC: Mutation analysis of cell-free DNA and single circulating tumor cells in metastatic breast cancer patients with high circulating tumor cell counts. *Clin Cancer Res* 2017, 23:88–96
32. Bardia A, Iafate JA, Sundaresan T, Younger J, Nardi V: Metastatic breast cancer with ESR1 mutation: clinical management considerations from the molecular and precision medicine (MAP) tumor board at Massachusetts General Hospital. *Oncologist* 2016, 21:1035–1040
33. Yu M, Bardia A, Aceto N, Bersani F, Madden MW, Donaldson MC, Desai R, Zhu H, Comaills V, Zheng Z, Wittner BS, Stojanov P, Brachtel E, Sgroi D, Kapur R, Shioda T, Ting DT, Ramaswamy S, Getz G, Iafate AJ, Benes C, Toner M, Maheswaran S, Haber DA: Cancer therapy. Ex vivo culture of circulating breast tumor cells for individualized testing of drug susceptibility. *Science* 2014, 345:216–220
34. Clatot F, Perdrix A, Augusto L, Beaussire L, Delacour J, Calbrix C, Sefrioui D, Vially P-J, Bubenheim M, Moldovan C, Alexandru C, Tennevet I, Rigal O, Guillemet C, Leheurteur M, Gouéran S, Petrau C, Théry J-C, Picqueton J-M, Veyret C, Frébourg T, Jardin F, Sarafan-Vasseu N, Di Fiore F: Kinetics, prognostic and predictive values of ESR1 circulating mutations in metastatic breast cancer patients progressing on aromatase inhibitor. *Oncotarget* 2016, 7:74448–74459

35. Fribbens C, O'Leary B, Kilburn L, Hrebien S, Garcia-Murillas I, Beaney M, Cristofanilli M, Andre F, Loi S, Loibl S, Jiang J, Bartlett CH, Koehler M, Dowsett M, Bliss JM, Johnston SR, Turner NC: Plasma ESR1 mutations and the treatment of estrogen receptor-positive advanced breast cancer. *J Clin Oncol* 2016, 34:2961–2968
36. Schiavon G, Hrebien S, Garcia-Murillas I, Cutts RJ, Pearson A, Tarazona N, Fenwick K, Kozarewa I, Lopez-Knowles E, Ribas R, Nerurkar A, Osin P, Chandarlapaty S, Martin LA, Dowsett M, Smith IE, Turner NC: Analysis of ESR1 mutation in circulating tumor DNA demonstrates evolution during therapy for metastatic breast cancer. *Sci Transl Med* 2015, 7:313ra182
37. Zhang Q, Borg Å, Wolf DM, Borg A, Oesterreich S, Fuqua SA: An estrogen receptor mutant with strong hormone-independent activity from a metastatic breast cancer. *Cancer Res* 1997, 57:1244–1249
38. Weir HM, Bradbury RH, Lawson M, Rabow AA, Buttar D, Callis RJ, Curwen JO, de Almeida C, Ballard P, Hulse M, Donald CS, Feron LJJ, Karoutchi G, MacFaul P, Moss T, Norman RA, Pearson SE, Tonge M, Davies G, Walker GE, Wilson Z, Rowlinson R, Powell S, Sadler C, Richmond G, Ladd B, Pazolli E, Mazzola AM, D'Cruz C, De Savi C: AZD9496: an oral estrogen receptor inhibitor that blocks the growth of ER-positive and ESR1-mutant breast tumors in preclinical models. *Cancer Res* 2016, 76:3307–3318
39. Wang Y, Lonard DM, Yu Y, Chow DC, Palzkill TG, Wang J, Qi R, Matzuk AJ, Song X, Madoux F, Hodder P, Chase P, Griffin PR, Zhou S, Liao L, Xu J, O'Malley BW: Bufalin is a potent small-molecule inhibitor of the steroid receptor coactivators SRC-3 and SRC-1. *Cancer Res* 2014, 74:1506–1517
40. Polzer B, Medoro G, Pasch S, Fontana F, Zorzino L, Pestka A, Andergassen U, Meier-Stiegen F, Czyz ZT, Alberter B, Treitschke S, Schamberger T, Sergio M, Bregola G, Doffini A, Gianni S, Calanca A, Signorini G, Bolognesi C, Hartmann A, Fasching PA, Sandri MT, Rack B, Fehm T, Giorgini G, Manaresi N, Klein CA: Molecular profiling of single circulating tumor cells with diagnostic intention. *EMBO Mol Med* 2014, 6:1371–1386
41. Babayan A, Hannemann J, Spötter J, Müller V, Pantel K, Joosse SA: Heterogeneity of estrogen receptor expression in circulating tumor cells from metastatic breast cancer patients. *PLoS One* 2013, 8: e75038
42. Paoletti C, Larios JM, Muniz MC, Aung K, Cannell EM, Darga EP, Kidwell KM, Thomas DG, Tokudome N, Brown ME, Connelly MC, Chianese DA, Schott AF, Henry NL, Rae JM, Hayes DF: Heterogeneous estrogen receptor expression in circulating tumor cells suggests diverse mechanisms of fulvestrant resistance. *Mol Oncol* 2016, 10:1078–1085

3.2 Manuskript 2:

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Label-Free Enrichment and Molecular Characterization of Viable Circulating Tumor Cells from Diagnostic Leukapheresis Products

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INTRODUCTION: Circulating tumor cells (CTCs) may be used to improve cancer diagnosis, prognosis, and treatment. However, because knowledge regarding CTC biology is limited and the numbers of CTCs and CTC-positive cancer patients are low, progress in this field is slow. We addressed this limitation by combining diagnostic leukapheresis (DLA) and microfluidic enrichment to obtain large numbers of viable CTCs from metastasized breast cancer patients.

METHODS: DLA was applied to 9 patients, and 7.5 mL of peripheral blood was drawn. CTCs were enriched with the ParsortixTM system. The quality of CTCs from fresh and cryopreserved DLA products was tested, and CTCs were cultured in vitro. Single uncultured and cultured CTCs were isolated by micromanipulation to determine different parameters, such as genomic aberrations and mutation profiles of selected tumor-associated genes. Expression levels of estrogen receptor and HER2/neu were monitored during in vitro culture.

RESULTS: Viable CTCs from peripheral blood and fresh or frozen DLA products could be enriched. DLA increased the likelihood of successful CTC culture. Cryopreserved DLA products could be stored with minimal CTC loss and no overt reduction in the tumor cell quality and viability during an observation period of up to 3 years. The analyzed parameters did not change during in vitro culture. DLA samples with high CTC numbers and lower ratios of apoptotic CTCs were more likely to grow in culture.

CONCLUSIONS: The increased CTC numbers from fresh or cryopreserved DLA products facilitate multiple func-

tional and molecular analyses and, thus, could improve our knowledge of their biology.

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Solid tumors and metastases release circulating tumor cells (CTCs)⁵ into the circulatory system. These CTCs can genotypically and phenotypically differ from the primary tumor (1, 2). Most CTCs are either eliminated quickly or do not proliferate or perish owing to a shortage of nutrients (3). However, some CTCs survive and may extravasate into different tissues where they may persist and eventually initiate metastatic growth. Theoretically, CTCs can be easily obtained by drawing peripheral blood (PB). Thus, CTCs have a promising potential to improve diagnosis and treatment accuracy as a “liquid biopsy” (4, 5). CTC quantities in 7.5 mL of PB determined with the FDA-approved CellSearch[®] assay are correlated with decreased progression-free and overall survival in primary (6) and metastasized breast cancer patients (7, 8). In addition to the pure quantification of CTCs, their molecular analysis could provide information about their origin and biology, which could be used for drug selection. However, to date, this predictive utility of CTCs has not been demonstrated (9).

In particular, viable CTCs could be highly beneficial for obtaining further insight into their source and may increase the clinical utility of liquid biopsies. They could be used in drug testing and to investigate the functional properties and mechanisms of metastasis formation (10). Although a few CTC cell lines have already been established from different cancer entities, culturing CTCs still remains a challenging task, and culture success has

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⁵ Nonstandard abbreviations: CTC, circulating tumor cell; PB, peripheral blood; DLA, diagnostic leukapheresis; MNCs, mononuclear cells; WBCs, white blood cells; CK, cytokeratin; WGA, whole genome amplification; aCGH, array comparative genome hybridization; ER, estrogen receptor; HER2, HER2/neu.

mainly been limited to CTCs from a very few patients carrying remarkably high CTC numbers (11–15).

For the routine clinical use of cultured CTCs, improving the CTC yields per patient is necessary and could broaden the number of patients from which CTC cultures can be successfully derived. This major challenge can be solved by the implementation of diagnostic leukapheresis (DLA) in the CTC detection work flow. Leukapheresis is a standard procedure routinely used in the clinic to enrich mononuclear cells (MNCs) from blood for various applications, including stem cell harvest. During DLA, MNCs are continuously and extracorporeally separated by density from other blood components with constant centrifugation. By enlarging the analyzed blood volume up to several liters, DLA increases the CTC number obtained per patient and the patients' CTC positivity rates (16).

The primary aim of this project was to assess the feasibility of culturing CTCs from fresh and frozen DLA products compared to PB. CTC enrichment from DLA product with the microfluidic Parsortix system was established and optimized with MCF7 cells to maintain the viability of enriched tumor cells for further culture approaches. Furthermore, molecular analyses of cultured and uncultured CTCs were performed.

Materials and Methods

Further protocols can be found in the Supplemental Methods in the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol65/issue4>.

DIAGNOSTIC LEUKAPHERESIS

DLA was performed as previously described for 1 h (16). This study was carried out in accordance with the Good Clinical Practice guidelines and was approved by the Ethics Committee of the Medical Faculty of the Heinrich Heine University Düsseldorf (Ref-No: 3460). All patients enrolled in this study provided written informed consent. The clinical patient data are presented in Table 1 in the online Data Supplement. Aliquots of the DLA product were used for CTC enumeration and CTC culture or frozen and stored for future use.

For cryopreservation, 10^8 MNCs from the DLA samples were frozen in 2 mL of freezing medium consisting of 45% RPMI 1640 medium (Thermo Fisher Scientific Inc.), 45% HSA (Octapharma AG), and 10% DMSO (Sigma-Aldrich Corporation) after platelet washing at 200g for 10 min and then stored in liquid nitrogen.

To determine whether long-term storage affects tumor cell recovery and quality, 5000 KYSE30 cells were added into DLA products from healthy donors and cryopreserved for over 5 years.

Frozen DLA samples were rapidly thawed in a 37 °C water bath and filtered through a 100- μ m sieve before enriching CTCs with CellSearch (Menarini Group) or Parsortix™ system (Angle plc).

ENUMERATION OF CTCs IN PB AND DLA PRODUCTS

The enrichment and enumeration of the CTCs was performed with the CellSearch Circulating Epithelial Cell Kit (Menarini) according to the manufacturer's instructions.

ENRICHMENT OF VIABLE CTCs FROM PB OR DLA PRODUCTS

Viable CTCs were enriched from 7.5 mL of PB or DLA product adjusted to 2×10^8 MNCs with the Parsortix system. The system was operated according to the manufacturer's instructions after a sterilization step with 70% ethanol before sample analysis. Parsortix cassettes with a 6.5- μ m gap were used, and 100 mbar of pressure was applied. Before the Parsortix enrichment, DLA product was diluted in PBS (1:20).

To determine the capturing and harvesting rates of the Parsortix system, 100 MCF7 cells were prelabeled with CellTracker green (Thermo Fisher Scientific) and added into fresh or frozen CTC-negative DLA product with a FACS system (MoFlo XDP sorter, Beckman Coulter Inc.). Subsequently, the number of prelabeled cells captured in the Parsortix cassette during enrichment and the number of released cells after harvesting were counted by fluorescence microscopy.

The efficiency of the Parsortix enrichment of patients' CTCs was compared to the performance of CellSearch Profile Kit. Then, the enriched cell fractions were stained in parallel for DAPI, CK, EpCAM, and CD45 to identify and enumerate the enriched CTCs.

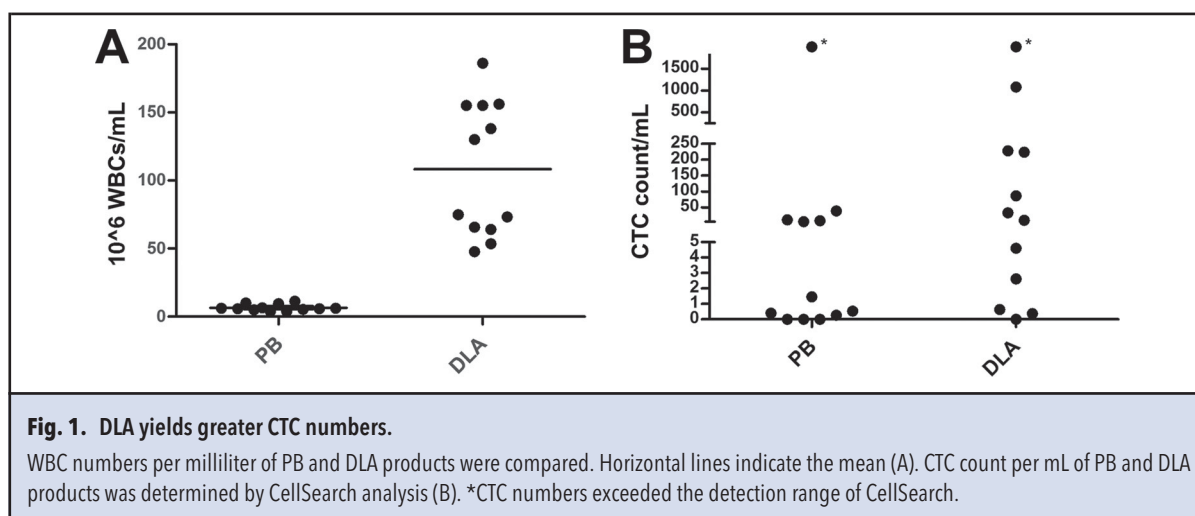
CTC CULTURE

CTCs were grown in Tumor Sphere Medium (13). Following this protocol, CTCs were cultured in low attachment plates (Corning Inc.) with RPMI 1640 medium supplemented with $1 \times B27$ (Thermo Fisher Scientific), 20 ng/mL human epidermal growth factor (Sigma-Aldrich), 20 ng/mL fibroblast growth factor (Sigma-Aldrich), and 1% penicillin-streptomycin (Thermo Fisher Scientific) in a humidified atmosphere with 5% CO₂ and 4% O₂.

Results

DLA YIELDS HIGHER CTC NUMBERS

During 12 DLAs of metastasized breast cancer patients, MNCs of a mean volume of 3.41 ± 0.91 L of blood were enriched. Compared to the number of MNCs obtained in 7.5 mL PB, DLA led to increased numbers of MNCs, which could be screened for CTCs. In PB, a mean of $6.67 \times 10^6 \pm 2.40 \times 10^6$ white blood cells (WBCs) per mL was observed, whereas the DLA products contained a



mean of $108.28 \times 10^6 \pm 49.36 \times 10^6$ WBCs per mL (Fig. 1A).

CTCs were detected in 11 PB and matching DLA product samples with CellSearch analysis. CTC numbers per mL were increased by a mean of 20.7 ± 9.9 -fold in DLA products. One patient whose CTC numbers exceeded the CellSearch detection range was excluded from this calculation. Additionally, in 2 DLA samples with corresponding CTC-negative PB samples, at least 1 CTC was detected (Fig. 1B). Of note, no significant difference was observed between CTC enrichment and WBC enrichment (P value = 0.558, determined by paired 2-tailed t -test).

PARSORTIX™ SYSTEM ENABLES TUMOR CELL ENRICHMENT FROM DLA PRODUCTS

Subsequently, the ability to culture CTCs enriched from DLA products and patient-matched PB samples was compared with the Parsortix system. In this system, CTCs are captured on the basis of their less deformable nature and larger size compared to those of other cellular blood components.

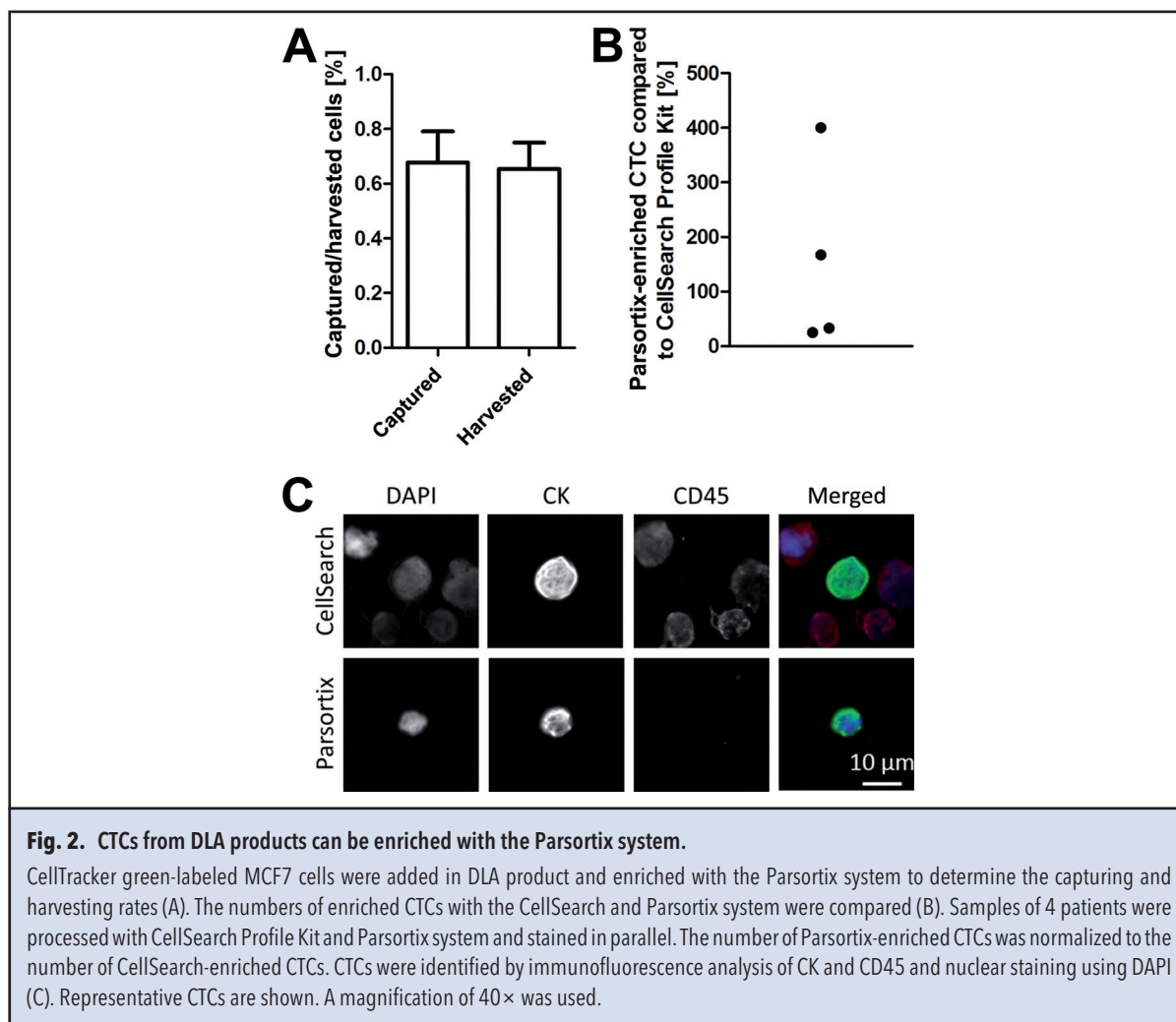
First, the Parsortix system was adapted to process DLA products. The efficacy of enriching tumor cells from DLA products was determined with CellTracker green-labeled MCF7 breast cancer cells that had been added into DLA products. A capturing rate of $67.7 \pm 11.3\%$ and a harvesting rate of $65.3 \pm 9.7\%$ were determined, which led to a total recovery rate of 44.2% of supplemented MCF7 cells (Fig. 2A).

Subsequently, the CTC enrichment rate of the Parsortix system using DLA samples from 4 patients was compared to the performance of the CTC enrichment approach using CellSearch. With the Parsortix system, CTCs were enriched by 0.2- to 4-fold compared to those using CellSearch (Fig. 2B). Immunofluorescence analysis

of the Parsortix-enriched samples exceeding the CTC numbers obtained with CellSearch confirmed the presence of CTCs with low EpCAM expression (see Fig. 1 in the online Data Supplement). Notably, the Parsortix-enriched CTCs had a significantly smaller mean size of $10.01 \pm 1.96 \mu\text{m}$ than the mean size of $12.55 \pm 1.36 \mu\text{m}$ observed with CellSearch (P value = 0.0015, determined by 2-tailed t -test; Fig. 2C, and see Fig. 2A in the online Data Supplement). The same finding was observed with added MCF7 cells. This effect was independent of the different pressures used to process the samples in the Parsortix system (see Fig. 2B in the online Data Supplement).

CTCs FROM DLA PRODUCTS CAN BE ISOLATED BY THE PARSORTIX SYSTEM FOR SUBSEQUENT CULTURING

After enriching CTCs from DLA products with the Parsortix system, we aimed to determine whether the CTC viability was affected and whether these cells could be used for further functional studies. First, the effect of different pressures on the viability of enriched MCF7 breast cancer cells was determined by measuring the mitochondrial reductase activity (MTT assay). Reducing the pressure from 100 to 50 mbar did not increase the viability of the enriched tumor cells (see Fig. 2C in the online Data Supplement). Then, CTCs enriched and harvested as described above were tested for in vitro cultivation. Growing cells were isolated and stained for neoplastic and hematopoietic markers after 1 week. Cells displaying a nuclear DAPI signal, CK positivity (CK 4, 5, 6, 8, 10, 13 or 18), and CD45 negativity by immunofluorescence microscopy were identified as CTCs. Cultivable CTCs could be obtained in 2 of the 7 DLA samples classified as "CTC positive" by CellSearch. In sample #3, growing CTCs were obtained from both PB and the DLA product. In sample #1, we obtained growing CTCs



from the DLA product but not the PB. These 2 samples had the highest CTC counts according to CellSearch analysis (Table 1). Cultured CTCs formed clusters and could be cultured and expanded for several months for sample #3 (see Fig. 3A in the online Data Supplement). Notably, despite EpCAM-independent enrichment, immunofluorescence analysis revealed EpCAM positivity of all cultured CTCs (see Fig. 3B in the online Data Supplement). Short tandem repeat analyses confirmed patient origin of the cultured CTCs (see Fig. 3C in the online Data Supplement). Furthermore, array comparative genome hybridization (aCGH) analyses of amplified genomes from single isolated CTCs cultured for 3 months were performed. Several genomic aberrations, such as smaller gains and larger losses, especially in chromosomes 1p, 6, 16q, and 17p, were detected in the cultured CTCs, confirming their malignant origin (Fig. 3). It is worth mentioning that without previous Parsortix enrichment, blood cells rapidly and massively overgrew the CTC cultures.

CRYOPRESERVATION MAINTAINS CTC QUALITY AND VIABILITY

In total, a mean yield of 51.7 mL (maximum yield, 122 mL; minimum yield, 35 mL) was achieved per DLA. Only a small portion of the total volume is used for routine analyses, and the remaining DLA product can be cryopreserved after platelet washing and stored for future analysis and cross-validation experiments. Platelet washing was required for successful processing of thawed DLA products with the Parsortix system. Without platelet washing, processing of thawed DLA products with Parsortix resulted in clogging.

We aimed to (a) determine the CTC loss due to cryopreservation, (b) compare the quality of CellSearch-enriched CTCs from fresh and cryopreserved DLA products, and (c) investigate whether CTCs enriched from cryopreserved DLA samples could be cultured.

First, the enrichment of tumor cells from cryopreserved DLA products was tested with EpCAM-positive KYSE30 cells that had been added into DLA products from healthy donors more than 5 years ago and have been

Table 1. CTC positivity rates in PB and corresponding DLA products and the success of CTC culture.^a

Sample ID	PB		DLA product		
	CTC count	Culture success	Fresh		Cryopreserved
			CTC count	Culture success	Culture success
#1	34	–	320	+	+
#2	0	–	1	–	ND ^b
#3	^c	+	^c	+	+
#4	11	–	50	–	–
#5	0	–	2	–	ND
#6	2	–	14	–	–
#7	0	–	0	–	ND
#8	3	–	11	–	–
#9	291	ND	2913	ND	+
#10	63	ND	223	ND	–
#11	83	ND	296	ND	–
#12	4	ND	6	ND	–

^a Culture success of CTCs obtained from PB and fresh or frozen DLA products was compared. Samples with cultivable CTCs are marked with "+". CTCs were enumerated by CellSearch® analysis of a sample with the same volume in parallel.

^b ND, not determined.

^c CTC numbers exceeded the detection range of CellSearch®.

cryopreserved since. The recovery rate with CellSearch was 81.2% ± 4.9%. Then, CTC count and morphology of cryopreserved patient samples were compared to those of matched samples that had been analyzed immediately after the DLA procedure by CellSearch analysis. The morphology of detected CTCs was compared by evaluating DAPI and cytokeratin (CK) signals, and the CTCs were grouped into 4 classes according to whether the nuclei and cytoplasm were intact or fragmented (see Fig. 4 in the online Data Supplement). A mean total CTC loss of 16.6 ± 11.2% was observed. However, the number of CTCs with an intact nucleus and intact cytoplasm was reduced by a mean of only 7.6% ± 4.8% (Fig. 4A).

The genomic integrity of single KYSE30 cells and CTCs from cryopreserved DLA products was analyzed by whole-genome amplification (WGA) and following Ampli1™ quality control. The analyzed KYSE30 cells showed a mean GII of 3.77. Furthermore, no reduction in GII of amplified genomic DNA of the CTCs was observed on cryopreservation (Fig. 4B).

On thawing, 86.2% ± 7.3% of all MNCs from cryopreserved DLA products from breast cancer patients were viable determined by propidium iodide staining. To compare cultivation performance of CTCs enriched from cryopreserved and fresh DLA products, 9 samples of cryopreserved DLA product containing more than 5 CTCs per 1 × 10⁸ MNCs as determined by CellSearch analysis were chosen. Analysis of the corresponding fresh samples had been performed in 5 samples. We observed no differences in Parsortix enrichment of thawed cryopreserved DLA products. CTCs originating from 3 of

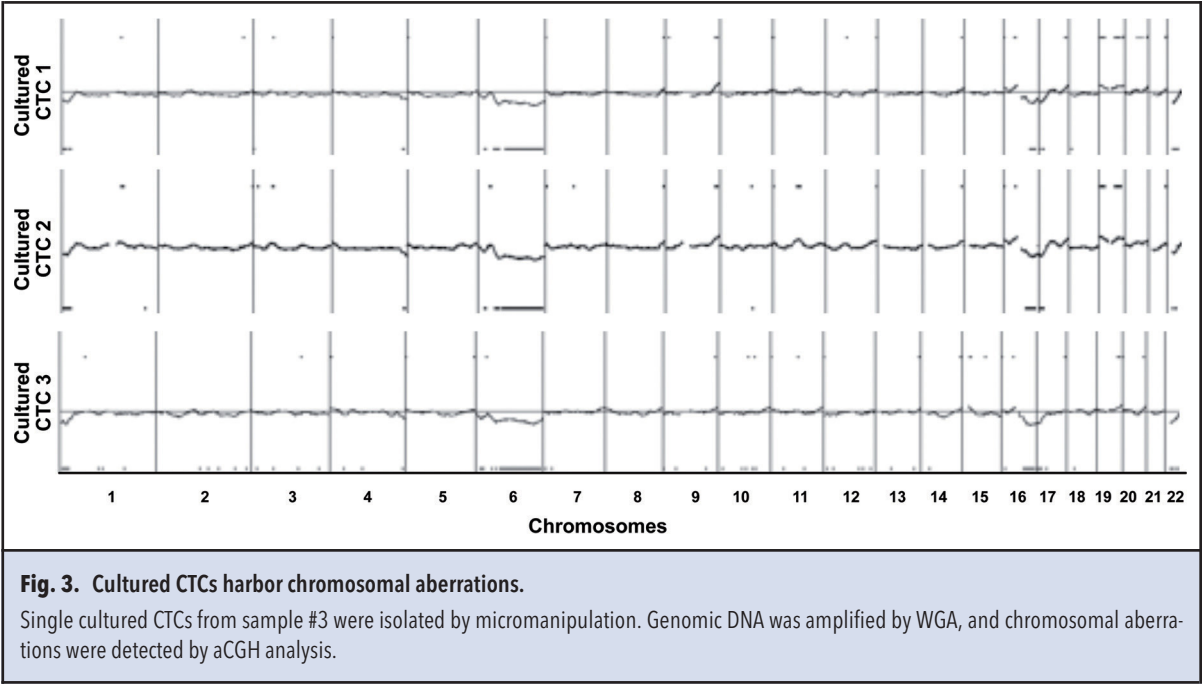


Fig. 3. Cultured CTCs harbor chromosomal aberrations. Single cultured CTCs from sample #3 were isolated by micromanipulation. Genomic DNA was amplified by WGA, and chromosomal aberrations were detected by aCGH analysis.

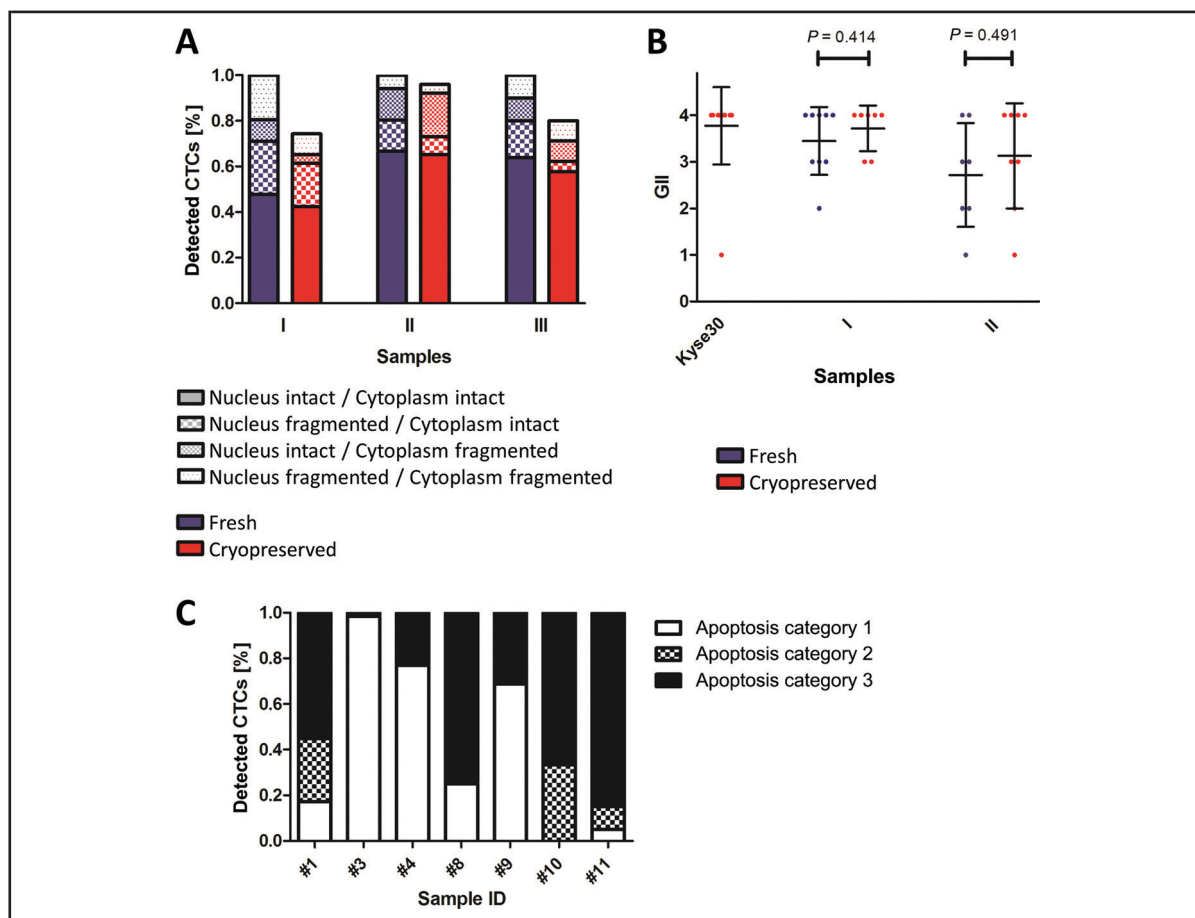


Fig. 4. Cryopreservation of DLA products only slightly impairs CTC quantity and quality.

CTCs from fresh and cryopreserved DLA products were enumerated by CellSearch analysis (A). Numbers of CTCs in thawed DLA products were normalized to CTC numbers in fresh samples. Morphology of nuclei and cytoplasm was analyzed, and the cells were grouped into 4 categories (see Fig. 4 in the online Data Supplement). Genomes of single tumor cells—either supplemented KYSE30 cells or patient CTCs—isolated from fresh and cryopreserved DLA products were amplified by WGA (B). GII was determined by Ampli1 QC. *P* values were determined by 2-tailed *t*-test. No significant difference was observed in GII between the fresh and cryopreserved tumor cells. In all cases, very few numbers of 0-band samples were obtained most likely owing to cell loss during the single-cell isolation process and are, therefore, not shown. CTCs enriched with the CellSearch Profile Kit from thawed DLA product were stained by immunofluorescence analysis for caspase-cleaved CK to determine apoptosis (C). CTCs were grouped into 3 “apoptotic categories” according to the detected nuclear staining and caspase-cleaved CK pattern (see Fig. 5 in the online Data Supplement).

these 9 samples were successfully cultured. As described above for the other 2 samples, the third frozen sample containing cultivable CTCs also had particularly high CTC numbers in the DLA product (Table 1). These results were consistent with the culture success using fresh samples and confirmed viability of cryopreserved CTCs. Of note, no differences in growth morphology between cultured CTCs from fresh or cryopreserved DLA products were observed.

To compare culture success to CTC quality, CTCs that had been enriched with the same DLA products by the CellSearch Profile Kit were analyzed for caspase-

cleaved CK as a surrogate for apoptosis by immunofluorescence analysis. First, specificity of the antibody (M30) was tested with MCF7 cells that had been treated with apoptosis-inducing bisphosphonate zoledronic acid (see Fig. 5A in the online Data Supplement). Three apoptotic states were defined according to the intensity of the M30 signal and the appearance of intact or fragmented cytoplasm as determined by the CK pattern (see Fig. 5B in the online Data Supplement). Applying this classification to CTCs from the DLA products, heterogeneous degrees of apoptosis were observed: 1.6%–100% of the CTCs exhibited signs of apoptosis. As expected, the DLA samples

with cultivable CTCs contained higher ratios of nonapoptotic CTCs, whereas the samples with comparatively high CTC numbers that were not cultivable showed higher ratios of apoptotic CTCs (Fig. 4C).

CULTURED CTCs MAINTAIN THEIR PHENOTYPE AND GENOTYPE

If cultured CTCs are to be used for further characterization, such as chemosensitivity assays, it is critical for these CTCs to maintain their phenotypic and genotypic characteristics and heterogeneity during culture. Thus, we analyzed chromosomal aberrations and mutations in 50 major oncogenes and tumor suppressor genes in the cultured CTCs from sample #3 after 3 months of expansion. Analysis of chromosomal aberrations by aCGH analysis revealed largely identical aberrations. In all cultured and uncultured CTCs, the same prominent losses in chromosomes 1p, 6, 16q, and 17p were detected (Fig. 5A, and see Fig. 6 in the online Data Supplement). Targeted panel sequencing revealed that mutation profiles of the cultured and uncultured CTCs from this patient were identical (see Table 2 in the online Data Supplement).

In addition, the expression of estrogen receptor (ER) and HER2/neu (HER2) was determined over 4 weeks of culture by CellSearch analysis. These CTCs were compared to uncultured CTCs isolated directly from the DLA product. Heterogeneous ER expression was observed in single cells, and the percentage of ER positivity was unaltered during culture. Specifically, two-thirds of both uncultured and cultured CTCs were determined to be ER-positive (Fig. 5, B and C). Although the primary tumor was HER2 negative, 90% of the uncultured CTCs showed expression of HER2. During culture, the proportion of HER2-positive CTCs slightly increased to 95% and remained stable (Fig. 5, D and E).

Discussion

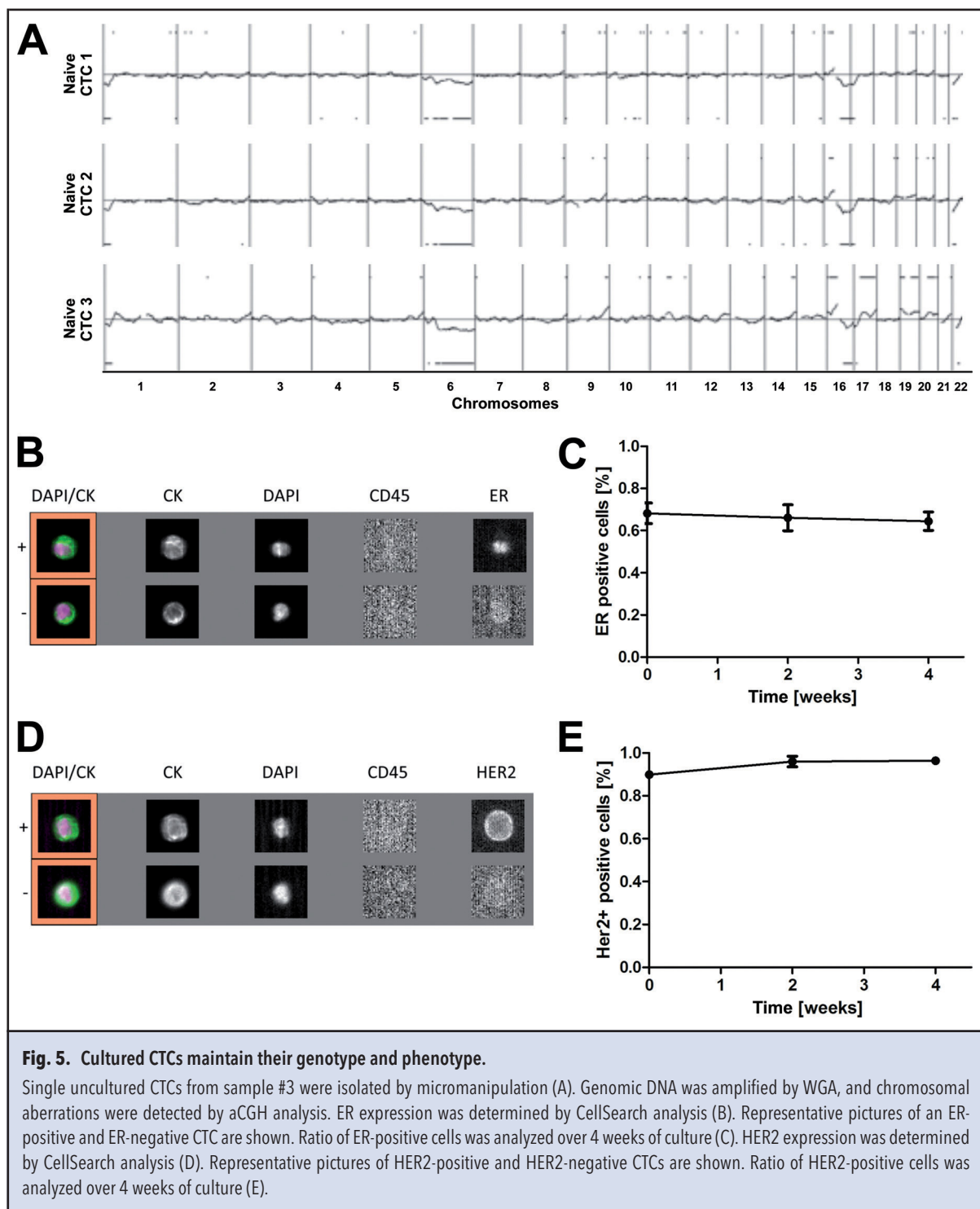
Low CTC numbers in PB detected with different technologies are challenging for CTC analyses in general and CTC culture in particular. In contrast, DLA of larger blood volumes provides considerably more MNCs and by this much higher numbers of CTCs per cancer patient. In addition, DLA results in higher CTC positivity rates (16). These advantages of DLA may enable the diagnostic deficiencies of PB to be surmounted, which will be crucial for the successful implementation of CTC analyses in clinical diagnostics and patient management (17). Culturing CTCs from blood is still mainly limited to cancer patients with remarkably high CTC numbers (11, 12). Because of the higher numbers of CTCs obtained from DLA products, we attempted to culture CTCs from DLA products.

Although DLA provides higher CTC numbers, it is only a preenrichment step. In our selected cases, CTCs

were not enriched relative to WBCs during DLA. Thus, a CTC enrichment technology is required after DLA to separate the CTCs from other blood-borne cells for further characterization (16), which is especially important for in vitro culture because these blood cells would outgrow the rare CTC populations. Viable CTCs can be enriched on the basis of their surface protein production by positive or negative selection, based on their physical properties or based on their functional properties (10). Among several available technologies, we chose the label-free Parsortix system, which separates CTCs from other blood components by size and deformability while keeping the tumor cells viable (18, 19). Hence, the tumor cells are enriched regardless of the expression of specific surface markers, also enabling the capture of EpCAM-negative cells (20). Here, we observed enrichment of EpCAM-negative CTCs from DLA products, which could play a role in successfully deriving CTC cultures (21). However, we did not observe growing EpCAM-negative CTC clones despite the coseeding of both EpCAM-positive and EpCAM-low or EpCAM-negative CTCs. In our experiments, we showed that CTC enrichment with the Parsortix system not only keeps the captured CTCs viable but also enables subsequent in vitro culture by effectively depleting other blood components. Our experiments also demonstrate that CTCs can retain their viability and capacity to grow in vitro during the DLA procedure. Furthermore, our observations in sample #1, in which growing CTCs could only be derived from DLA product but not from PB, indicate that using the DLA product might be advantageous for CTC culture; this advantage is most likely due to higher CTC numbers in the DLA product, which may be critical for obtaining enough of the few CTCs for in vitro culture.

All breast cancer patients with cultivable CTCs had high CTC numbers. Their primary tumors were of luminal subtype, and the patients showed metastatic spread to the bone. Notably, the optimal culture conditions were determined with CTCs from patient VIII, and therefore, these conditions might be particularly suitable for CTCs derived from tumors of a similar subtype. Of note, similar observations were reported by Yu et al., who developed the Tumor Sphere Medium for CTC culture (13). However, a primary tumor of luminal subtype and bone metastasis also correlate with a higher rate of CTC positivity (7, 22).

Reasonably, a lower proportion of apoptotic CTCs in DLA product was found to predict culture success. This finding is not in contradiction to the clinical situation in which high numbers or even high rates of apoptotic CTCs correlate with poor, rather than good, prognoses (23–25). One explanation for this observation may be that in the first case, the viability of CTCs is a requisite for their in vitro growth. In the second scenario, the presence of apoptotic CTCs is most likely a byproduct of fast-growing



metastasis, which influences prognosis (26). This result points toward a different relevance of CTCs depending on the readouts with which they are associated.

Despite our DLA approach, only CTCs from a few patients could be successfully cultured. However, to fur-

ther understand the biology of CTCs, it is critical to combine data from more cultivable CTCs derived from a larger patient cohort. Thus, cryopreservation becomes an important requirement. Cryopreservation of leukapheresis products is widely used for preserving hematopoietic

stem cells for future use (27, 28). We tested the storage of cryopreserved DLA products and observed that more than 80% of supplemented cell line cells could be recovered with the work flow described here after banking for over 5 years. Furthermore, we observed a particularly low loss of tumor cells with higher quality. Moreover, the morphology and quality were unaffected by cryopreservation, and CTCs from the same fresh and cryopreserved patient samples could be cultured in vitro. Thus, cryopreservation allows long-term storage and easy distribution of uncultured primary CTCs, for example, between different clinical and research sites.

In one sample followed as an example for 3 months, the cultured CTCs were remarkably stable with no differences in the distribution of chromosomal aberrations or mutations compared to their uncultured counterparts. The *AKT1*⁶ E17K mutation, which has been hypothesized to be a disease driver in certain breast cancer patients (29), has been observed in most primary and cultured CTCs. Although only few genomic aberrations were found in this case, detected losses have previously been associated with breast cancer (30–32). Furthermore, the ratios of HER2- and ER-expressing CTCs remain stable during culture.

We acknowledge that some of our data resulted from the in-depth analysis of CTCs obtained from 1 patient suffering from extremely high numbers of CTCs. Therefore, our data may not be applicable to all CTCs or CTCs from all breast cancer subtypes. However, such cases provide an opportunity for investigations that are still in their initial phase and must be complemented by data obtained from breast cancer and other tumor patients.

In summary, we have presented data suggesting that DLA is an alternative source of higher numbers of CTCs for further in vitro culturing. Successful CTC culturing was observed even in 1 case in which no CTCs could be cultivated from PB. Viable CTCs from both PB and fresh or frozen DLA products can be enriched with the Par-

sortix system. Cryopreservation of DLA products enables their long-term storage with a low CTC loss and no effect on tumor cell quality or viability. Culturing CTCs from DLA has the potential to promote the power of functional studies for a better understanding of the biology of CTCs and increasing the number of patients who could benefit from ex vivo drug testing.

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⁶ Human Genes: *AKT1*, AKT serine/threonine kinase 1.

References

1. Yu M, Stott S, Toner M, Maheswaran S, Haber DA. Circulating tumor cells: approaches to isolation and characterization. *J Cell Biol* 2011;192:373–82.
2. Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, et al. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. *Clin Cancer Res* 2005;10:6897–904.
3. Vanharanta S, Massagué J. Origins of metastatic traits. *Cancer Cell* 2013;24:410–21.
4. Pantel K, Alix-Panabières C. Circulating tumour cells in cancer patients: challenges and perspectives. *Trends Mol Med* 2010;16:398–406.
5. Barradas AMC, Terstappen LWMM. Towards the biological understanding of CTC: capture technologies, definitions and potential to create metastasis. *Cancers* 2013; 5:1619–42.
6. Janni WJ, Rack B, Terstappen LWMM, Pierga J-Y, Taran FA, Fehm T, et al. Pooled analysis of the prognostic relevance of circulating tumor cells in primary breast cancer. *Clin Cancer Res* 2016;22:2583–93.
7. Bidard FC, Peeters DJ, Fehm T, Nolé F, Gisbert-Criado R, Mavroudis D, et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. *Lancet Oncol* 2014; 15:406–14.
8. Cristofanilli M, Hayes DF, Budd GT, Ellis MJ, Stopeck A, Reuben JM, et al. Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. *J Clin Oncol* 2005;23:1420–30.
9. Banys-Paluchowski M, Krawczyk N, Fehm T. Potential role of circulating tumor cell detection and monitoring in breast cancer: a review of current evidence. *Front Oncol* 2016;6:1–9.
10. Pantel K, Alix-Panabières C. Functional studies on viable circulating tumor cells. *Clin Chem* 2016;62:328–34.
11. Baccelli I, Schneeweiss A, Riethdorf S, Stenzinger A, Schillert A, Vogel V, et al. Identification of a population of blood circulating tumor cells from breast cancer patients that initiates metastasis in a xenograft assay. *Nat Biotechnol* 2013;31:539–44.

12. Cayrefourcq L, Mazard T, Joosse S, Solassol J, Ramos J, Assenat E, et al. Establishment and characterization of a cell line from human circulating colon cancer cells. *Cancer Res* 2015;75:892-901.
13. Yu M, Bardia A, Aceto N, Bersani F, Madden MW, Donaldson MC, et al. Ex vivo culture of circulating breast tumor cells for individualized testing of drug susceptibility. *Science* 2014;345:216-20.
14. Zhang Z, Shiratsuchi H, Lin J, Chen G, Reddy RM, Azizi E, et al. Expansion of CTCs from early stage lung cancer patients using a microfluidic co-culture model. *Onco-target* 2014;5:12383-97.
15. Gao D, Vela I, Sboner A, Iaquinta PJ, Wouter R, Arora VK, et al. Organoid cultures derived from patients with advanced prostate cancer. *Cell* 2014;159:176-87.
16. Fischer JC, Niederacher D, Topp SA, Honisch E, Schumacher S, Schmitz N, et al. Diagnostic leukapheresis enables reliable detection of circulating tumor cells of nonmetastatic cancer patients. *Proc Natl Acad Sci* 2013;110:16580-5.
17. Stoecklein NH, Fischer JC, Niederacher D, Terstappen LWMM. Challenges for CTC-based liquid biopsies: low CTC frequency and diagnostic leukapheresis as a potential solution. *Expert Rev Mol Diagn* 2016;16:147-64.
18. Xu L, Mao X, Imrali A, Syed F, Mutsvangwa K, Berney D, et al. Optimization and evaluation of a novel size based circulating tumor cell isolation system. *PLoS One* 2015;10:1-23.
19. Hvichia GE, Parveen Z, Wagner C, Janning M, Quidde J, Stein A, et al. A novel microfluidic platform for size and deformability based separation and the subsequent molecular characterization of viable circulating tumor cells. *Int J Cancer* 2016;138:2894-904.
20. Lampignano R, Yang L, Neumann MHD, Franken A, Fehm T, Niederacher D, et al. A novel workflow to enrich and isolate patient-matched EpCAMhigh and EpCAM-low/negative CTCs enables the comparative characterization of the PIK3CA status in metastatic breast cancer. *Int J Mol Sci* 2017;18:1885.
21. Zhang L, Ridgway LD, Wetzel MA, Ngo J, Yin W, Kumar D, et al. The identification and characterization of breast cancer CTCs competent for brain metastasis. *Sci Transl Med* 2013;5.
22. De Gregorio A, Friedl TWP, Huober J, Scholz C, De Gregorio N, Rack B, et al. Discordance in human epidermal growth factor receptor 2 (HER2) phenotype between primary tumor and circulating tumor cells in women with HER2-negative metastatic breast cancer. *JCO Precis Oncol* 2017;2:1-12.
23. Smerage JB, Budd GT, Doyle GV, Brown M, Paoletti C, Muniz M, et al. Monitoring apoptosis and Bcl-2 on circulating tumor cells in patients with metastatic breast cancer. *Mol Oncol* 2013;7:680-92.
24. Deutsch TM, Riethdorf S, Nees J, Hartkopf AD, Schönfisch B, Domschke C, et al. Impact of apoptotic circulating tumor cells (aCTC) in metastatic breast cancer. *Breast Cancer Res Treat* 2016;160:277-90.
25. Jansson S, Bendahl P-O, Larsson A-M, Aaltonen KE, Rydén L. Prognostic impact of circulating tumor cell apoptosis and clusters in serial blood samples from patients with metastatic breast cancer in a prospective observational cohort. *BMC Cancer* 2016;16:433.
26. Rupa JD, De Bruïne AP, Gerbers AJ, Leers MPG, Nap M, Kessels AGH, et al. Simultaneous detection of apoptosis and proliferation in colorectal carcinoma by multiparameter flow cytometry allows separation of high and low-turnover tumors with distinct clinical outcome. *Cancer* 2003;97:2404-11.
27. Fisher V, Khuu H, David-Ocampo V, Byrne K, Pavletic S, Bishop M, et al. Analysis of the recovery of cryopreserved and thawed CD34+ and CD3+ cells collected for hematopoietic transplantation. *Transfusion* 2014;54:1088-92.
28. Lecchi L, Giovanelli S, Gagliardi B, Pezzali I, Ratti I, Marconi M. An update on methods for cryopreservation and thawing of hemopoietic stem cells. *Transfus Apher Sci* 2016;54:324-36.
29. Rudolph M, Anzeneder T, Schulz A, Beckmann G, Byrne AT, Jeffers M, et al. AKT1E17K mutation profiling in breast cancer: prevalence, concurrent oncogenic alterations, and blood-based detection. *BMC Cancer* 2016;16:1-12.
30. Novello CC, Courjal F. Loss of heterozygosity on the long arm of chromosome 6 in breast cancer: possibly four regions of deletion. *Clin Cancer Res* 1996;2:1601-6.
31. Brunelli M, Nottegar A, Bogina G, Calì A, Cima L, Eccher A, et al. Monosomy of chromosome 17 in breast cancer during interpretation of HER2 gene amplification. *Am J Cancer Res* 2015;5:2212-21.
32. Rakha EA, Green AR, Powe DG, Roylance R, Ellis IO. Chromosome 16 tumor-suppressor genes in breast cancer. *Genes Chromosomes Cancer* 2006;45:527-35.

3.3 Manuskript 3:

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Multiparametric circulating tumor cell analysis to select targeted therapies for breast cancer patients

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Simple Summary

Liquid biopsies may act as a dynamic tool for identification of targets for precision therapy while circumventing limitations of tissue biopsies. In opposite to most liquid biopsy-related studies that are analyzing limited patient material for only one parameter this study is based on a longitudinal and multiparametric analysis of circulating tumor cells (CTCs). A metastatic breast cancer patient was followed over a period of three years and analyses of the genome, RNA profiling, and in-vitro drug testing on cultured CTCs were performed in a unique manner. We show that combining the strengths of multiple technologies for analysis yielded maximum information on the ongoing disease and, eventually, allowed choosing an effective therapy, which led to a massive reduction of CTC numbers. This approach provides a concept for future detailed longitudinal and multiparametric CTC analyses.

Abstract

Background: The analysis of liquid biopsies e.g. circulating tumor cells (CTCs) is an appealing diagnostic concept for targeted therapy selection. In this proof-of-concept study, we aimed to perform multiparametric analyses of CTCs to select targeted therapies for metastatic breast cancer patients. **Methods:** First, CTCs of five metastatic breast cancer patients were analyzed by whole exome sequencing (WES). Based on the results, one patient was selected and monitored by longitudinal and multiparametric liquid biopsy analyses over more than three years, including WES, RNA profiling and in vitro drug testing of CTCs. **Results:** Mutations addressable by targeted therapies were detected in all patients, including mutations that were not detected in biopsies of the primary tumor. For the index patient, the clonal evolution of the tumor cells was retraced

49 and resistance mechanisms were identified. The AKT1 E17K mutation was uncovered as the driver
50 of the metastatic process. Drug testing on the patient's CTCs confirmed the efficacy of drugs
51 targeting the AKT1 pathway. During a targeted therapy chosen based on the CTC characterization
52 and including the mTOR inhibitor everolimus, CTC numbers dropped by 97.3% and the disease
53 remained stable as determined by computer tomography/magnetic resonance imaging. Conclusion:
54 These results illustrate the strength of a multiparametric CTC analysis to choose and validate
55 targeted therapies to optimize cancer treatment in future. Furthermore, from a scientific point of
56 view, such studies promote the understanding of the biology of CTCs during different treatment
57 regimens.

58

59 Keywords

60 AKT1 / Breast Cancer / Circulating Tumor Cells / Targeted Therapy / Whole Exome Sequencing

Introduction

In the last decades, targeted therapy has become the preferred treatment approach in many cancers. However, obtaining information to choose targeted therapies is challenging: biopsies of recurrent or metastatic lesions are invasive and can often not be performed when clinical conditions have worsened or when a tumor is inaccessible [1]. Furthermore, the genomic profile of biopsy tissues provides a picture limited to a single point in space and time, and may thus under-represent intratumoral heterogeneity [2]. Such factors limit the predictive utility of tissue biopsies, worsened still by the continuous evolution of the tumor cells in response to endogenous and exogenous selective pressures [3]. To overcome this challenge, the idea of deriving information about the primary tumor (PT) or metastatic lesions from liquid biopsies which may act as a dynamic diagnostic tool is an appealing concept [4]. Potential targets are circulating tumor cells (CTCs), cell-free DNA (cfDNA) which contains circulating tumor DNA (ctDNA), microRNA signatures, extracellular vesicles, tumor-educated platelets, proteins, and metabolites [5]. Clinical utility has been demonstrated for three cfDNA-based tests that are approved by the U. S. Food and Drug Administration (FDA): the cobas *EGFR* Mutation Test v2 [6], the Epi proColon test [7], and the therascreen *PIK3CA* RGQ PCR kit [8]. Furthermore, the CellSearch system has been approved by the FDA for detection of CTCs in patients with metastatic breast, prostate, and colorectal cancer.

CTCs are shed into the blood by tumor tissue and are commonly considered as precursor cells for metastasis formation [4]. Elevated CTC counts correlate with shortened progression free survival (PFS) and overall survival (OS) in metastatic breast cancer and other metastatic cancers [9]. The CTC count is also a prognostic factor in non-metastatic breast cancer and other non-metastatic cancers [10]. Furthermore, CTC analysis allows monitoring of treatment response [11].

84 The predictive utility of CTCs is currently investigated. Whereas some trials such as the SWOG
85 S0500 trial question a clinical utility [12–14], others point towards their clinical utility: In the STIC
86 CTC trial first-line treatment of estrogen receptor (ER)-positive metastatic breast cancer patients,
87 either with hormone therapy or chemotherapy, was determined by clinicians or by the baseline
88 CTC level. Patients whose treatment was escalated to chemotherapy based on CTC count had a
89 significantly longer PFS and showed a trend towards a longer OS. Patients whose therapy was
90 deescalated based on a low CTC count had no worse outcome [15].

91 Here, we present a proof-of-concept study that explores the mutation status combined with further
92 multiparametric characterizations of CTCs to provide additional CTC-based tailored
93 recommendations in clinical practice based on the idea that an in-depth analysis will be highly
94 informative about the evolution of the tumor and lead to an understanding of cancer biology in
95 general and CTC biology in particular. First, whole-exome sequencing (WES) was performed on
96 CTCs from five metastatic breast cancer patients. Next, one patient whose CTCs harbored a
97 mutation targetable with drugs approved by the FDA and the European Medicines Agency (EMA)
98 was selected and the mutation-based treatment recommendation was validated with a detailed
99 multiparametric liquid biopsy analysis over a time period of more than three years. The information
100 gained on the disease and its evolution was used to choose an effective targeted therapy, thus
101 highlighting the potential offered by liquid biopsies.

Materials and Methods

Patients

Five patients with high CTC counts were selected from the Augusta study (approved by the Ethics Committee of the Medical Faculty of the Heinrich Heine University Düsseldorf; Ref-No: 3430) and DETECT III study (NCT01619111; <https://clinicaltrials.gov/ct2/show/NCT01619111>). All patients provided their informed consent for the use of their blood samples for CTC analysis and for translational research projects. Clinical patient data are shown in Table 1.

By whole exome sequencing of the CTCs, mutations targetable with drugs approved by the FDA were detected in four patients. From those patients, one was selected to further validate the treatment recommendation by a detailed multiparametric liquid biopsy analysis over a period of more than three years.

The Caucasian 65-year-old female patient 1 was first diagnosed with a multicentric right-sided breast cancer. The breast cancer was ER as well as progesterone receptor (PR) positive and negative for HER2. Staging revealed absence of metastatic lesions. The patient was treated with breast conserving therapy and sentinel lymph node biopsy (pT2 pN0 (0/2 sn) G2 R0 L0 V0, ER-positive, PR-positive, HER2-negative) followed by adjuvant local radiotherapy. In addition, she was treated for five years with an aromatase inhibitor (AI; anastrozole). After 7 years, the patient was first diagnosed with bone metastatic lesions. Therefore, she restarted an endocrine therapy with another AI (letrozole) in combination with the RANKL inhibitor denosumab. A stable disease was observed for 18 months, until she first presented to our department with a newly diagnosed vesical metastatic lesion. Histologic examination showed a positive ER and PR status and a negative HER2 status. A subsequently performed CellSearch analysis revealed 5500 CTCs per 7.5 ml blood. The

endocrine therapy was switched to the AI exemestane. Because of osteonecrosis of the jaw, denosumab was suspended. Two months later, CTC numbers increased to approximately 50000 per 7.5 ml blood. In line, a staging computer tomography (CT) scan demonstrated liver metastatic lesions, progression of bone lesions and suspicion of bone marrow infiltration. The endocrine therapy was stopped and a chemotherapy with weekly epirubicin was started. CTC counts decreased to 3 – 47 CTCs per 7.5 ml blood. Staging CTs revealed a stable disease of vesical and bone lesions. Hepatic lesions were not radiographically visible anymore. After reaching the cumulative overall dose, epirubicin treatment was switched to fulvestrant (SERD), palbociclib (CDK4/6 inhibitor) and denosumab. CTC counts were measured alongside therapy and fluctuated between 25 and 217 CTCs per 7.5 ml blood. After 13 months, CTC analysis revealed a continuous increase of CTC numbers and CT staging diagnosed new cervical metastatic lesions, a progression of the vesical metastatic lesion, ascites as well as new hepatic metastatic lesions. Moreover, a peritoneal carcinomatosis could not be excluded. A biopsy of the cervical lymph node revealed a positive ER, negative PR and negative HER2 status of the tumor cells. The treatment regime was switched to weekly paclitaxel. However, weekly taxol was stopped after two administrations because of the further increasing CTC count to 6030 and strong adverse side effects (Figure 2, Supplemental Figure S1).

Intervention

Based on the multiparametric characterization of the CTCs, 5 mg/day everolimus was applied in combination with 20mg/day tamoxifen and denosumab following clinical guidelines [16].

Primary parameter for monitoring CTC-based therapy were the CTC numbers in peripheral blood, monthly determined by the FDA-approved CellSearch system. In addition, clinical response was measured by computer tomography/magnetic resonance imaging 2.5 months after intervention.

Enrichment and enumeration of CTCs

CTCs were enriched from peripheral blood or DLA product. DLA was performed as previously described [17,18].

Enrichment and enumeration of CTCs was performed using the CellSearch Circulating Epithelial Cell Kit (Menarini, Florence, Italy) according to manufacturer's instructions. 7.5 ml blood or 2×10^8 white blood cells (WBCs) from DLA product were used for enumeration of CTCs. The expression of ER α (antibody clone ER119.3) was determined by the CellSearch CXC Kit (Menarini).

Enrichment of CTCs without subsequent automated immunofluorescent characterization was performed using the CellSearch Epithelial Cell Profile Kit (Menarini) according to the manufacturer's instructions.

Immunofluorescence analysis

For immunofluorescence analysis cells were fixed with CellSave reagent, permeabilized with 0.1% Triton X-100 (Merck, Darmstadt, Germany), and stained for nucleic acid (DAPI; F. Hoffmann-La Roche, Basel, Switzerland), CK (clone C11, Alexa Fluor 488 conjugated, Cat#: GTX11212, GeneTex, Irvine, United States or clones C11/AE1/AE3, TRITC conjugated, Cat#: CKALLRMB000S, Aczon, Monte San Pietro, Italy), CD45 (clone 35-ZS, Alexa Fluor 647

conjugated, Cat#: sc-1178 AF647, Santa Cruz Biotechnology, Dallas, United States), and caspase cleaved cytokeratin (M30 Cytodeath, FITC conjugated, Cat#: 10800, VLVbio, Nacka, Sweden), Ki67 (clone D3B5, Cat#: 9129S, Cell Signaling Technology, Danvers, United States), phosphorylated Akt (clone D9E, Cat#: 4060, Cell Signaling Technology), or phosphorylated mTOR (clone D9C2, Cat#5536, Cell Signaling Technology). For Ki67 analysis a goat anti-rabbit IgG antibody (Cat# A-11012, Thermo Fisher Scientific, Waltham, United States), for phosphorylated Akt and mTOR a donkey anti-rabbit IgG antibody (Cat# A-21206, Thermo Fisher Scientific) was used as the secondary antibody.

CTC isolation

Single CTCs were isolated from CellSearch cartridge mainly by micromanipulation with the CellCelector (ALS, Jena, Germany) [19]. For isolation of ER α positive and negative CTCs the DEParray (Menarini) was used.

Larger numbers of CTCs were obtained by FACS sorting (MoFlo XDP sorter, Beckman Coulter, Brea, United States) from DLA product. CTCs were identified by staining for EpCAM (clone VU1D9, Alexa Fluor 488 conjugated, Cat#: 5488S, Cell Signaling Technology), CD45 (clone 35-ZS) and Hoechst 33342 (Thermo Fisher Scientific).

Whole-genome amplification

Chromosomal DNA of single isolated cells was amplified by whole genome amplification (WGA) with the Ampli1 WGA Kit (Menarini). Afterwards DNA integrity was determined with the Ampli1 QC Kit (Menarini).

Whole-exome sequencing

DNA was purified from FFPE tissue with the GeneRead DNA FFPE Kit (Qiagen, Venlo, Netherlands). Furthermore, tumor content was determined by staining with h malaun/eosin and immunohistochemistry. Slides were analyzed by experienced pathologists.

DNA from FACS sorted CTC pellets was extracted using QIAamp DNA Micro Kit (Qiagen).

WES of sorted CTCs (patient 1) or pooled WGA products from CTCs (patients 2, 3, 4, and 5) and matched WBCs was performed using the Agilent Sure Select XT Kit V7 (Agilent, Santa Clara, United States) (patient 1), V6 (Agilent) (patients 4 and 5), or the Illumina IDT Exome Analysis Kit (Illumina, San Diego, United States) (patients 2 and 3). Matched PTs were sequenced using the Agilent Sure Select XT V7 (patient 1) or the Illumina IDT Exome Analysis Kit (patient 3). The integrity of extracted DNA from PTs of patients 2, 4, and 5 was not sufficient to perform WES or no tissue was available. The libraries were sequenced on either a HiSeq 3000 system (Illumina) or a NovaSeq 6000 system (Illumina).

After sequencing, data were uploaded to the Molecular Health Guide platform (Molecular Health, Heidelberg, Germany) and identified single nucleotide variants were analyzed. Variants exceeding a variant allele frequency (VAF) of 5% and exceeding a coverage of 100  with a coverage of that position exceeding 40  in the reference sample were considered reliable.

Clonal reconstruction

The PT, CTCs from time point 3 (T3) and tumor cells from the bladder metastasis and lymph node metastasis were considered for this analysis. Regions of copy-number neutrality common to all of these samples were determined on the basis of WES copy number profiles obtained with

CopywriteR [20] and B-allele frequency analysis. Somatic mutations falling in these regions were selected for analysis with Cloe [21]. Mutations were first clustered with Cloe's CRP, and Cloe run on the resulting 30 meta-mutations with 6 to 20 clones. Results that achieved the highest log-likelihood were reported.

Sequencing of *ESR1* and *AKT1* from single CTCs

ESR1 mutation analysis was performed as previously described [22]. The *AKT1* E17K mutation was analyzed by Sanger sequencing. The amplification was performed by semi nested PCR. Used primers are shown in Supplemental Table S4.

Sequencing of *PIK3CA* hotspots by ddPCR

Droplet digital PCR of PTs and single isolated cells was performed by using a QX200™ Droplet Digital PCR system (Bio-Rad Laboratories, Hercules, United States). Primers and probes are shown in Supplemental Tables S5 and S6. For droplet generation the QX200 droplet generator (Bio-Rad Laboratories) was used. Samples were subjected into the Droplet Reader (Bio-Rad Laboratories). Data were analyzed using QuantaSoft analysis software (Bio-Rad Laboratories). MCF7 and T-47D cells were used as a negative and positive control to gate the threshold for finding mutation. Negative controls with no DNA were included at each run.

Array comparative genome hybridization

WGA products were processed for aCGH as previously described [23]. 1 µg DNA was processed for aCGH. As reference, the WGA product of a single GM14667 cell or DNA from matched normal tissue was used. For data analysis, the output image files were normalized and fluorescence ratios for each probe were determined using Feature Extraction software (Agilent Technologies Inc.; version 10.7.3.1, Protocol CGH_1105_Oct09). Data were visualized and analyzed with the Genomic Workbench 6.5.0.18 software by applying the ADM-2 algorithm with a threshold of 6.0. The centralization algorithm was set to a threshold of 4.0 with a bin size of 10. To identify copy number alterations, an aberration filter with a minimum log₂ ratio of ±0.3 and a minimum of 100 consecutive probes was set.

FISH analysis

CTCs enriched by CellSearch were spun on slides and detected using a pan cytokeratin antibody (Cat#: 1835, Biotium, Fremont, United States). As secondary antibody, a donkey anti-rabbit IgG antibody (Cat# A-31573, Thermo Fisher Scientific) was used. Afterwards slides were pretreated with SSC Wash Buffer (Zytovision, Bremerhaven, Germany), pepsin (Zytovision), and formaldehyd (Merck, Darmstadt, Germany). DNA was then denatured and ZytoLight SPEC ESR1/CEN 6 Dual Color Probes (Zytovision) were hybridized overnight at 37 °C. For washing Wash Buffer A (Zytovision) was used.

Multiplex ESR1-ddPCR from cfDNA and data analysis

For screening of *ESR1* mutations plasma was collected from EDTA vacutainers (Becton, Dickinson and Company, Franklin Lakes, United States) immediately after blood draw and cryo-conserved. The cfDNA was extracted from 2 ml plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen). Screening for *ESR1* mutations was performed as previously described by using a multiplex ddPCR [24]. Samples were considered positive if the merged replicates presented a minimum of 3 E380Q mutant droplets or 8 exon 8 mutant droplets and if the average mutant allele frequency was higher than 0.1%.

RNA sequencing

40.000 CTCs were sorted from DLA product fixed with 4% paraformaldehyde. RNA sequencing was performed in duplicates using the TruSeq RNA Access Library Kit (Illumina). Libraries were sequenced on a NextSeq 500 system (Illumina). Data were analyzed and TPM values were determined using the CLC Genomics Workbench (Qiagen). Reads were mapped by applying the following parameters: mismatch cost: 2; insertion/deletion cost: 3; length fraction: 0.8; similarity fraction: 0.8; global alignment: no; strand specific: both; maximum number of hits per read: 5.

CTC culture

Viable CTCs were enriched from a cryo-conserved DLA product by using the Parsortix system and were cultured as previously reported [25]. Briefly, CTCs were cultured in low attachment plates (Corning, Corning, United States) with RPMI 1640 medium supplemented with 1× B27 (Thermo Fisher Scientific), 20 ng/mL human epidermal growth factor (Merck), 20 ng/mL fibroblast growth

275 factor (Merck), and 1% penicillin-streptomycin (Thermo Fisher Scientific) in a humidified
276 atmosphere with 5% CO₂ and 4% O₂.

277 For drug testing 100 CTCs were seeded per well of a 96-well plate after ten days of pre-culture.
278 The cells were treated with capivasertib (MedChem Express, Monmouth Junction, United States),
279 everolimus (Merck), epirubicin (Merck), and paclitaxel (Merck). Each drug concentration was
280 tested in triplicates. After incubation for 6 days, cells were spun on glass slides, stained for
281 cytokeratin, and numbers were determined by counting. As references, cell lines MDA-MB-231,
282 SK-BR-3, T-47D and MCF7 were used (ATCC, Manassas, United States; catalog numbers: MDA-
283 MB 231: HTB-26, SK-BR-3: HTB-30, T-47D: HTB133, and MCF7: HTB-22). Cells were
284 authenticated via short tandem repeat analysis and regularly tested negative for *Mycoplasma*.

285

286 Statistical analysis

287 Statistical analyses were performed using GraphPad Prism (Graphpad Software, San Diego, United
288 States). *P*-values < 0.05 were considered statistically significant.

Results

Whole-exome sequencing of CTCs to provide CTC-based treatment recommendations

CTCs from five metastatic breast cancer patients were isolated and analyzed by WES. All patients had a PT of luminal subtype. At the time of CTC mutation analysis, the patients' CTC counts per 7.5 ml of blood were between 94 and approximately 50.000 (Table 1).

Mutations targetable by specific therapies were identified in CTCs from all patients (Figure 1A, Supplemental Figure S2). In patient 1, we identified an *ESR1* E380Q mutations with a VAF of 80.0% and an *AKT1* E17K mutation with a VAF of 65.3%. The *ESR1* E380Q mutation leads to a conformational change of the protein and to a ligand-independent ER activation, thereby conferring resistance to aromatase inhibition [26]. Despite this, second-generation selective estrogen receptor degrader such as fulvestrant retain activity [27]. The *AKT1* E17K mutation hyperactivates the mTOR pathway. This pathway could be targeted by mTOR inhibitors like everolimus or AKT-inhibitors like ipatasertib and capivasertib [28,29]. Furthermore, a *CDH1* Q23* mutation was observed in both the PT with a VAF of 47.1% and in CTCs with a VAF of 78.6%. CDH1 is a negative regulator of beta-catenin and inactivates the canonical WNT signaling pathway which normally inhibits cell proliferation and differentiation [30]. A nonsense mutation might lead to an activation of WNT signaling and therefore might be targeted with WNT-pathway inhibitors, which have shown efficacy in pre-clinical and clinical trials in multiple cancer types [31].

The analysis of the CTCs from patient 2 revealed a mutation in the tumor suppressor gene *TP53*. Nonsense mutations in *TP53* are likely to confer loss of its tumor suppressor activity [32]. Preclinical and clinical data suggest that the Wee1-inhibitor adavosertib may increase sensitivity towards DNA-damaging agents in tumors with loss of *TP53* [33]. In clinical studies antiangiogenic

311 agents targeting VEGF/VEGFR signaling such as bevacizumab and pazopanib improved the
312 outcome of patients with *TP53* mutations [34,35]. Furthermore, preclinical and initial clinical data
313 showed that the p53-reactivating drug APR-246 may be effective in *TP53*-mutated cancers and
314 may have synergistic or additive effects with other anti-cancer agents, such as chemotherapy [36].

315 Analysis of the CTCs from patients 3, 4 and 5 revealed the presence of *PIK3CA* mutations. The
316 *PIK3CA* gene encodes for the p110 α subunit of the phosphoinositide-3-kinase (PI3K) which
317 promotes cell proliferation and survival by activation of the PI3K/AKT signaling pathway [37].
318 Patient 3 harbored the mutations H1047R and N345K with VAFs of 100%, patient 4 had a H1047L
319 mutation at a VAF of 48%, and in patient 5 the mutation E545K was detected with a VAF of 72%.
320 The detected variants strongly activate the downstream pathway [38] and offer the chance to be
321 targeted by PI3K inhibitors. For the treatment of hormone receptor-positive, HER2-negative
322 advanced breast cancers harboring such variants, alpelisib plus fulvestrant is indicated [39]. HER2-
323 positive tumors of patients owing this variant showed resistance to trastuzumab [40]. Additionally,
324 preclinical models with this variant are sensitive to the mTOR inhibitors everolimus and sirolimus
325 [41]. Furthermore, the CTCs from patient 3 harbored a nonsense mutation in the *FANCA* gene.
326 FANCA is involved in DNA damage repair and in the maintenance of genomic stability. Nonsense
327 mutations in FANCA are likely to disrupt function and lead to deficiencies in DNA repair [42]. In
328 preclinical studies, loss of functional FANCA sensitized cells to PARP inhibitors such as olaparib
329 [43].

330 In addition to the CTC analysis, mutational analyses of the PTs were performed by WES for the
331 patients 1 and 3 and ddPCR on the mutated *PIK3CA* positions for the patients 4 and 5. The clinically
332 targetable mutations of *ESR1* and *AKT1* were not detected in the PT of patient 1. In the PT of
333 patient 3 the *PIK3CA* mutation H1047R was present in only a minor part of the cells as indicated

by a VAF of 13%, whereas the *PIK3CA* mutation N345K was not detected (Figure 1B, Supplemental Figure S3).

In time matched needle biopsies of patients 4 and 5, the mutational status of the *PIK3CA* gene was investigated by ddPCR. In both cases, *PIK3CA* mutations similar to those in the CTCs were detected (Supplemental Table S1).

Analysis of mutations and chromosomal aberrations during the course of the disease

To validate the above findings and eventually treat the patient accordingly, we performed a detailed longitudinal and multiparametric analysis on the CTCs of patient 1. CTC based treatments were not considered for the other patients because, first, the clinical situation of some of the patients worsened quickly or, second, the PI3K inhibitor alpelisib was not yet approved by the EMA.

During the course of treatment mutations and chromosomal gains and losses were analyzed comprehensively in the PT, the bladder metastasis from T0, the cervical lymph node metastasis from T33, as well as single CTCs from T3, T13, T21, T29, and T33 (Figure 2, Figure 3 A, Supplemental Figure S4). According to array comparative genome hybridization (aCGH), the PT showed losses of parts of chromosome 1p, of a large part of chromosome 6, and of parts of chromosomes 16q, 17p and 22. This profile overlapped with the whole exome sequencing data. The bladder metastasis and lymph node metastasis had an aberration profile similar to that of the PT, although the lymph node metastasis was detected and sampled 31 months after the bladder metastasis. An additional loss of chromosome 3p was detected in both metastases. CTCs at T3 exhibited largely the same aberrations as the PT and these aberrations were widely congruous. The genetic profiles of CTCs from T13 and from the later time points T21 and T29 were largely

consistent with the profiles detected in CTCs isolated at earlier time points. However, individual CTCs at T29 also exhibited a loss on chromosome 3p as seen in the bladder metastasis and lymph node metastasis. At T33, this chromosomal loss was no longer observed while gains on chromosomes 13q and 16q and a loss of chromosome 18 were acquired. However, in some CTCs, at least within the applied detection thresholds, the partial loss of chromosome 6 disappeared, which was so far present in almost all analyzed CTCs and tissue samples: instead, these CTCs were characterized by only a minor loss on chromosome 6p. Furthermore, a loss in the X chromosome was identified in CTCs collected at T33. The aberrations that were detected at the single-cell level were also confirmed by WES of pooled WGA products.

The evolution of the tumor cells was further investigated by clonal reconstruction, which highlighted the dynamics of different tumor clones during the course of the disease. Eight clones were identified based on mutations and placed in a branched phylogeny. The majority of mutations was found at low levels in all samples (Supplemental Figure S5). These mutations modelled with clones on the left branch (tumor clones 1 - 3) (Figure 3 B, Supplemental Figure S6). The PT sample is mainly composed of tumor clone 4, the progenitor of clones on the right branch. Subsequent samples showed an expansion of clones that are descendants of clone 4 (Figure 3 B, Figure 3 C). At T13 the majority of the CTCs stemmed from tumor clone 8. This remained a minority tumor clone in blood samples taken at the following time points, until it became dominant again in CTCs when the tumor acquired resistance to palbociclib/fulvestrant and later on did not respond to paclitaxel.

Next, the presence of clinically relevant mutations during the course of the disease was analyzed. Unlike the PT, where no clinically relevant mutations were detected by WES, DNA analysis of the bladder and lymph node metastases showed the activating *AKT1* mutation E17K. Similarly, this

AKT1 mutation was present in CTCs ever since (Supplemental Table S2). Moreover, CTCs from T3 harbored the *ESR1* mutation E380Q with a VAF of 80.0%. The VAF of this mutation reduced to 12.8% at T13, and was undetectable in CTCs isolated at later time points (Figure 3 E).

Both, *ESR1*-mutant and *ESR1*-wildtype CTCs could be ER α -positive or ER α -negative (Figure 4 A, Figure 4 B). The observed *AKT1* and *ESR1* mutations were also detected at the transcriptomic level. The *ESR1* mutation E380Q was observed with a frequency of 83%. The *AKT1* E17K transcript showed a frequency of 74.5% (Figure 4 C). In line with the aberrations detected in chromosome 6 in CTCs from T3, FISH analysis confirmed the absence of one *ESR1* allele and one centromere of chromosome 6 (Figure 4 D).

To analyze the lack of detection of *ESR1* mutations on CTCs at later points of the course of the disease further, an analysis of *ESR1* hotspot mutations at positions E380, Y537 and D538 was performed on ctDNA. At T3, the E380Q mutation was identified with a frequency of 0.2%. In concordance with the CTC analysis, no *ESR1* mutations were detected at T21. However, *ESR1* mutations could not be detected in the ctDNA at T13, contrary to the CTC results (Figure 4 E). Mutations at the positions Y537 and D538 were not detected at any time point (Supplemental Table S3).

In addition, the proportion of ER α -positive CTCs was investigated. At T3, 68% of the CTCs showed a nucleated ER α staining. At T21, during therapy with palbociclib and fulvestrant, the proportion of ER α -positive cells dropped to 17% (Figure 4 F, Figure 4 G).

CTCs exhibit no indications for proliferation but show reduced apoptosis

Patients with several thousand CTCs per 7.5 ml blood are extremely rare. To investigate the reason behind such high numbers, we analyzed the expression of markers related to proliferation and apoptosis in the PT and CTCs from T3. In comparison to tumor cells from the PT, in CTCs the expression levels of *PCNA* and *MKI67* was reduced 495.5 fold (p -value 0.0008, two-tailed t -test) and 81.9 fold (p -value 0.0054, two-tailed t -test), respectively (Figure 5 A). The Absence of *Ki67* in CTCs was confirmed by immunofluorescence analysis (Figure 5 B).

Staining signals for cleaved cytokeratin 18 were only observed in a remarkable small proportion of 1.6% of the CTCs (Figure 5D, Supplemental Figure S7). In line, a trend towards a lower expression of transcripts such as *BAX*, *BAK*, *BAD*, *BCL2*, *BCL2L2* and *MCL1* coding for apoptosis related proteins was observed in the CTCs compared to the PT (Figure 5 C).

Finally, analysis of the PI3K/AKT1/mTOR pathway, which is involved in cell proliferation, stress response and apoptosis, revealed a 9.23 fold reduced expression of the negative regulator of PI3K-dependent Akt signaling *PTEN* in the CTCs (p -value 0.0001, two-tailed t -test). Expression of *AKT1* was not reduced in CTCs compared to the PT although CTCs did not show any indications for proliferation. This suggests increased AKT1 signaling not only based on the presence of the *AKT1* E17K mutation but also a constantly high abundance of both the transcript and the protein. *PIK3CA* and *PIK3CB*, which act upstream of AKT1, were both significantly downregulated: *PIK3CA* 45.60 fold (p -value 0.0003, two-tailed t -test) and *PIK3CB* 10.98 fold (p -value 0.0020, two-tailed t -test) (Figure 5 E). To assess activation of AKT1 and mTOR via phosphorylation, immunofluorescence analysis for phosphorylated Akt1 (Ser473) and phosphorylated mTOR (Ser2448) was performed on CTCs from T3. A positive staining was observed for 56.9% of the CTCs for phosphorylated AKT1 and for 12.4% of the CTCs for phosphorylated mTOR (Figure 5 F, Figure 5 G).

***In vitro* treatment of cultured CTCs**

Based on the mutation analysis and the expression analysis, AKT1 was deemed a disease driver in this patient. Therefore, AKT1 or mTOR inhibitors were considered as potential drugs. The efficacy of the AKT1 inhibitor capivasertib and the mTOR inhibitor everolimus were tested on CTCs *in vitro* and compared to the cytostatic drugs epirubicin and paclitaxel using cultured CTCs from a diagnostic leukapheresis (DLA) product obtained at T3. As references the triple-negative cell line MDA-MB-231, the ER α -positive and *PIK3CA*-mutated cell lines T-47D and MCF7, and the *ERBB2*-amplified cell line SK-BR-3 were used. All cell lines perished in presence of epirubicin and paclitaxel, but only T-47D, MCF7 and SK-BR-3 responded to capivasertib and everolimus. For drug testing, CTCs from T3 were short-term cultured. These CTCs were highly sensitive to epirubicin treatment validating the clinicians' decision for treatment at this time point. Paclitaxel was as effective as in the reference cell lines. Furthermore, the CTCs responded to the treatment with capivasertib and everolimus targeting the AKT1/mTOR pathway (Figure 5 H).

CTC-based treatment

Based on the above findings, treatment of the patient was switched after taxol to the mTOR-inhibitor everolimus, combined with the selective estrogen receptor modulator tamoxifen according to current therapeutic guidelines. Within 17.5 weeks, the CTC count dropped by 97.3% from 6030 CTC to 165 CTC per 7.5 of blood (Figure 2). Staging 2.5 months after treatment with everolimus plus tamoxifen and denosumab showed a stable disease situation of the metastatic lesions. The chromosomal aberrations detected on the remaining CTCs at time point T36 were largely similar to those detected on CTCs at T33 (Figure 5 I). The *AKT1* E17K mutation was also still detected in the majority of these CTCs (Supplemental Table S2).

Discussion

CTCs are considered as a prognostic marker in metastatic breast cancer. However, their clinical utility is still under investigation and has not been conclusively demonstrated. Here, we tested their clinical utility in a proof-of-concept study starting with CTC WES from five metastatic luminal breast cancer patients.

Therefore, the CTCs were enriched from blood or DLA product with the FDA approved CellSearch system. Single CTCs were isolated, their DNA was amplified and high quality WGA products were pooled for WES. By this approach, mutations that can be addressed by FDA approved therapies were detected in the *PIK3CA*, the *ESR1* and the *AKT1* genes in four patients, including mutations that were not detected in the PT. Tumor cells with *PIK3CA* mutations could be targeted by the PI3K inhibitor alpelisib plus fulvestrant in ER α positive tumors. Mutations leading to a constitutive activity of the ER α convey a resistance to aromatase inhibition and may be treated with the selective estrogen receptor degrader fulvestrant. Tumor cells with an activating *AKT1* mutation can be targeted by AKT1 inhibitors or agents blocking mTOR, a downstream member of the AKT1 signaling pathway. The therapies were recommended to the clinicians and turned out to be especially clinically relevant for one patient.

In the following, a longitudinal and multiparametric liquid biopsy analysis of one index patient was performed to track down the developmental history of the tumor. Clustering analysis of mutations identified by WES led to the identification of eight tumor cell clones belonging to two major branches. While it looks like that there are two independent origins of the tumor, the most likely explanation is that the mutations common to both branches were not included in the analysis because they were not called, filtered out or have been affected by copy number alterations. Nearly all tumor clones were already present in the PT. Furthermore, in tissue from both analyzed

metastases, several different tumor cell clones have been detected. This suggests the seeding of multiple tumor cell clones to the metastatic niche, either by co-seeding of multiple single tumor cells [44] or by seeding of tumor cell clusters consisting of tumor cells of multiple clones, that might have survival advantages and an enhanced metastatic potential compared to single CTCs [45]. Indeed, some CTC clusters were observed by CellSearch analysis at most time points. Of note, the bladder metastasis and lymph node metastasis shared very similar copy number aberration profiles leading to the assumption of an early seeding event. The CTC populations at all time points consisted of different tumor cell clones. This heterogeneity is underlined by the aCGH analysis of single CTCs, also showing different copy number aberration profiles especially at T21 and T33. At T3 and T13 the *ESR1* mutation E380Q was identified. The mutation was absent in the tissue biopsies and no CTCs with this mutation were found upon epirubicin treatment. We assume that the *ESR1* mutant subclones that developed during estrogen deprivation were widely eradicated by the chemotherapy with epirubicin. During the epirubicin therapy, the majority of the analyzed CTCs belonged to the tumor clone eight. This observation leads to the assumption that this clone was especially resistant towards chemotherapy. During therapy with palbociclib and fulvestrant clone 8 became less abundant. However, at T31 most of the analyzed CTCs belonged to the tumor cell clone 8 and were later resistant towards a chemotherapy with paclitaxel.

Our analysis identified the *AKT1* E17K mutation as the putative driver of the tumor. This mutation was first detected in a biopsy of the bladder metastasis and found in the majority of the CTCs ever since, as well as in a lymph node metastasis biopsied at T31. AKT1 is a member of the serine-threonine kinase class and plays a key role in cellular processes, including growth, proliferation, survival, and angiogenesis. The E17K mutation leads to a pathologic association of *AKT1* with the plasma membrane and constitutive activation of the protein which, in turn, results in an increased level of AKT1 phosphorylation and activation of downstream molecules independent of upstream

events, e.g. stimulation by growth factors [46]. For breast cancer patients, *AKT1* E17K mutation frequencies between 1.4 % and 8.2% have been described in tissue [47].

Based on our findings a therapy with the mTOR inhibitor everolimus in combination with tamoxifen was recommended to the patient when a progress of the tumor was observed under therapy with palbociclib and fulvestrant. The patient initially rejected it and preferred a chemotherapy with paclitaxel. However, during the treatment with paclitaxel the clinical situation worsened, the CTC numbers dramatically increased and the patient was suffering from severe side effects. Thus, the patient agreed to the liquid biopsy-based therapy eventually. Based on the detection of this mutation and the following characterization of the CTCs, the patient was treated with the mTOR inhibitor everolimus. Alternatively, patients with activating *AKT1* mutations could be treated with specific AKT1 inhibitors. Such drugs like capivasertib are currently tested in clinical trials. The efficacy of capivasertib on luminal breast cancer has been shown in combination with paclitaxel in the BEECH trial and in combination with fulvestrant in the FAKTION trial [48,49]. Especially patients with an *AKT1* mutated tumor could benefit from AKT1 inhibition by capivasertib [28]. However, AKT1 inhibitors are not yet approved for treatment of breast cancers. Here we could show that the CTCs harboring the *AKT1* E17K mutation respond to capivasertib *in vitro*. The patient responded well to the liquid biopsy-based therapy and a reduction of the CellSearch determined CTC count by 97.3% was observed during a time period of 17.5 weeks, in which no progression of the metastasis was detected. The chromosomal aberrations uncovered on CTCs by aCGH did not differ from those before the everolimus / tamoxifen therapy leading to the assumption that within that observation period no new and potentially resistant tumor clone has occurred.

516 The efficacy of a treatment which is based on a liquid biopsy mutation analysis is currently
517 investigated in several clinical trials: In the PADA-1 trial *ESR1* mutated patients are treated with
518 CDK4/6 inhibition in combination with fulvestrant [50]. In the multiple parallel cohort trial
519 plasmaMATCH targetable mutations in several genes are identified and patients are treated
520 accordingly [51]. The SOLAR-1 trial is analyzing the efficacy of alpelisib on tumor harboring
521 activating *PIK3CA* mutations and could already show a benefit of such patients which led to the
522 approval of alpelisib and the therascreen PIK3CA RGQ PCR kit by the FDA [39].

523 In all the trials mentioned above mutations are identified by ctDNA analysis. Although digital
524 PCR-based methods have demonstrated to have suitable clinical sensitivity considering that digital
525 PCR and BEAMing can detect somatic point mutations at a sensitivity range of 1% to 0.001%,
526 these technologies require prior knowledge of the region of interest to detect known mutations
527 given the need for the PCR assay to be designed accordingly [1]. Whether the analysis of ctDNA
528 or CTCs is superior has been discussed extensively with the conclusion that both analytes might
529 be complementary [52]. Here we show the detection of the *ESR1* E380Q mutation in CTCs
530 although no *ESR1* mutations have been detected from ctDNA at the same time. This might be
531 because the analysis of a higher number of CTCs from DLA product enables to identify mutations
532 in minor subclones. Furthermore, the analysis of CTCs offers the potential to detect novel
533 mutations as therapeutic targets.

534 However, the analysis of CTCs is challenging due to low CTC numbers and a significant part of
535 cancer patients without detectable CTCs. This limitation could at least partly be solved by our
536 approach of using DLA to pre-enrich MNCs from several liters of the patients' blood. DLA
537 increases the number of CTC positive patients and the CTC yield per patient [17,18]. Furthermore,
538 its implementation into the clinic is unproblematic and no adverse events are observed [17].

However, the characterization of CTCs at single cell level has so far mainly been limited to proof-of-concept studies and it remains unclear to which extent a limited number of CTCs reflects the heterogeneity of the tumor [53].

To maximize the clinical utility of liquid biopsies, implementation of novel multiparametric strategies to combine information from multiple sources might play a key role [5]. Especially functional analysis of living CTCs could add another dimension to conventional liquid biopsies and can help to deepen the understanding on the CTCs' biology [54]. As previously described, DLA facilitates to obtain high numbers of viable CTCs for culture that can be cryo conserved for later usage [25]. Although culturing CTCs is still highly challenging, we were successful to enrich living cells from the blood and from the DLA product at T3. Due to the high CTC count at that time, there was no need for a time-consuming pre culture that could potentially lead to artefacts in the response to the tested drugs by long-term *in vitro* culture. The observed results from a panel of tested drugs matched with the observations of the clinical situation: The cultured CTCs responded to therapies targeting the *AKT1* E17K mutation and showed a high sensitivity towards epirubicin. However, we also observed the CTCs to be sensitive towards paclitaxel, which is contradictory with the clinical observation. We assume this to be the case because the resistance towards a later chemotherapy was mainly provided by CTCs belonging to tumor clone 8. This tumor clone was widely underrepresented at T3 when the CTCs for the drug test were isolated compared to the time when the tumor was treated with paclitaxel, but was selected during epirubicin treatment.

We are aware that our 'index patient' may only represent a minority of all breast cancer patients. She had, at least at some time points of blood collection, extraordinary high numbers of CTCs in her blood circulation. We assume that this was due to reduced apoptosis and greater survival mediated by the activation of the Akt1 protein. High CTC numbers simplify their analysis.

562 However, due to recent developments in single cell analysis, clinical relevant information on the
563 genome, transcriptome or proteome can be gained from single CTCs [55,56]. Mutation data on
564 CTCs from patients 2, 3, 4, and 5 were gained from pooled WGA products from 6 to 15 cells.
565 Mutation analyses of the CTCs from patient 1 at later time points were also generated from WGA
566 products from few single CTCs.

Conclusions

In this study, we show a workflow for WES of amplified DNA from low CTC numbers to identify clinically relevant mutations, combined with a detailed multiparametric liquid biopsy analysis that can personalize and optimize patient therapy based on liquid biopsies. We propose that in a clinical setting such an analysis should be started with a screening for CTC positive patients. For patients with lower CTC numbers DLA should be considered. Next, the CTCs DNA should be amplified and analyzed by whole exome sequencing. In case of the detection of mutations, that can be targeted with drugs that are clinically suitable for the particular patient in a major portion of the tumor cell, we recommend a longitudinal and multiparametric analysis to validate the findings. Such an analysis can exploit the strengths of liquid biopsies to be performed regularly over a period of time without any clinical complications as well as the opportunities of CTCs as a multiparametric analyte, ultimately enabling a drug test if CTCs can be cultured in vitro. If the CTCs respond to the selected drug, the CTC-based therapy can be applied to the patients.

In conclusion, we present here a longitudinal multiparametric liquid biopsy analysis of a breast cancer patient and demonstrated the potential of this approach to identify actionable alterations that can guide and validate individualized treatment decisions in real time, monitor treatment response and influence clinical outcome. Finally, from a scientific point of view, such studies can provide valuable additional insights into the biology and dynamic response of CTCs to different treatment regimens.

586 Abbreviations

- 587 Aromatase inhibitor (AI)
- 588 Cell-free DNA (cfDNA)
- 589 Circulating tumor cells (CTCs)
- 590 Circulating tumor DNA (ctDNA)
- 591 Computer tomography (CT)
- 592 Diagnostic leukapheresis (DLA)
- 593 Estrogen receptor (ER)
- 594 Human epidermal growth factor 2; HER2/neu (HER2)
- 595 Overall survival (OS)
- 596 Phosphoinositide-3-kinase (PI3K)
- 597 Primary tumor (PT)
- 598 Progesterone receptor (PR)
- 599 Progression free survival (PFS)
- 600 Time point (T)
- 601 U. S. Food and Drug Administration (FDA)
- 602 Variant allele frequency (VAF)
- 603 Whole-exome sequencing (WES)

604 Whole-genome amplification (WGA)

605

606

Author contributions

AF, DN, TF, HN conceived the project and provided project leadership. AF, FR, and HN wrote the manuscript. LY and MR were involved in single CTC isolation. BB and NHS performed aCGH analysis and analyzed the data. FM and NB performed clonal reconstruction and analyzed the data. EJ and CP performed ctDNA analysis and analyzed the data. NS supported sample collection. IE contributed to evaluation of tissue samples. FD analyzed CT/MRI data. FR, BJ, NK, JPC, EH, and HA contributed to data analysis. All authors read and approved the final manuscript.

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627 **Competing interests**

628 The authors declare no potential conflicts of interest.

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References

1. Perakis, S.; Speicher, M.R. Emerging concepts in liquid biopsies. *BMC Med.* **2017**, *15*.
2. Reinhardt, F.; Franken, A.; Fehm, T.; Neubauer, H. Navigation through inter- and intratumoral heterogeneity of endocrine resistance mechanisms in breast cancer: A potential role for Liquid Biopsies? *Tumor Biol.* **2017**, *39*.
3. Palmirotta, R.; Lovero, D.; Cafforio, P.; Felici, C.; Mannavola, F.; Pellè, E.; Quaresmini, D.; Tucci, M. Liquid biopsy of cancer: a multimodal diagnostic tool in clinical oncology. *Ther. Adv. Med. Oncol.* **2018**, *10*.
4. Pantel, K.; Alix-Panabières, C. Circulating tumour cells in cancer patients: Challenges and perspectives. *Trends Mol. Med.* **2010**, *16*, 398–406.
5. Heitzer, E.; Haque, I.S.; Roberts, C.E.S.; Speicher, M.R. Current and future perspectives of liquid biopsies in genomics-driven oncology. *Nat. Rev. Genet.* **2019**, *20*.
6. Weber, B.; Meldgaard, P.; Hager, H.; Wu, L.; Wei, W.; Tsai, J.; Khalil, A.; Nexø, E. Detection of EGFR mutations in plasma and biopsies from non-small cell lung cancer patients by allele-specific PCR assays. *BMC Cancer* **2014**, *14*.
7. Grützmann, R.; Molnar, B.; Pilarsky, C.; Habermann, J.K.; Schlag, P.M.; Saeger, H.D.; Miehke, S.; Stolz, T.; Model, F.; Roblick, U.J.; et al. Sensitive Detection of Colorectal Cancer in Peripheral Blood by Septin 9 DNA Methylation Assay. *PLoS One* **2008**, *3*.
8. FDA Oncology Update. *Am. Heal. Drugs Benefits* **2019**, *12*, 198–200.
9. Bidard, F.C.; Peeters, D.J.; Fehm, T.; Nolè, F.; Gisbert-Criado, R.; Mavroudis, D.;

- 650 Grisanti, S.; Generali, D.; Garcia-Saenz, J.A.; Stebbing, J.; et al. Clinical validity of
651 circulating tumour cells in patients with metastatic breast cancer: A pooled analysis of
652 individual patient data. *Lancet Oncol.* **2014**, *15*, 406–414.
- 653 10. Janni, W.J.; Rack, B.; Terstappen, L.W.M.M.; Pierga, J.-Y.; Taran, F.-A.; Fehm, T.; Hall,
654 C.; de Groot, M.R.; Bidard, F.-C.; Friedl, T.W.P.; et al. Pooled Analysis of the Prognostic
655 Relevance of Circulating Tumor Cells in Primary Breast Cancer. *Clin. Cancer Res.* **2016**,
656 *22*, 2583–2593.
- 657 11. Cristofanilli, M.; Budd, G.T.; Ellis, M.J.; Stopeck, A.; Matera, J.; Ph, R.; Miller, M.C.;
658 Reuben, J.M.; Ph, D.; Doyle, G. V; et al. Circulating Tumor Cells, Disease Progression,
659 and Survival in Metastatic Breast Cancer. *N. Engl. J. Med.* **2004**, *351*, 781–791.
- 660 12. Smerage, J.B.; Barlow, W.E.; Hortobagyi, G.N.; Winer, E.P.; Leyland-jones, B.;
661 Srkalovic, G.; Tejwani, S.; Schott, A.F.; Rourke, M.A.O.; Lew, D.L.; et al. Circulating
662 Tumor Cells and Response to Chemotherapy in Metastatic Breast Cancer: SWOG S0500.
663 *J. Clin. Oncol.* **2014**, *32*, 3483–3490.
- 664 13. Pestrin, M.; Bessi, S.; Puglisi, F.; Minisini, A.M.; Masci, G.; Battelli, N.; Ravaioli, A.;
665 Gianni, L.; Di, R.; Carlo, M.; et al. Final results of a multicenter phase II clinical trial
666 evaluating the activity of single-agent lapatinib in patients with HER2- negative metastatic
667 breast cancer and HER2-positive circulating tumor cells. A proof-of-concept study. *Breast*
668 *Cancer Res. Treat.* **2012**, *134*, 283–289.
- 669 14. Stebbing, J.; Payne, R.; Reise, J.; Frampton, A.E.; Avery, M.; Woodley, L.; Leo, A. Di;
670 Pestrin, M.; Krell, J.; Coombes, R.C. The Efficacy of Lapatinib in Metastatic Breast
671 Cancer with HER2 Non-Amplified Primary Tumors and EGFR Positive Circulating

- 672 Tumor Cells: A Proof-Of-Concept Study. *PLoS One* **2013**, 8.
- 673 15. Bidard Clinical utility of circulating tumor cell count as a tool to chose between first line
674 hormone therapy and chemotherapy for ER+ HER2- metastatic breast cancer: Results of
675 the phase III STIC CTC trial. *SABCS* **2018**.
- 676 16. Thill, M.; Liedtke, C.; Müller, V.; Janni, W.; Schmidt, M. AGO Recommendations for the
677 Diagnosis and Treatment of Patients with Advanced and Metastatic Breast Cancer: Update
678 2018. *Breast Care* **2018**, 13, 209–215.
- 679 17. Fehm, T.N.; Meier-Stiegen, F.; Driemel, C.; Jäger, B.; Reinhardt, F.; Naskou, J.; Franken,
680 A.; Neubauer, H.; Neves, R.P.L.; van Dalum, G.; et al. Diagnostic Leukapheresis for CTC
681 Analysis in Breast Cancer Patients: CTC Frequency, Clinical Experiences and
682 Recommendations for Standardized Reporting. *Cytom. Part A* **2018**, 93, 1213–1219.
- 683 18. Fischer, J.C.; Niederacher, D.; Topp, S.A.; Honisch, E.; Schumacher, S.; Schmitz, N.;
684 Zacarias Fohrding, L.; Vay, C.; Hoffmann, I.; Kasprowicz, N.S.; et al. Diagnostic
685 leukapheresis enables reliable detection of circulating tumor cells of nonmetastatic cancer
686 patients. *Proc. Natl. Acad. Sci.* **2013**, 110, 16580–16585.
- 687 19. Neumann, M.H.D.; Schneck, H.; Decker, Y.; Schömer, S.; Franken, A.; Endris, V.; Pfarr,
688 N.; Weichert, W.; Niederacher, D.; Fehm, T.; et al. Isolation and characterization of
689 circulating tumor cells using a novel workflow combining the CellSearch® system and the
690 CellCelector™. *Biotechnol. Prog.* **2017**, 33, 125–132.
- 691 20. Kuilman, T.; Velds, A.; Kemper, K.; Ranzani, M.; Bombardelli, L.; Hoogstraat, M.;
692 Nevedomskaya, E.; Xu, G.; de Ruiter, J.; Lolkema, M.P.; et al. CopywriteR : DNA copy
693 number detection from off-target sequence data. *Genome Biol.* **2015**, 16.

- 694 21. Marass, F.; Mouliere, F.; Yuan, K.; Rosenfeld, N.; Markowetz, F. A phylogenetic latent
695 feature model for clonal deconvolution. *Ann. Appl. Stat.* **2016**, *10*, 2377–2404.
- 696 22. Franken, A.; Honisch, E.; Reinhardt, F.; Meier-Stiegen, F.; Yang, L.; Jaschinski, S.;
697 Esposito, I.; Alberter, B.; Polzer, B.; Huebner, H.; et al. Detection of ESR1 Mutations in
698 Single Circulating Tumor Cells on Estrogen Deprivation Therapy but Not in Primary
699 Tumors from Metastatic Luminal Breast Cancer Patients. *J. Mol. Diagnostics* **2019**, *22*,
700 111–121.
- 701 23. Möhlendick, B.; Bartenhagen, C.; Behrens, B.; Honisch, E.; Raba, K.; Knoefel, W.T.;
702 Stoecklein, N.H. A Robust Method to Analyze Copy Number Alterations of Less than 100
703 kb in Single Cells Using Oligonucleotide Array CGH. *PLoS One* **2013**, *8*.
- 704 24. Jeannot, E.; Darrigues, L.; Michel, M.; Pierga, M.S.J.; Rampanou, A.; Melaabi, S.;
705 Benoist, C.; Bièche, I.; El, R.; Aurélien, A.; et al. A single droplet digital PCR for ESR1
706 activating mutations detection in plasma. *Oncogene* **2020**.
- 707 25. Franken, A.; Driemel, C.; Behrens, B.; Meier-stiegen, F.; Endris, V.; Stenzinger, A.;
708 Niederacher, D.; Fischer, J.C.; Stoecklein, N.H.; Ruckhaeberle, E.; et al. Label-Free
709 Enrichment and Molecular Characterization of Viable Circulating Tumor Cells from
710 Diagnostic Leukapheresis Products. *Clin. Chem.* **2019**, *65*, 549–558.
- 711 26. Toy, W.; Shen, Y.; Won, H.; Green, B.; Sakr, R. a; Will, M.; Gala, K.; Fanning, S.; King,
712 T. a; Hudis, C.; et al. ESR1 ligand binding domain mutations in hormone-resistant breast
713 cancer. *Nat. Genet.* **2013**, *45*, 1439–1445.
- 714 27. Fribbens, C.; O’Leary, B.; Kilburn, L.; Hrebien, S.; Garcia-Murillas, I.; Beaney, M.;
715 Cristofanilli, M.; Andre, F.; Loi, S.; Loibl, S.; et al. Plasma ESR1 Mutations and the

- 716 Treatment of Estrogen Receptor-Positive Advanced Breast Cancer. *J. Clin. Oncol.* **2016**,
717 34, 2961–2968.
- 718 28. Hyman, D.M.; Smyth, L.M.; Donoghue, M.T.A.; Westin, S.N.; Bedard, P.L.; Emma, J.;
719 Bando, H.; El-khoueiry, A.B.; Mita, A.; Schellens, J.H.M.; et al. AKT Inhibition in Solid
720 Tumors With AKT1 Mutations. *J. Clin. Oncol.* **2019**, 35, 2251–2262.
- 721 29. Zardavas, D.; Fumagalli, D.; Loi, S. Phosphatidylinositol 3-kinase/AKT/mammalian target
722 of rapamycin pathway inhibition: a breakthrough in the management of luminal
723 (ER+/HER2-) breast cancers? *Curr. Opin. Oncol.* **2012**, 24, 623–634.
- 724 30. Heuberger, J.; Birchmeier, W. Interplay of cadherin-mediated cell adhesion and canonical
725 Wnt signaling. *Cold spring Harb. Perspect. Biol.* **2010**, 2.
- 726 31. Serafino, A.; Sferrazza, G.; Colini Baldeschi, A.; Nicotera, G.; Andreola, F.; Pittaluga, E.;
727 Pierimarchi, P. Developing drugs that target the Wnt pathway: recent approaches in cancer
728 and neurodegenerative diseases. *Expert Opin. Drug Discov.* **2017**, 12, 169–186.
- 729 32. Mantovani, F.; Collavin, L.; Del Sal, G. Mutant p53 as a guardian of the cancer cell. *Cell*
730 *Death Differ.* **2019**, 26, 199–212.
- 731 33. Leijen, S.; van Geel, R.M.J.M.; Sonke, G.S.; Jong, D. De; Rosenberg, E.H.; Marchetti, S.;
732 Pluim, D.; Werkhoven, E. Van; Rose, S.; Lee, M.A.; et al. Phase II Study of WEE1
733 Inhibitor AZD1775 Plus Carboplatin in Patients With TP53 -Mutated Ovarian Cancer
734 Refractory or Resistant to First-Line Therapy Within 3 Months. *J. Clin. Oncol.* **2016**, 34,
735 4354–4361.
- 736 34. Wheler, J.J.; Janku, F.; Naing, A.; Li, Y.; Stephen, B.; Zinner, R.; Subbiah, V.; Fu, S.;
737 Karp, D.; Falchook, G.S.; et al. TP53 Alterations Correlate with Response to

- 738 VEGF/VEGFR Inhibitors: Implications for Targeted Therapeutics. *Mol. Cancer Ther.*
739 **2016**, *15*, 2475–2485.
- 740 35. Koehler, K.; Liebner, D.; Chen, J.L. TP53 mutational status is predictive of pazopanib
741 response in advanced sarcomas. *Ann. Oncol.* **2016**, *27*, 539–543.
- 742 36. Lehmann, S.; Bykov, V.J.N.; Ali, D.; Andrén, O.; Cherif, H.; Tidefelt, U.; Ugglä, B.;
743 Yachnin, J.; Juliusson, G.; Moshfegh, A.; et al. Targeting p53 in Vivo: A First-in-Human
744 Study With p53-Targeting Compound APR-246 in Refractory Hematologic Malignancies
745 and Prostate Cancer. *J. Clin. Oncol.* **2012**, *30*, 3633–3639.
- 746 37. Janku, F.; Yap, T.A.; Meric-Bernstam, F. Targeting the PI3K pathway in cancer: are we
747 making headway? *Nat. Rev. Clin. Oncol.* **2018**, *15*, 273–291.
- 748 38. Zhao, L.; Vogt, P.K. Helical domain and kinase domain mutations in p110 α of
749 phosphatidylinositol 3-kinase induce gain of function by different mechanisms. *Proc. Natl.*
750 *Acad. Sci. U. S. A.* **2008**, *105*, 2652–7.
- 751 39. André, F.; Ciruelos, E.; Rubovszky, G.; Campone, M.; Loibl, S.; Rugo, H.S.; Iwata, H.;
752 Conte, P.; Mayer, I.A.; Kaufman, B.; et al. Alpelisib for PIK3CA-Mutated, Hormone
753 Receptor–Positive Advanced Breast Cancer. *N. Engl. J. Med.* **2019**, *380*, 1929–1940.
- 754 40. Chandarlapaty, S.; Sakr, R.A.; Giri, D.; Patil, S.; Heguy, A.; Morrow, M.; Modi, S.;
755 Norton, L.; Rosen, N.; Hudis, C.; et al. Frequent Mutational Activation of the PI3K-AKT
756 Pathway in Trastuzumab-Resistant Breast Cancer. *Clin. Cancer Res.* **2012**, *18*, 6784–6791.
- 757 41. Weigelt, B.; Warne, P.H.; Downward, J. PIK3CA mutation, but not PTEN loss of function,
758 determines the sensitivity of breast cancer cells to mTOR inhibitory drugs. *Oncogene*
759 **2011**, *30*, 3222–3233.

- 760 42. Nakanishi, K.; Yang, Y.; Pierce, A.J.; Taniguchi, T.; Digweed, M.; D'Andrea, A.D.;
761 Wang, Z.; Jasin, M. Human Fanconi anemia monoubiquitination pathway promotes
762 homologous DNA repair. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 1110–5.
- 763 43. Wilkes, D.C.; Sailer, V.; Xue, H.; Cheng, H.; Collins, C.C.; Gleave, M.; Wang, Y.;
764 Demichelis, F.; Beltran, H.; Rubin, M.A.; et al. A germline FANCA alteration that is
765 associated with increased sensitivity to DNA damaging agents. *Cold Spring Harb. Mol.*
766 *Case Stud.* **2017**, *3*.
- 767 44. Heyde, A.; Reiter, J.G.; Naxerova, K.; Nowak, M.A. Consecutive seeding and transfer of
768 genetic diversity in metastasis. *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116*, 14129–14137.
- 769 45. Giuliano, M.; Shaikh, A.; Lo, H.C.; Arpino, G.; Placido, S. De; Zhang, X.H.; Cristofanilli,
770 M.; Schiff, R.; Trivedi, M. V Perspective on Circulating Tumor Cell Clusters: Why It
771 Takes a Village to Metastasize. *Cancer Res.* **2018**, *78*, 845–853.
- 772 46. Carpten, J.D.; Faber, A.L.; Horn, C.; Donoho, G.P.; Briggs, S.L.; Robbins, C.M.;
773 Hostetter, G.; Boguslawski, S.; Moses, T.Y.; Savage, S.; et al. A transforming mutation in
774 the pleckstrin homology domain of AKT1 in cancer. *Nature* **2007**, *448*, 439–44.
- 775 47. Troxell, M.L. PIK3CA/AKT1 Mutations in Breast Carcinoma: a Comprehensive Review
776 of Experimental and Clinical Studies Clinical & Experimental Pathology. *J. Clin. Exp.*
777 *Pathol.* **2012**.
- 778 48. Turner, N.C.; Alarcón, E.; Armstrong, A.C.; Philco, M.; López Chuken, Y.A.; Sablin, M.-
779 P.; Tamura, K.; Gómez Villanueva, A.; Pérez-Fidalgo, J.A.; Cheung, S.Y.A.; et al.
780 BEECH: a dose-finding run-in followed by a randomised phase II study assessing the
781 efficacy of AKT inhibitor capivasertib combined with paclitaxel in patients with estrogen

- 782 receptor-positive advanced or metastatic breast cancer. *Ann. Oncol.* **2019**, *30*, 774–780.
- 783 49. Jones, R.H.; Carucci, M.; Casbard, A.C.; Butler, R.; Alchami, F.; Bale, C.J.; Bezecny, P.;
784 Joffe, J.; Moon, S.; Twelves, C.; et al. Capivasertib (AZD5363) plus fulvestrant versus
785 placebo plus fulvestrant after relapse or progression on an aromatase inhibitor in metastatic
786 ER-positive breast cancer (FAKTION): A randomized, double-blind, placebo-controlled,
787 phase II trial. *J. Clin. Oncol.* **2019**, *37*, 1005.
- 788 50. Bidard, F.C.; Sabatier, R.; Berger, F.; Pistilli, B.; Dalenc, F.; De La Motte Rouge, T.;
789 Frenel, J.-S.; Dubot, C.; Ladoire, S.; Ferrero, J.-M.; et al. PADA-1: A randomized, open
790 label, multicentric phase III trial to evaluate the safety and efficacy of palbociclib in
791 combination with hormone therapy driven by circulating DNA ESR1 mutation monitoring
792 in ER-positive, HER2-negative metastatic breast cancer. *J. Clin. Oncol.* **2018**, *36*.
- 793 51. Turner, N.; Bye, H.; Kernaghan, S.; Proszek, P.; Fribbens, C.; Moretti, L.; Morden, J.;
794 Snowden, C.; Macpherson, I.; Wardley, A.; et al. Abstract OT1-06-03: The
795 plasmaMATCH trial: A multiple parallel cohort, open-label, multi-centre phase II clinical
796 trial of ctDNA screening to direct targeted therapies in patients with advanced breast
797 cancer (CRUK/15/010). *Cancer Res.* **2018**, *78*.
- 798 52. Lianidou, E.; Pantel, K. Liquid biopsies. *Genes Chromosom. Cancer* **2019**, *58*, 219–232.
- 799 53. Alberter, B.; Klein, C.A.; Polzer, B. Single-cell analysis of CTCs with diagnostic
800 precision: opportunities and challenges for personalized medicine. *Expert Rev. Mol.*
801 *Diagn.* **2016**, *16*, 25–38.
- 802 54. Faugeroux, V.; Pailler, E.; Oulhen, M.; Deas, O.; Brulle-Soumare, L.; Hervieu, C.; Marty,
803 V.; Alexandrova, K.; Andree, K.C.; Stoecklein, N.H.; et al. Genetic characterization of a

804 unique neuroendocrine transdifferentiation prostate circulating tumor cell-derived eXplant
805 model. *Nat. Commun.* **2020**, *11*, 1–16.

806 55. Sinkala, E.; Sollier-Christen, E.; Renier, C.; Rosàs-Canyelles, E.; Che, J.; Heirich, K.;
807 Duncombe, T.A.; Vlassakis, J.; Yamauchi, K.A.; Huang, H.; et al. Profiling protein
808 expression in circulating tumor cells using microfluidic western blotting. *Nat. Commun.*
809 **2017**, *8*.

810 56. Gorges, T.M.; Kuske, A.; Röck, K.; Mauermann, O.; Müller, V.; Peine, S.; Verpoort, K.;
811 Novosadova, V.; Kubista, M.; Riethdorf, S.; et al. Accession of Tumor Heterogeneity by
812 Multiplex Transcriptome Profiling of Single Circulating Tumor Cells. *Clin. Chem.* **2016**,
813 *62*, 1504–1515.

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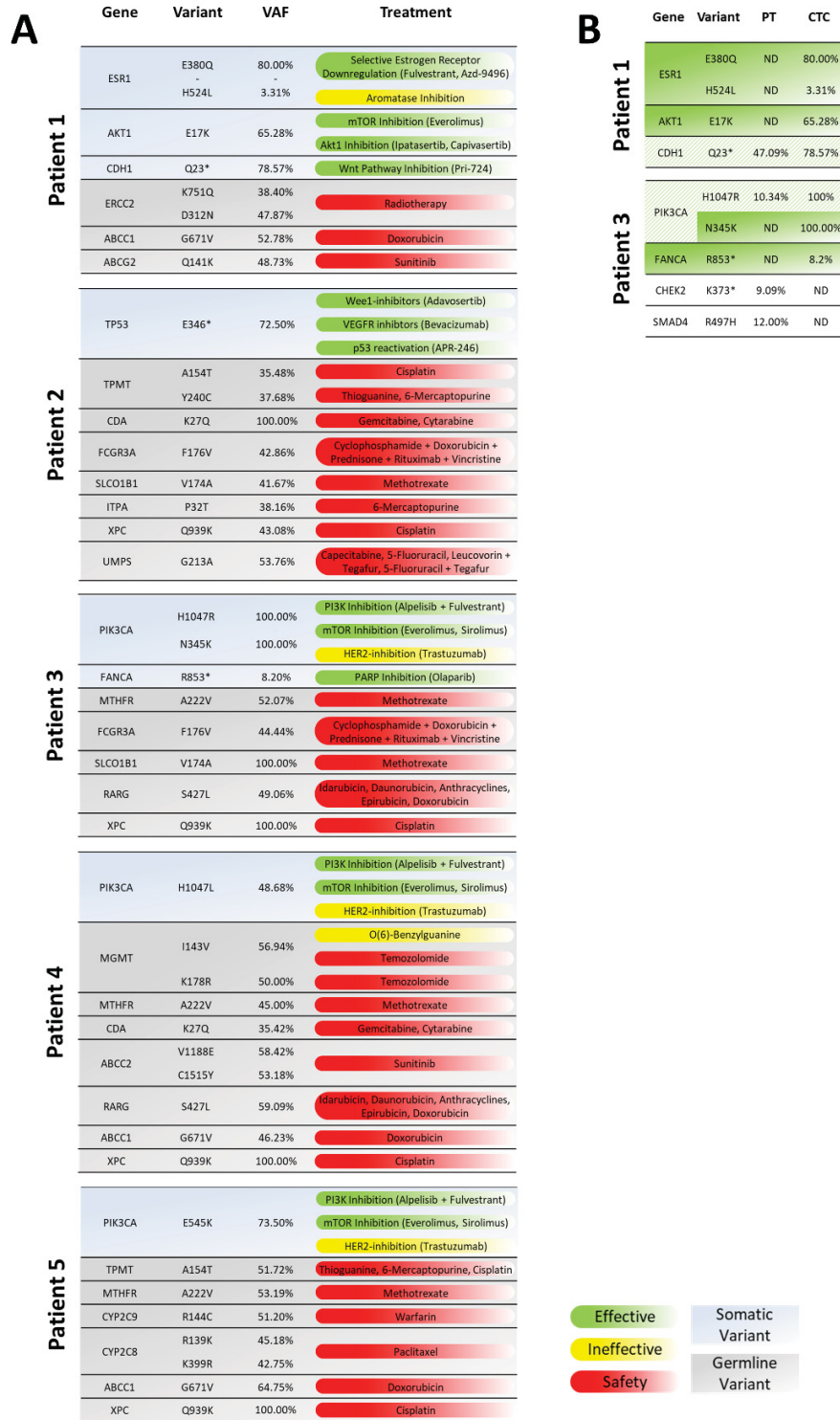
Table 1: Clinical data

* Clinical data from the beginning of the observation period at T3 when whole exome sequencing (WES) was performed. Later, patient 1 developed metastatic lesions in the liver and lymph nodes and received chemotherapy – first epirubicin, then paclitaxel.

[†] determined by CellSearch system

M (metastasis sites); G (grading); ER (estrogen receptor); PR (progesterone receptor); lob (lobular); NST (no special type); LN (lymph node); BM (bone marrow); WGA (whole genome amplification); DLA (diagnostic leukapheresis)

Patient ID	Age at CTC sequencing	Type	M	G	ER	PR	HER2/neu	Chemotherapy	Radiation	Endocrine Therapy	CTC count per 7,5 ml blood	Cells used for WES	Material CTCs
Patient 1	74	Invasive-lob	Bone, bladder *	2	+	+	-	- *	+	+	Approx. 50,000	20,000 CTCs	DLA product
Patient 2	70	Invasive-lob	Bone, liver	2	+	-	-	+	+	+	2,687	8 CTCs (WGA)	Blood
Patient 3	64	Invasive-lob	Bone, LN, ovary, pleura	2	+	+	-	+	+	+	583	6 CTCs (WGA)	Blood
Patient 4	72	Invasive-lob	Bone, BM	2	+	+	-	+	+	+	8,000	15 CTCs (WGA)	Blood
Patient 5	51	NST	Bone, liver	2	+	+	-	+	+	+	94	9 CTCs (WGA)	Blood



822

823 Figure 1: Mutations detected by whole-exome sequencing of CTCs and the therapies targeting them

824 (A) Only clinically relevant mutations detected in the CTCs are shown. The second exon of the
825 *AKT1* gene was not covered in the WES of all WGA samples. Because this exon contains amino
826 acid E17, frequently mutated in metastatic breast cancer, it was sequenced also with targeted
827 Sanger sequencing (Supplemental Figure S2). For each detected mutation, treatments and their
828 efficacies and safety concerns are listed (effective: potentially effective treatment option;
829 ineffective: potentially ineffective treatment; safety: potential safety concern). (B) Concordance of
830 mutations between primary tumor (PT) and CTCs. Only clinically targetable mutations are listed.
831 Variants that were detected only in CTCs but not in the PT are shown in green. Variants that were
832 detected in a minor subclone of the PT compared to the CTCs are indicated in green hatched.
833 nd (not detected)

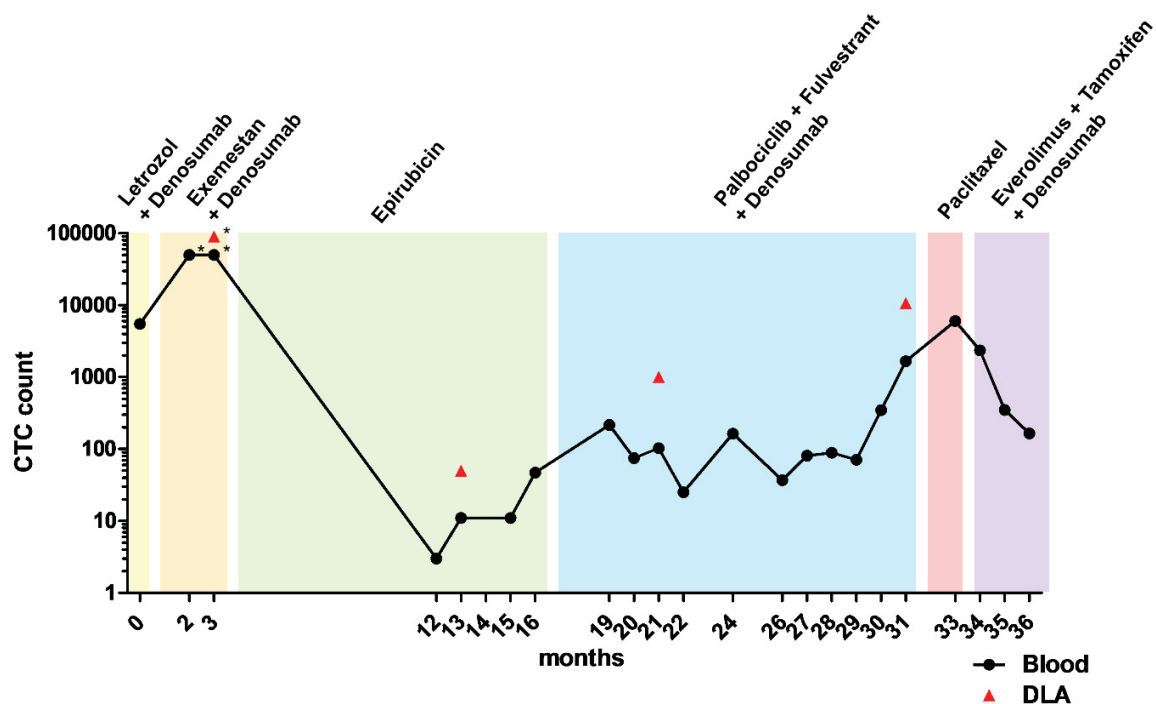


Figure 2: CTC count dynamics of patient 1 during the course of the disease

CTCs from patient 1 were counted from 7.5 ml blood or 2 ml diagnostic leukapheresis product (DLA) using the CellSearch system. Each color indicates the duration of a different line of therapy. Time point T0 corresponds to the first presentation of the patient to our department, approximately seven years after the resection of the primary tumor.

* The CTC count exceeded the CellSearch detection range. CTC count was estimated by dilution.

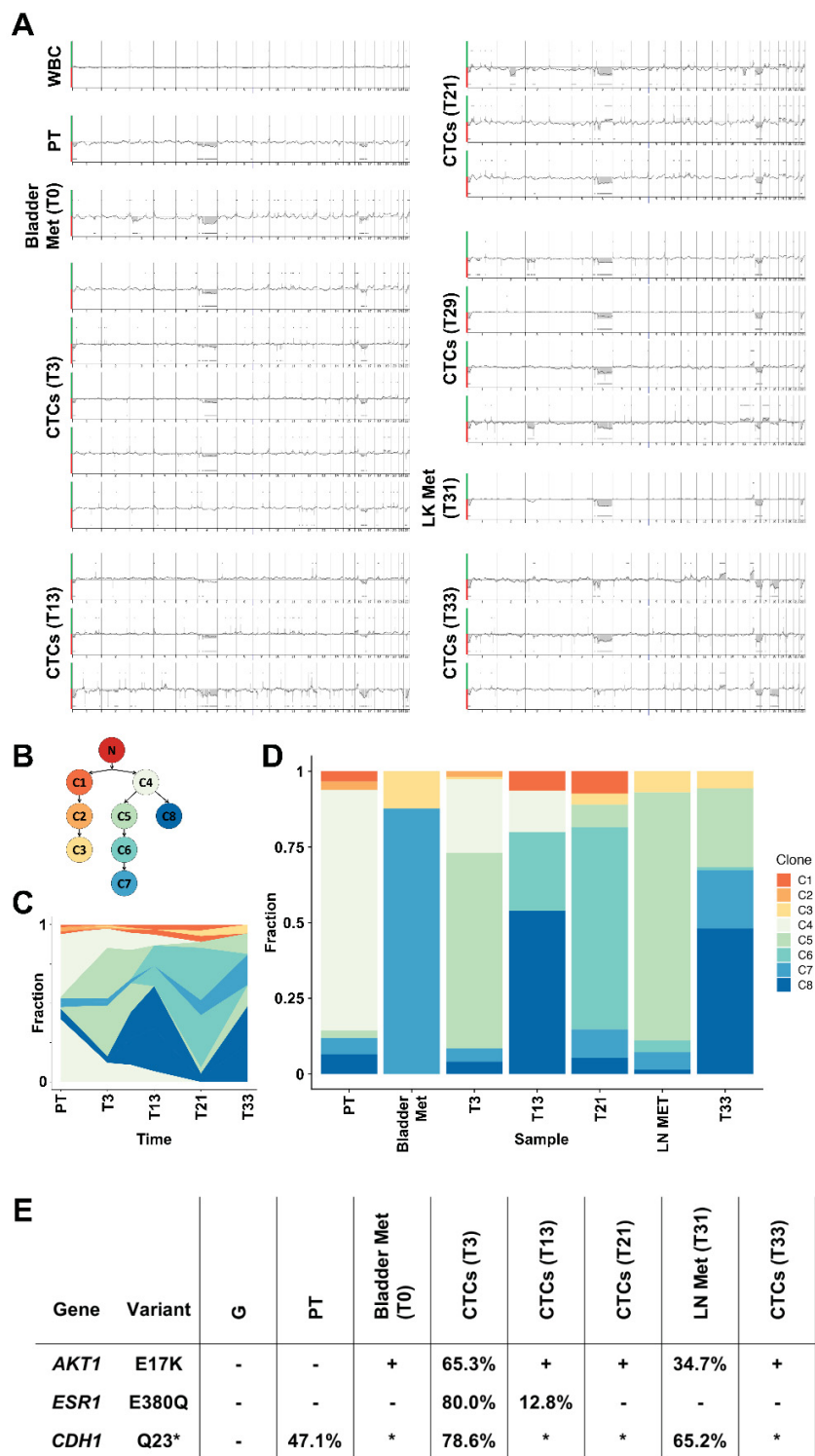


Figure 3: Clonal evolution of the tumor of patient 1

843 (A) Chromosomal aberrations as determined by array comparative genome hybridization. Green
844 indicates a signal above the baseline representing a copy number gain. Red indicates a signal below
845 the baseline representing a copy number loss. (B) Clonal reconstruction from the whole-exome
846 sequencing data revealed eight tumor clones. (C & D) Clonal dynamics of the tumor cell clones
847 during the course of the disease. (E) Presence and dynamics of clinically relevant mutations. Values
848 indicate variant allele frequencies. Positive results (no allele fraction) come from Sanger
849 sequencing (Supplemental Table S2). * The *CDH1* Q23 position was not covered by whole-exome
850 sequencing on amplified DNA samples. No information about the mutational status in those
851 samples is available.

852 CTC (circulating tumor cell); WBC (white blood cell); PT (primary tumor); LN (lymph node); Met
853 (metastasis); G (germline)

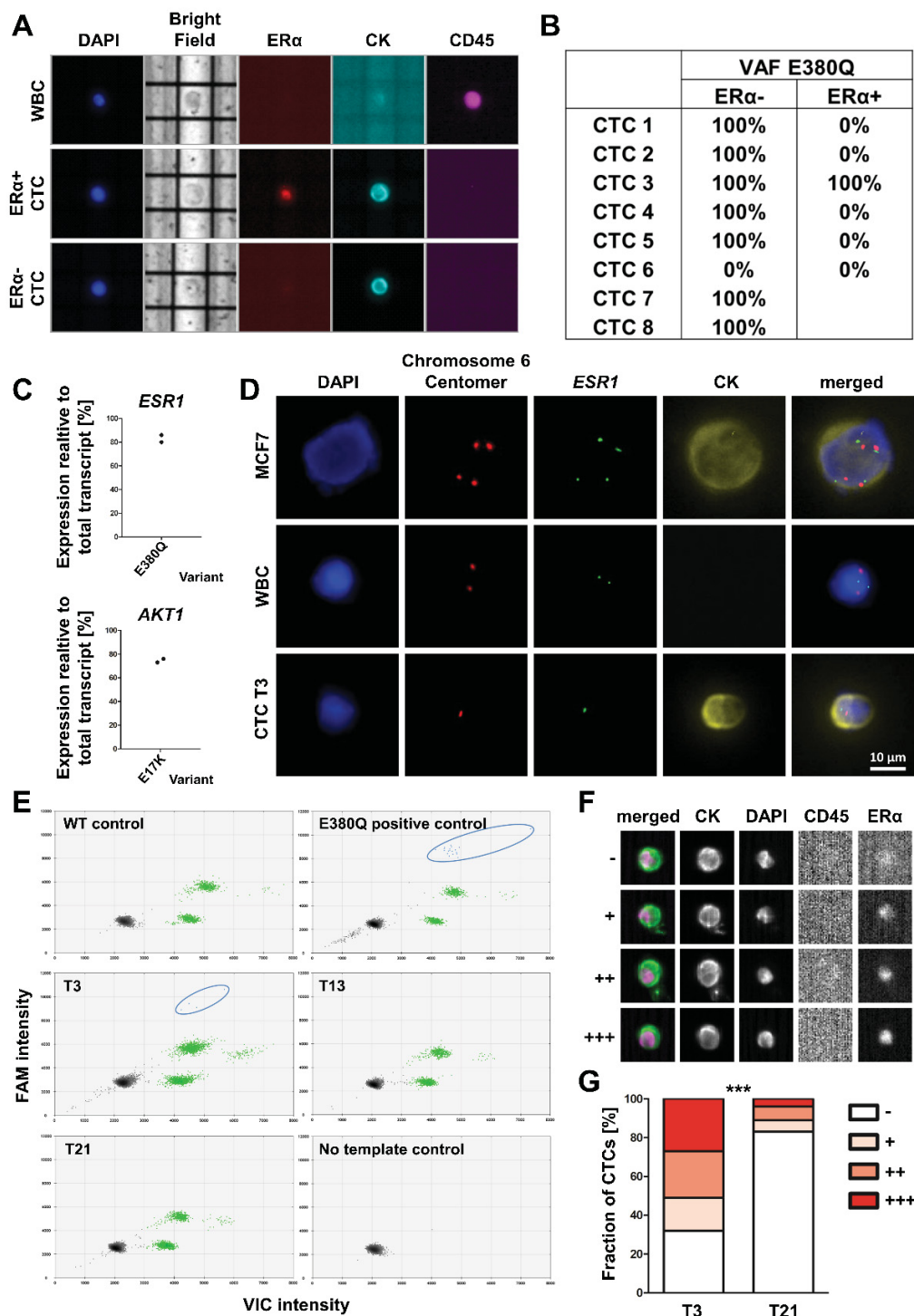


Figure 4: Analysis of *ESR1* mutations and ERα expression during the course of the disease of patient 1

857 (A & B) The *ESR1* gene from single ER α positive or negative circulating tumor cells (CTCs) from
858 T3 was sequenced (images were generated with the DEParray system). (C) Frequencies of the
859 variant allele on transcriptome level were determined by RNA sequencing of the primary tumor
860 and CTCs from T3 (2 replicates each). (D) *ESR1* copy numbers determined by fluorescence in situ
861 hybridization. Signals detected with a probe for the *ESR1* gene are shown in green. As a reference,
862 a probe for the centromere region of chromosome 6 was used (red). Tumor cells were identified by
863 cytokeratin positivity (original magnification 40 $\times$). (E) ddPCR analysis of *ESR1* E380Q mutation
864 in ctDNA. Droplets harboring a mutation are highlighted with circles (blue). (F & G) ER α
865 expression analyzed with the CellSearch system. CTCs were classified into four groups based on
866 the ER α staining intensity (images were generated with the CellSearch system). The statistical
867 significance level was determined by a Fisher's exact test by comparing ER α -positive (+/+/+/+) and
868 ER α -negative CTCs (p -value < 0.001).

869 WBC (white blood cell); WT (wildtype)

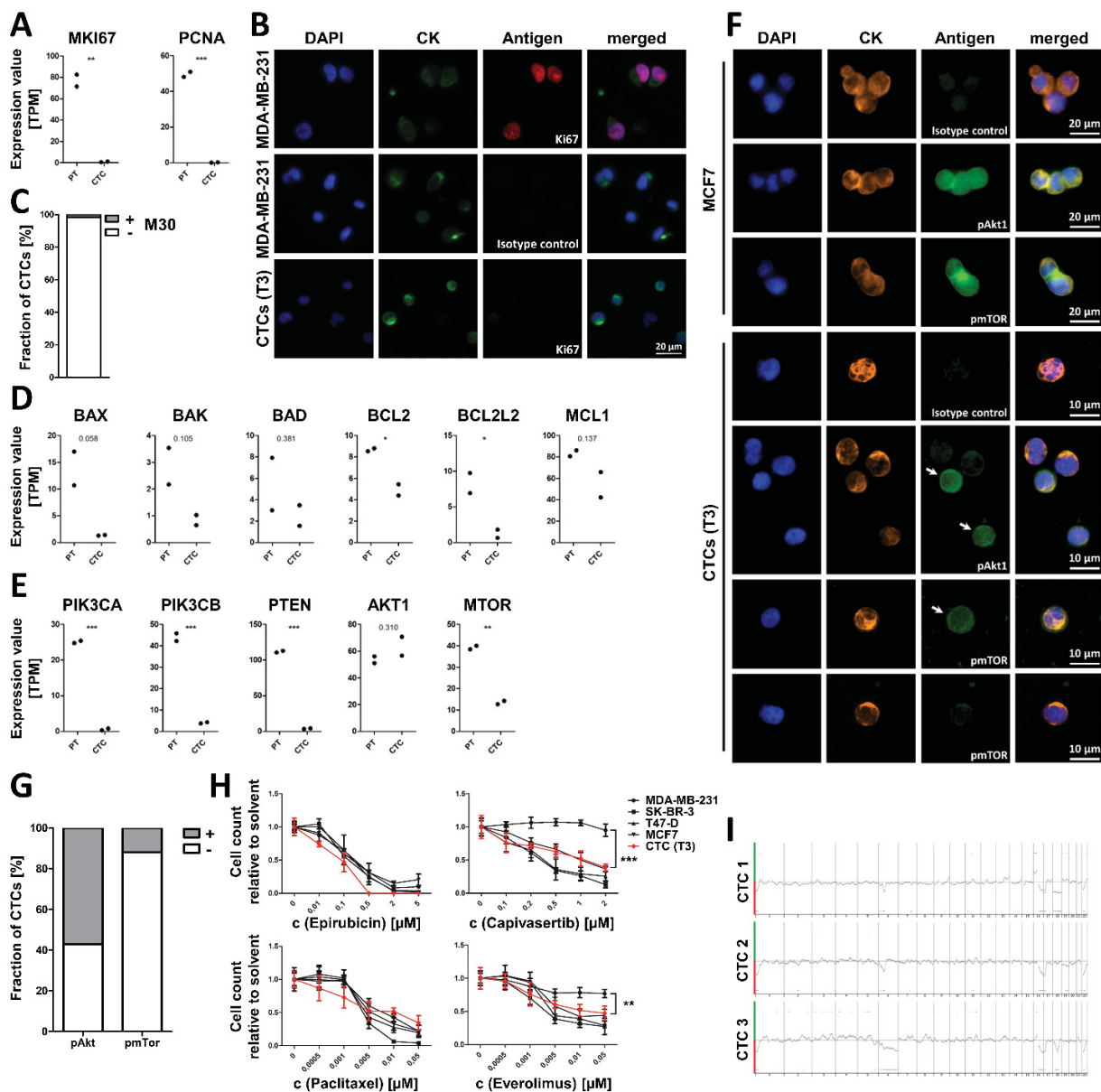


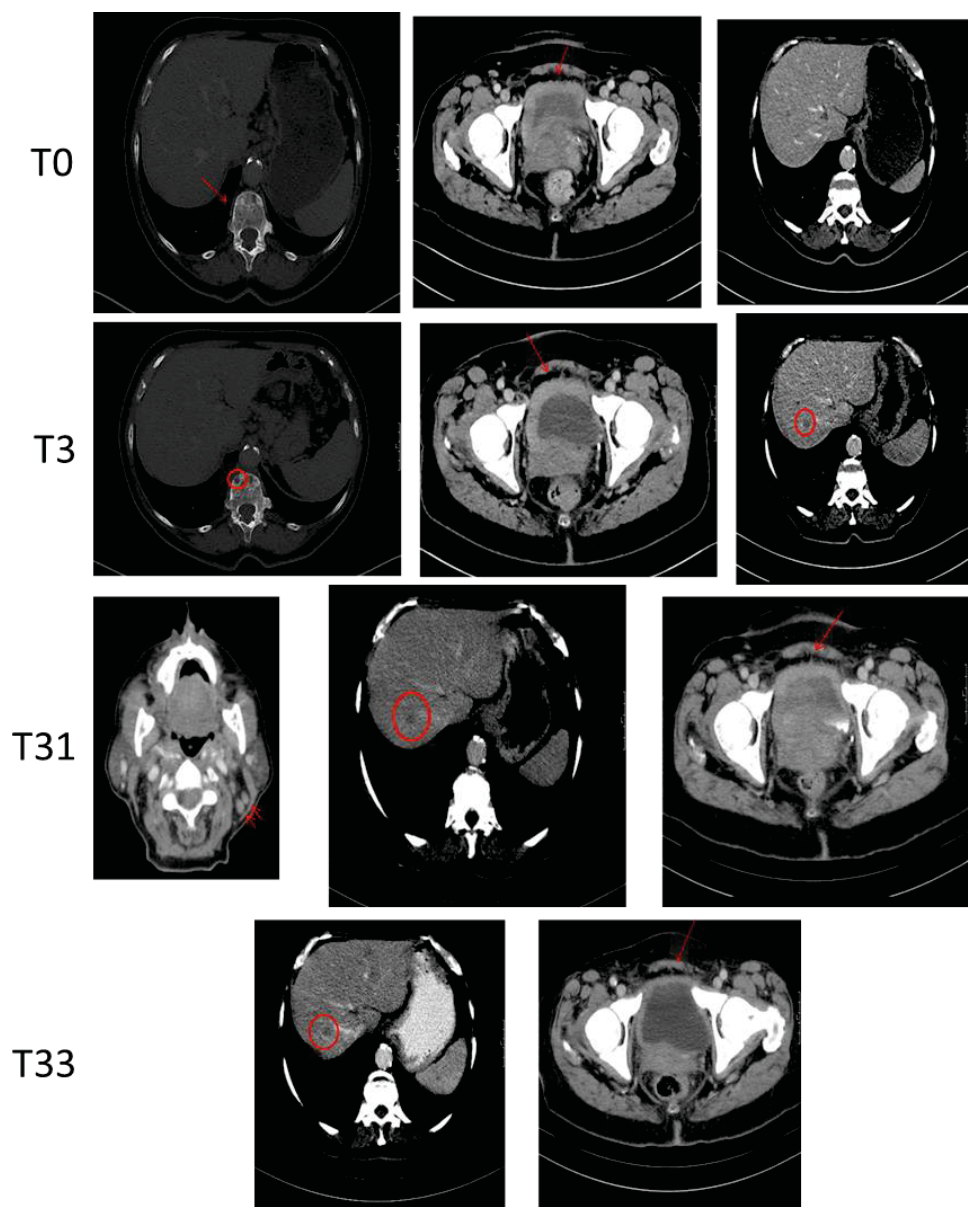
Figure 5: Analysis to predict the efficacy of a treatment targeting the Akt1/mTOR pathway in patient 1

Gene expression data were generated by RNA sequencing of bulk circulating tumor cells (CTCs) and the primary tumor (2 replicates each). Statistical significance levels were determined with a two-tailed *t*-test. Selected genes are involved in proliferation (A), regulation of apoptosis (D), and regulation of the PI3K/AKT1/mTOR pathway (E). (B) Expression of the proliferation marker Ki67

analyzed by immunocytofluorescence analysis. Ki67 positive cells from the MDA-MB-231 cell line served as references (original magnification 40×). (C) Apoptosis status as determined by staining for caspase cleaved cytokeratin 18 (M30) (Supplemental Figure S7). Positive cells were deemed apoptotic. (F) Immunofluorescence analysis of phosphorylated AKT1 (Ser473) and phosphorylated mTOR (Ser2448). Cells from the MCF7 cell line served as positive control. Positive CTCs are highlighted with arrows (original magnification 40×). (G) Ratio of CTCs positive/negative for phosphorylated AKT1 and phosphorylated mTOR. (H) The drugs epirubicin, capivasertib, paclitaxel, and everolimus were tested with *in vitro* cultured CTCs. As references, cells from SK-BR-3, T47-D, MCF7 (all sensitive to treatment with capivasertib and everolimus; shown in grey), and MDA-MB-231 cell lines (resistant to treatment with capivasertib and everolimus; shown in black) were used. Statistical significance levels were determined by a two-way ANOVA with posthoc Bonferroni test. Error bars show standard deviation. (I) Copy number aberration profiles of remaining CTCs at T36. Green indicates a signal below the baseline representing a copy number gain. Red indicates a signal below the baseline representing a copy number loss.

*** indicates a p -value < 0.001, ** indicates a p -value < 0.01, * indicates a p -value < 0.05; TPM (transcripts per million); CK (cytokeratins)

895 Supplemental data



896

897 Figure S1: Staging CT analysis of patient 1

898 Staging CTs were performed regularly during the course of the disease. Discussed areas are

899 highlighted with red circles.

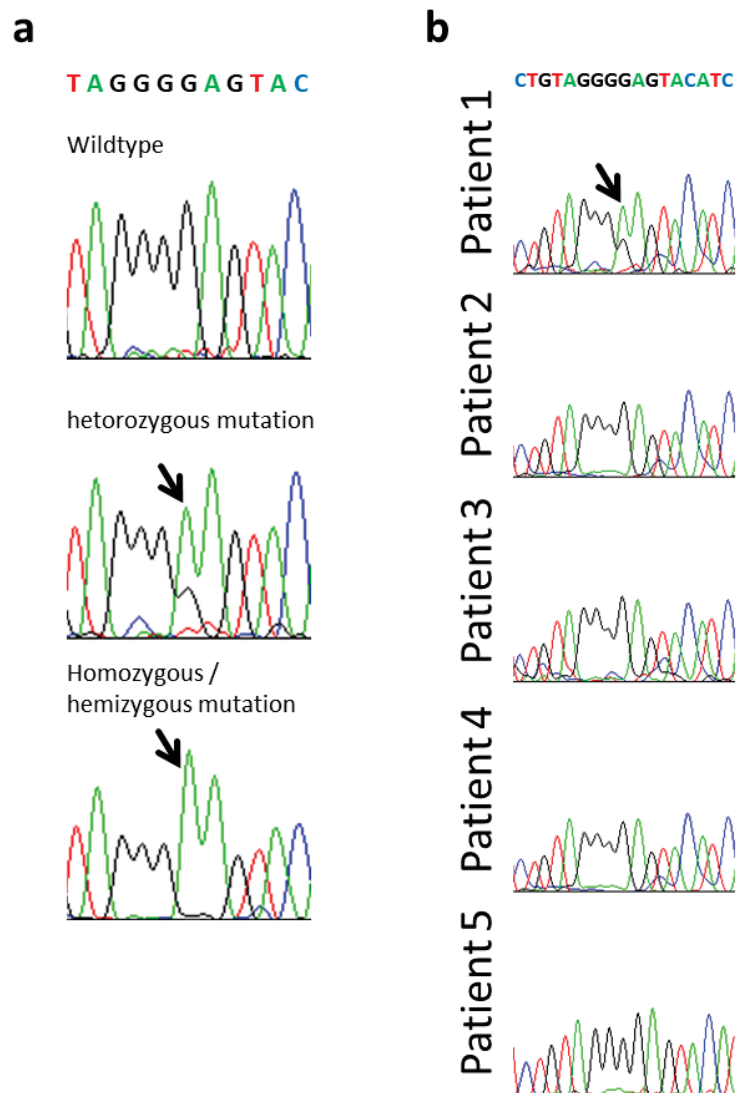


Figure S2: Mutation analysis of the *AKT1* E17K hotspot

(a) References for E17 hotspot mutation in *AKT1*. (b) Sanger sequencing results on pooled whole-genome amplification products from single CTCs. Patient 1 shows a heterozygous mutation, highlighted with an arrow.

	Gene	Variant	VAF	Treatment
Patient 1	CDH1	Q23*	47.09%	Wnt Pathway Inhibition (Pri-724)
	ERCC2	K751Q	31.53%	Radiotherapy
		D312N	43.90%	Radiotherapy
	ABCC1	G671V	45.68%	Doxorubicin
	ABCG2	Q141K	45.08%	Sunitinib
Patient 3	PIK3CA	H1047R	10.34%	PI3K Inhibition (Alpelisib + Fulvestrant)
				mTOR Inhibition (Everolimus, Sirolimus)
				HER2-inhibition (Trastuzumab)
	CHEK2	K373*	9.09%	PARP Inhibition
	SMAD4	R497H	12.00%	5-Fluorouracil
	MTHFR	A222V	52.07%	Methotrexate
	FCGR3A	F176V	44.44%	Cyclophosphamide + Doxorubicin + Prednisone + Rituximab + Vincristine
	SLCO1B1	V174A	100.00%	Methotrexate
	RARG	S427L	49.06%	Idarubicin, Daunorubicin, Anthracyclines, Epirubicin, Doxorubicin
	XPC	Q939K	100.00%	Cisplatin

Effective

Ineffective

Safety

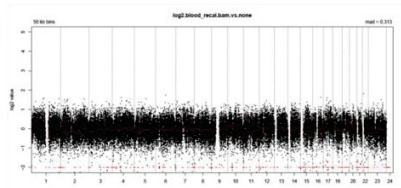
Somatic Variant

Germline Variant

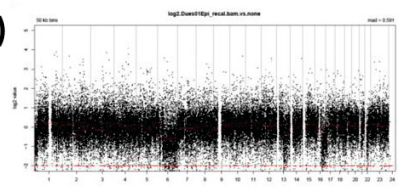
Figure S3: Whole exome sequencing of the primary tumor of patients 1 and 3

For each detected mutation, treatments and their efficacies and safety concerns are listed (effective: potentially effective treatment option; ineffective: potentially ineffective treatment; safety: potential safety concern).

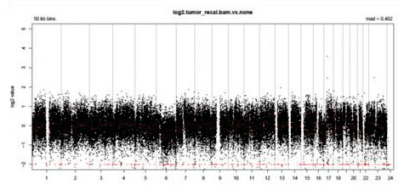
Germline



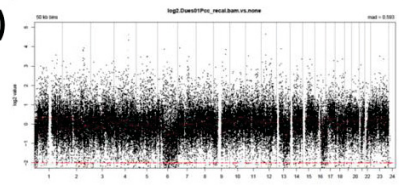
CTC (T13)



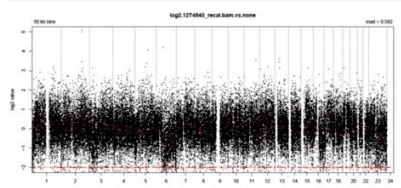
PT



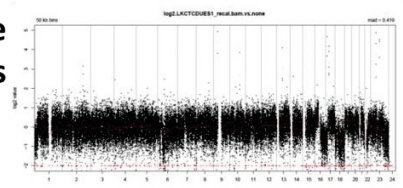
CTC (T21)



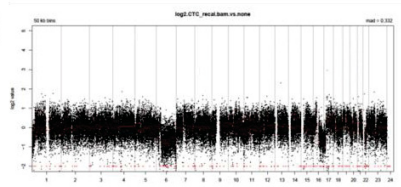
Bladder
Metastasis



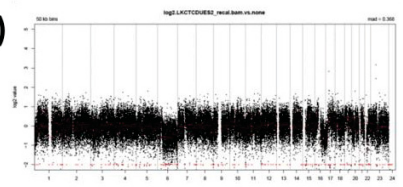
Lymph Node
Metastasis



CTC (T3)



CTC (T31)



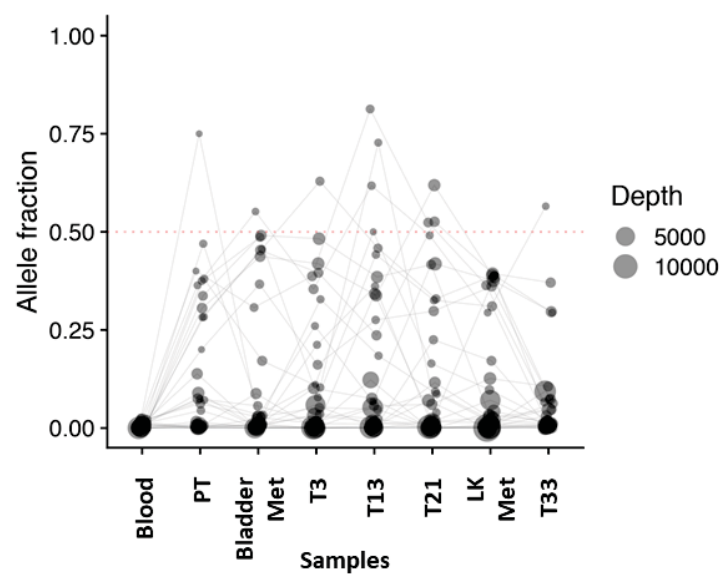
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912 Figure S4: Copy number profiles determined by whole-exome sequencing

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916

917 Figure S5: Allele fractions of clustered mutations

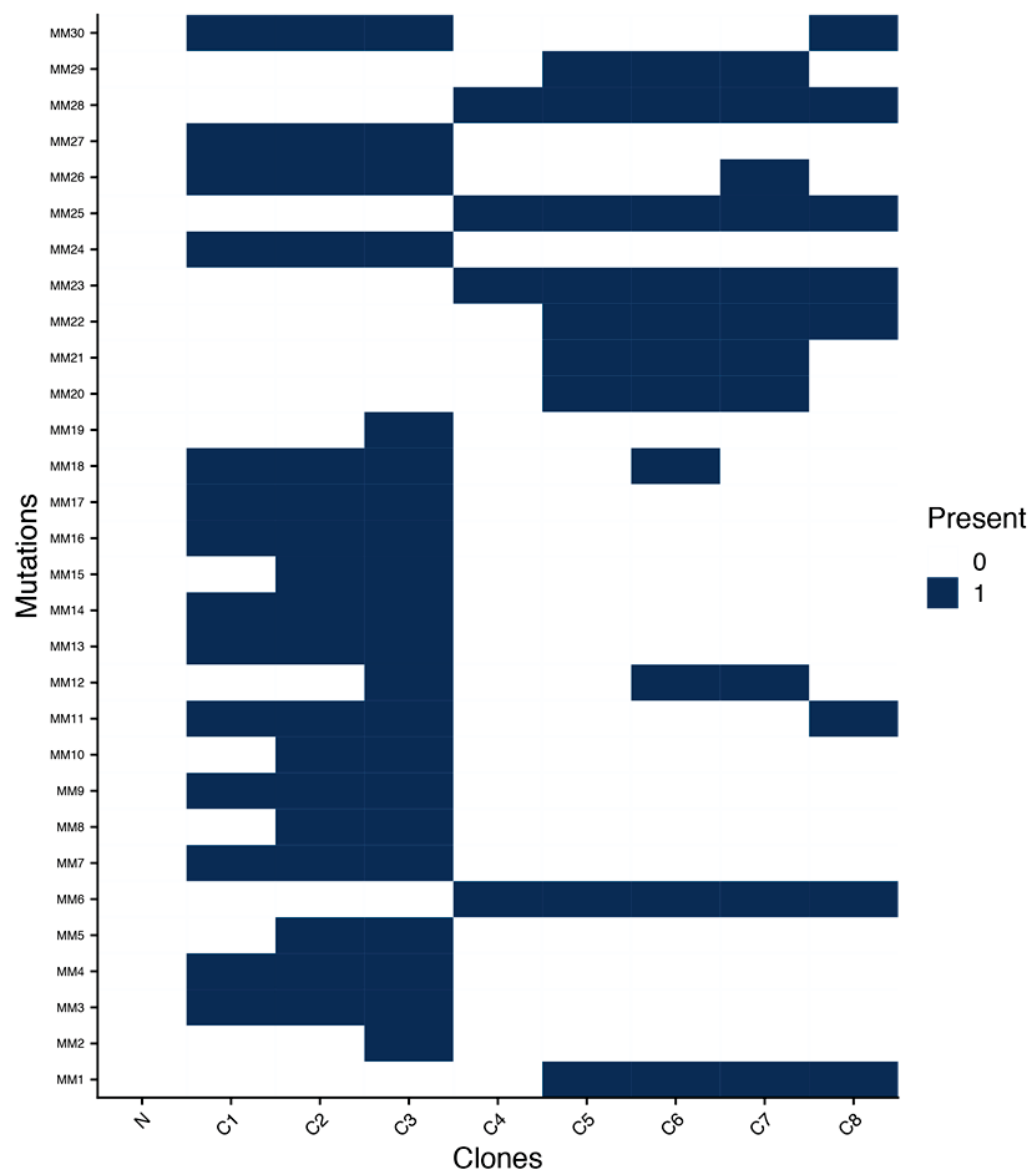
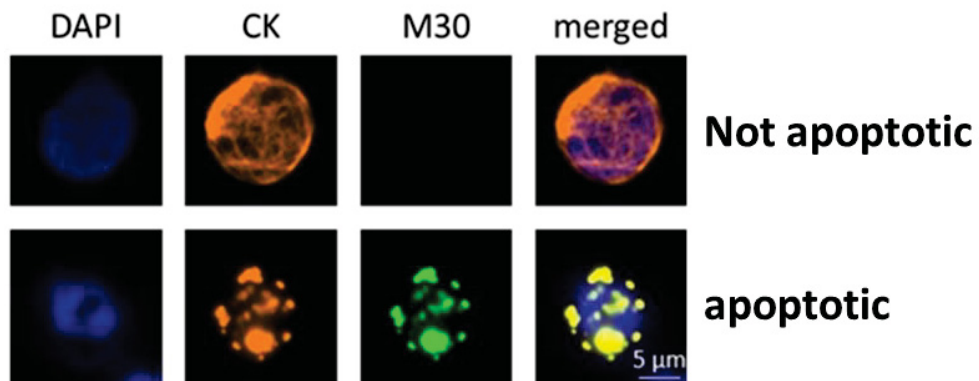


Figure S6: Genotypes of the identified tumor clones, in terms of clustered mutations



920

921 Figure S7: Analysis of apoptotic CTCs

922 The CTCs were stained for caspase cleaved cytokeratin 18 (M30). Positive cells were considered

923 as apoptotic (original magnification 40×).

924 Table S1: Analysis of *PIK3CA* mutations by ddPCR

925 DNA extracted from a time matched needle biopsy of a metastasis from patients 4 and 5 was
926 analyzed by ddPCR. The expected mutations were detected in the CTCs of patients 3, 4 and 5.
927 Tissue of a time matched biopsy of patient 3 was not available (na).

	Tissue Biopsy	CTCs
Patient 3 (H1047R)	na	100%
Patient 4 (H1047L)	43.1%	50.3 ± 0.4%
Patient 5 (E545K)	7.9% ± 6.6%	68.1% ± 4.5%

928

929 Table S2: *AKT1* E17K mutation on single CTCs of patient 1 during the observation period

930 *AKT1* E17K mutation on single CTCs from different time points were analyzed by targeted Sanger

931 sequencing. The varaint allele frequency is shown.

932 Het (heterozygous)

	T3	T12	T21	T31	T33	T37
CTC 1	100%	0%	0%	0%	Het	100%
CTC 2	Het	100%	100%	0%	100%	100%
CTC 3	100%	0%	100%	Het	0%	0%
CTC 4	100%	0%	0%	0%	Het	Het
CTC 5	100%	100%		Het	100%	0%
CTC 6	100%	100%		100%	0%	100%
CTC 7	0%	Het		0%	Het	0%
CTC 8	Het					100%
CTC 9	100%					100%

933

934 Table S3: Analysis of *ESR1* mutations in ctDNA by ddPCR

935 - indicates that no mutation was detected

	E380	Y537	D538
T3	0.2%	-	-
T13	-	-	-
T21	-	-	-

936

937 Table S4: Primers for sequencing of the *AKT1* region including the E17 mutational hotspot

Sequence	
Forward external	5‘ CACATCTGTCCTGGCACACC 3‘
Forward internal	5‘ AGTTCCTGCCTGGCTGCCTG 3‘
Reverse	5‘ GCCGCTCCTTGTAGCCAATG 3‘

938

939 Table S5: Primers for *PIK3CA* ddPCR

	Sequence
H1047R forward	5' GCAAGAGGCTTTGGAGTATTTCATG 3'
H1047R reverse	5' GCTGTTTAATTGTGTGGAAGATCCAA 3'
H1047L forward	5' GCAAGAGGCTTTGGAGTATTTCATG 3'
H1047L reverse	5' GCTGTTTAATTGTGTGGAAGATCCAA 3'
E545K forward	5' TCAAAGCAATTTCTACACGAGATCCT 3'
E545Kreverse	5' CTGTGACTCCATAGAAAATCTTTCTC 3'
E542K forward	5' GGGAAAATGACAAAGAACAGCTCAA 3'
E542K reverse	5` CTGTGACTCCATAGAAAATCTTTCTCCT 3'

940

941 Table S6: Probes for *PIK3CA* ddPCR

942 The following TaqMan probes with a 5' fluorophore and a 3' non-fluorescent quencher (NFQ)
943 were utilized

Sequence	
H1047R Wild type	5' (HEX)-CCACCATGATGTGCATC-(MGB NFQ) 3'
H1047R Mutant	5' (FAM)-CACCATGACGTGCATC-(MGB NFQ) 3'
H1047L Wild type	5' (HEX)-CCACCATGATGTGCATC-(MGBNFQ) 3'
H1047L Mutant	5' (FAM)-CACCATGAAGTGCATC-(MGB NFQ) 3'
E545K Wild type	5' (HEX)-CTCTCTGAAATCACTGAGCAG-(MGB NFQ) 3'
E545K Mutant	5' (FAM)-CTCTGAAATCACTAAGCAG-(MGB NFQ) 3'
E542K Wild type	5' (HEX)-CCTCTCTCTGAAATCA-(MGB NFQ) 3'
E542K Mutant	5' (FAM)-CCTCTCTCTAAAATCA-(MGB NFQ) 3'

944

3.4 Manuskript 4:

„A Multiplex PCR-Based Next Generation Sequencing-Panel to Identify Mutations for Targeted Therapy in Breast Cancer Circulating Tumor Cells.“

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Article

A Multiplex PCR-Based Next Generation Sequencing-Panel to Identify Mutations for Targeted Therapy in Breast Cancer Circulating Tumor Cells

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Abstract: Targeted therapy has become the preferred approach to treat most cancers, including metastatic breast cancer. Using liquid biopsies, which can act as a dynamic diagnostic tool, is an appealing concept to identify effective therapies. In order to identify mutations from circulating tumor cells (CTCs) on single cell level, we have developed a multiplex PCR-based next generation sequencing-panel. The CTCs were enriched using the CellSearch system and isolated by micromanipulation followed by whole genome amplification of their DNA. Afterwards, mutation hotspot regions in the *PIK3CA*, the *ESR1*, the *AKT1*, and the *ERBB2* genes were amplified and barcoded. Sequencing was performed on a MiSeq system. The assay was validated with cells from various cell lines displaying the expected mutations. Mutations that provide the basis for potential targeted therapies were detected in 10 out of 13 patients in all analyzed genes. In four patients, mutations in more than one gene were observed—either in the same cell or in different cells, suggesting the presence of different tumor cell clones, which might be targeted with combination therapies. This assay is a time and cost effective tool to investigate the most relevant genomic positions indicative for targeted therapies in metastatic breast cancer. It can support therapy decision to improve the treatment of cancer patients.

Keywords: breast cancer; circulating tumor cells; liquid biopsy; metastatic breast cancer; panel sequencing; targeted therapy; single cell analysis

1. Introduction

In the last decades, targeted therapy has become the preferred approach to treat most cancers. However, obtaining information to choose a targeted therapy is challenging: biopsies of recurrent or metastatic lesions are invasive and cannot be performed when clinical conditions have worsened or when a tumor is inaccessible [1]. Furthermore, the genomic profile of biopsy tissues provides a picture limited to a single point in space and time, and may, thus, under-represent intratumoral heterogeneity [2]. The predictive utility of tissue biopsies is limited by such factors, worsened still by the continuous evolution of the tumor in response to endogenous and exogenous selective pressures [3]. Deriving information about the primary tumor or metastatic lesions from liquid biopsies, which may act as a dynamic diagnostic tool, is an appealing concept to overcome this challenge [4,5].

Potential biomarkers are circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA). CTCs are shed into the blood by tumor tissue and are commonly considered as precursor cells for metastasis formation [6,7]. ctDNA as part of cell free DNA describes tumorous DNA circulating in the blood, which has been released from degraded tumor cells [8].

In breast cancer, clinical utility has been demonstrated for the theascreen *PIK3CA* RGQ PCR kit, which detects *PIK3CA* mutations in a tissue biopsy and/or in ctDNA obtained from a liquid biopsy in patients with an advanced or metastatic tumor [9]. The *PIK3CA* gene encodes for the p110 α subunit of the phosphoinositide-3-kinase (PI3K) which promotes cell proliferation and survival by activation of the PI3K/AKT signaling pathway [10]. Mutations, mostly located in its catalytic domain, can lead to an activation of downstream pathways [11].

Further mutations that could act as targets for therapy in breast cancer are located in the *ESR1*, the *AKT1* and the *ERBB2* genes: the *ESR1* gene codes for the estrogen receptor α (ER α), a nuclear hormone receptor that is involved in the regulation of gene expression affecting cellular proliferation and differentiation [12]. Mutations in the ligand binding domain of ER α lead to a conformational change of the protein and to a ligand-independent ER α activation, mostly acquired in resistance to estrogen deprivation therapy such as aromatase inhibition [13,14]. AKT1 is a member of the serine-threonine kinase class and plays a key role in cellular processes, including growth, proliferation, survival, and angiogenesis. Certain *AKT1* mutations lead to hyperactivation of the mTOR pathway [15]. The receptor tyrosine-protein kinase ERBB2 (HER2/neu (HER2)) activates the RAS/MAPK, PI3K/AKT, and JAK/STAT signaling pathways to promote cell proliferation and survival. Mutations in the hotspot regions coding for the extracellular domain and the kinase domain lead to an activation of the protein and its downstream pathways [16].

In order to identify mutations for targeted therapy in breast cancer using CTCs, which have been enriched using the CellSearch system, we developed a multiplex PCR-based next generation sequencing (NGS)-panel. The CellSearch system has been approved by the Food and Drug Administration (FDA) for detection of CTCs in patients with metastatic breast, prostate, and colorectal cancer.

2. Materials and Methods

2.1. Patients

We analyzed CTCs from 13 metastatic breast cancer patients. Patients were selected from the Augusta study cohort collected at the Department of Obstetrics and Gynecology, Duesseldorf, Germany (approved by the Ethics Committee of the Medical Faculty of the Heinrich Heine University Düsseldorf; Ref-No: 3430). All patients gave their informed consent for the use of their blood samples for CTC analysis and translational research. Patients' characteristics were anonymized by using sample identifiers. Clinical patient data are shown in Table 1.

2.2. Enrichment and Enumeration of CTCs

Enrichment and enumeration of CTCs was performed using the CellSearch Circulating Epithelial Cell Kit (Menarini, Florence, Italy) according to manufacturer's instructions. Briefly, blood was collected into CellSave Preservative Tubes (Menarini) and was processed within 96 h. 7.5 mL blood was used for enumeration of CTCs. Enrichment is based on immunomagnetic ferrofluid conjugated with an epithelial cell adhesion molecule-directed antibody. Subsequent characterization of CTCs was performed using immunofluorescent staining directed against cytokeratins to identify CTCs, CD45 to exclude leucocytes and DAPI to confirm nucleo-morphological integrity.

2.3. CTC Isolation

Single CTCs were isolated from the CellSearch cartridge by micromanipulation with the CellSelector (ALS, Jena, Germany). Cells were deposited in PCR tubes to perform whole genome amplification (WGA) as described previously [17].

2.4. Whole-Genome Amplification

DNA of single isolated cells was amplified by WGA with the Ampli1 WGA Kit (Menarini) according to the manufacturer's protocol. Afterwards DNA integrity was determined with the Ampli1 QC Kit (Menarini).

Table 1. Patient characteristics.

Characteristics	Total	in %
Patients	13	100
Age at blood draw		
Mean	61.2	
Median	61	
Range	46–78	
Tumor size		
pT1	4	30.8
pT2–4	8	61.5
na	1	7.7
Nodal status at time of diagnosis		
0	4	30.8
1–3	7	53.8
na	2	15.4
Metastasis status at time of diagnosis		
0	10	77.0
1	2	15.4
na	1	7.7
Histology		
NST	8	61.5
Invasive-lobular	5	38.5
Grading		
1–2	9	69.2
3	2	15.4
na	2	23.1
Subtype		
Luminal	11	84.6
Triple negative	2	15.4
CTC count (per 7.5 mL blood)		
Median	94	
Range	6–15,340	

NST, invasive carcinoma of no special type; na, data not available; CTC, circulating tumor cell.

2.5. Multiplex PCR-Based Next Generation Sequencing

DNA fragments were amplified and barcoded in two steps using KAPA2G Fast Multiplex Mix (Merck, Darmstadt, Germany). In the first step, fragments coding for mutation hotspot regions were amplified with gene specific primers (Table 2) containing primer binding sites for universal Multiplicom MID Dx primers (Agilent technologies, Santa Clara, CA, USA) in a multiplex PCR. 1 µL WGA product and in total 0.5 µM gene specific primers were applied (concentrations of all primers are shown in Table S1). The concentration of each primer is shown in Table S1. In the second step, Multiplicom MID Dx primers were used to attach unique barcodes for sequencing to the PCR products. 0.5 µL of Multiplicom MID Dx primers and 0.5 µL of first round PCR product were used. The applied PCR protocols are listed in Table S2.

Final PCR products were purified using Agencourt AMPure XP beads (Beckmann Coulter, Brea, CA, USA) and quantified with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) using the Broad Range Quant-iT dsDNA Assay Kit (Thermo Fisher Scientific).

DNA libraries of 0.0143 pM per sample were sequenced on a MiSeq system (Illumina, San Diego, CA, USA) with 151 bp paired end reads.

FASTQ files were analyzed with the Galaxy web platform, using the public server at usegalaxy.org (last access for the Galaxy web platform: March 2020) [18]. Reads were aligned to the human reference genome (hg38) with BWA-MEM (Galaxy Version 0.7.17.1) [19]. Standard parameters were applied. Aligned sequences were screened for mutations using the IG Viewer (Version 2.3.25) [20]. Identified mutations were covered by >100× and had a variant allele frequency (VAF) >12.5%.

As controls, DNA from the reference cell lines mentioned below as well as a negative control was included in every run.

Table 2. Primer overview.

	Forward Primer	Reverse Primer
<i>PIK3CA</i> Exon 5	5' AAGACTCGGCAGCATCTCCAGC ATTTCACAGCTACACCA 3'	5' GCGATCGTCACTGTTCTCCAGAT GTTCTCCTAACCATCTGA 3'
<i>PIK3CA</i> Exon 10	5' AAGACTCGGCAGCATCTCCAGGG AAAATGACAAAGAACAG 3'	5' GCGATCGTCACTGTTCTCCAATTT TAGCACTTACCTGTGAC 3'
<i>PIK3CA</i> Exon 21	5' AAGACTCGGCAGCATCTCCATTGA TGACATTGCATACATTCCG 3'	5' GCGATCGTCACTGTTCTCCAGTG GAAGATCCAATCCATTT 3'
<i>ESR1</i> Exon 5	5' AAGACTCGGCAGCATCTCCAT TGACCCTCCATGATCAGGT 3'	5' GCGATCGTCACTGTTCTCCAGC TACTCCTAAGCTACAGCC 3'
<i>ESR1</i> Exon 7	5' AAGACTCGGCAGCATCTCCATCT CTCACTCTCTCTCTGCG 3'	5' GCGATCGTCACTGTTCTCCAGATGT GGGAGAGGATGAGGA 3'
<i>ESR1</i> Exon 8	5' AAGACTCGGCAGCATCTCCAAGT AGTCCTTTCTGTGTCTTC 3'	5' GCGATCGTCACTGTTCTCCAAT GCGATGAAGTAGAGCCC 3'
<i>AKT1</i> Exon 3	5' AAGACTCGGCAGCATCTCCAGTA GAGTGTGCGTGGCTC 3'	5' GCGATCGTCACTGTTCTCCACCC CAAATCTGAATCCCGAG 3'
<i>ERBB2</i> Exon 8	5' AAGACTCGGCAGCATCTCCAGGC TACATGTTCTGATCTCC 3'	5' GCGATCGTCACTGTTCTCCAG GGTCTGAGGAAGGATAGGA 3'
<i>ERBB2</i> Exon 18	5' AAGACTCGGCAGCATCTCCAA AGTACACGATGCGGAGACT 3'	5' GCGATCGTCACTGTTCTCCAACCTT CACCTTCCTCAGCTC 3'
<i>ERBB2</i> Exon 19	5' AAGACTCGGCAGCATCTC CAATCCTCCTCTTTCTGCCAG 3'	5' GCGATCGTCACTGTTCTCCAAGTCTA GGTTTGCGGGAGTC 3'
<i>ERBB2</i> Exon 20	5' AAGACTCGGCAGCATCTC CATGGTTGTGATGGTTGGGAG 3'	5' GCGATCGTCACTGTTCTCCAGA CATGGTCTAAGAGGCAGC 3'

2.6. Validation Experiments with Spiked Cells

For validation of the workflow, 200 cells of the cell lines SK-BR-3, T47-D, and MCF7 (ATCC, Manassas, United States; catalog numbers: SK-BR-3: HTB-30, T-47D: HTB133, and MCF7: HTB-22) as well as long term cultured CTCs were spiked into 7.5 mL healthy donor blood. For detachment from the culture flask, an enzyme-free cell dissociation buffer (Thermo Fisher Scientific) was used. The tumor cells were enriched using the CellSearch, isolated using the CellSelector and the genome was amplified with the Ampli1 WGA Kit similarly to CTCs.

The SK-BR-3, T47-D, and MCF7 cells were cultured in RPMI 1640 containing 10% fetal calf serum, 25 mmol/L HEPES and 1% Penicillin-Streptomycin (all Thermo Fisher Scientific). Cells were grown at 37 °C in a humidified atmosphere with 5% CO₂ and were authenticated via short tandem repeat analysis. CTCs were cultured as previously described in low attachment plates (Corning, New York, NY, USA) in RPMI 1640 medium supplemented with 1× B27 (Thermo Fisher Scientific), 20 ng/mL hEGF (Merck), 20 ng/mL FGF (Merck), and 1% Penicillin-Streptomycin in a humidified atmosphere with 5% CO₂ and 4% O₂ [21]. Cultured cells were regularly tested negative for Mycoplasma infection.

2.7. Statistical Analysis

Statistical analyses were performed using GraphPad Prism (Graphpad Software, San Diego, CA, USA). *p*-values < 0.05 were considered statistically significant.

3. Results

3.1. Development of the Assay

We aimed to develop a multiplex PCR-based NGS panel to identify mutations in CTCs relevant for targeted therapies in metastatic breast cancer, considering that the genomic DNA of single CTCs was amplified using an MseI restriction-based method. We included hotspot regions in the *PIK3CA*, *ESR1*, *AKT1*, and *ERBB2* genes frequently mutated in breast cancers. In the *PIK3CA* gene, hotspot regions within the exons 5, 10, and 21 were covered. In the *ESR1* gene, the hotspot regions in exons 5, 7, and 8 were analyzed. In the *AKT1* gene, we investigated the hotspot regions in exon 3 and in the *ERBB2* gene our assay covers the hotspot regions in exons 8, 18, 19, and 20. For each amplicon, a panel of primers was tested and the most specific primer combination was chosen for the multiplex PCR (Table 2, Figure S1). Primer concentrations in the first PCR were titrated resulting in an equal ratio for each fragment of $7.0\% \pm 1.4\%$ to $10.2\% \pm 0.8\%$ after sequencing (Figure S2). In total, we achieved an on target rate of $95.3\% \pm 0.9\%$ of all reads.

Next, a group of CTCs of different quality determined by Ampli1 quality control was sequenced and the obtained coverage was compared. For cells with highest amplified DNA quality showing 4 bands in the quality control PCR a mean coverage of 10.2 ± 1.3 fragments per cell was observed (Figure 1). The coverage correlated with the quality determined with the quality control PCR. Cells displaying 3 or 4 bands in the quality control PCR had a significantly increased coverage compared to those with less than three bands ($p < 0.0001$ determined by two-tailed *t*-test). Thus, we focused on cells displaying at least 3 bands in the quality control PCR for further analysis to yield reliable results.

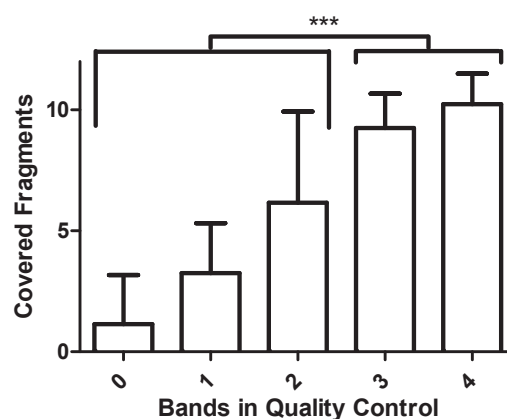


Figure 1. The number of covered fragments correlates with quality of WGA products. The *p*-value ($p < 0.0001$) was determined by two-tailed *t*-test. Error bars show standard deviation. For each group 10 to 30 cells were analyzed.

3.2. Validation of the Assay

To validate the assay, we performed spike in experiments using cells from four cell lines exhibiting different mutations. Those cells were processed applying the same workflow used later for clinical samples. As a negative control, cells from the Her2-enriched breast cancer cell line Sk-Br-3 harboring no mutations in the analyzed regions were analyzed. Cells from the luminal breast cancer cell lines T47-D and MCF7 were expected to show the *PIK3CA* mutations H1047R and E545K, respectively. Further, long term cultured CTCs derived from the DLA product of a metastatic luminal breast cancer patient harboring the *ESR1* mutation E380Q and the *AKT1* mutation E17K were analyzed. All investigated cells displayed the expected mutations demonstrating the reliability of our assay (Figure 2).

Cell line	Cell ID	PIK3CA		ESR1	AKT1
		E545K	H1047R	E380Q	E17K
Sk-BR-3	1				
	2				
	3				
	4				
	5				
	6				
T47-D	1		21%		
	2		24%		
	3		100%		
MCF-7	1	49%			
	2	58%			
	3	75%			
	4	57%			
	5	41%			
CTC (LTC)	1			100%	50%
	2			100%	100%
	3			100%	41%

Figure 2. Sequencing data of cells from reference cell lines. Displayed values show the variant allele frequency. Empty boxes indicate that no mutation was detected. LTC, long term cultured.

3.3. Analysis of CTCs from Metastatic Breast Cancer Patients to Identify Targeted Therapies

After validation with cell line cells we analyzed CTCs from 13 metastatic breast cancer patients. 11 of those patients had a primary tumor of luminal subtype and two patients were diagnosed with triple negative breast cancer. CellSearch analyses determined 6 to 15,340 CTCs per 7.5 mL blood. The median CTC count was 94 per 7.5 mL blood. For our study, 1 to 15 CTCs per patient were analyzed.

In seven patients, mutations in the *PIK3CA* gene were identified. Five of those mutations are well-described mutations located in the nucleotide triplets coding for the amino acid N345, E545, and H1047, respectively. *ESR1* mutations were detected in CTCs from two patients. One of those in the coding triplet for L536 is located in a well-known hotspot region. In CTCs from three patients, the *AKT1* mutation E17K was found, and in CTCs from five patients we discovered mutations in the *ERBB2* gene, including hotspot mutations in the nucleotide triplets coding for S310, L755, and V777 (Figure 3).

Mutations were detected in 16.7% (*ERBB2* A775T, patient 4) to 100% (e.g., *PIK3CA* H1047R, patient 8) of the CTCs analyzed per patient. In two cases, mutations were detected in two genes (*AKT1* and *ERBB2*, patient 6; *PIK3CA* and *ERBB2*, patient 8) per patient, in 1 patient we found mutations in three genes (*PIK3CA*, *ESR1*, and *AKT1*, patient 3). Those mutations either occurred in different CTCs (e.g., *AKT1* and *PIK3CA* mutations in CTCs from patient 3) or together in one cell (e.g., *PIK3CA* and *ERBB2* mutations in patient 8). One CTC from patient 3 harbored two activating mutations within the same gene (*PIK3CA* N345K and H1047R). Most mutations were detected with frequencies of 100% (e.g., *AKT1* E17K, patient 2) or about 50% (e.g., *PIK3CA* H1047R, patient 8).

Patient ID	CTC ID	PIK3CA						ESR1		AKT1	ERBB2				
		M318T	N345K	E545K	E547K	H1047L	H1047R	L536P	L541Q	E17K	S310F	L313P	L755S	A775T	V777L
Patient 1	1														
	2														
	3														
	4														
	5														
Patient 2	1									100%					
	2									100%					
	3														
	4									100%					
	5				16%					100%					
	6									100%					
	7									100%					
	8									100%					
	9									100%					
Patient 3	1									100%					
	2						100%		30%						
	3		100%												
	4														
	5		100%				100%								
Patient 4	1														
	2														
	3														
	4													34%	
	5														
	6														
Patient 5	2						80%								
	3														
	4						43%								
Patient 6	1												25%		
	2												70%		
	3									100%			74%		
	4												50%		
	5									38%					
	6														
	7												49%		
Patient 7	1														46%
Patient 8	1						51%								
	2						51%								
	3						52%				40%				
	4						34%				32%				
	5						49%				36%				
	6						51%				35%				
	7						50%								
Patient 9	1						79%						43%		
	2	13%					50%								
	3						34%								
	4														
	5														
	6						31%								
	7						22%								
	8						52%								
	9						59%								
	10						61%								
	11						60%								
	12						54%								
	13						85%								
	14														
	15														
Patient 10	1														
	2							100%							
	3														
	6														
Patient 11	1														
	2														
	3														
	4														
	5														
Patient 12	1			100%											
	2			100%											
	3														
	5			65%											
Patient 13	1			100%											
	2			70%											
	3			70%											
	4			65%											
	5			100%											
	6			75%											

Figure 3. Sequencing data from CTCs. Displayed values show the variant allele frequency. Empty boxes indicate that no mutation was detected.

4. Discussion

Mutations detected in cancer can help to choose an appropriate targeted therapy. In the advanced disease, analysis of a liquid biopsy can facilitate to identify such druggable targets. Various studies reported suitable tools for the analysis of single CTCs' WGA-products, either by investigation of single genomic positions by e.g., Sanger sequencing [22,23] or by sequencing multiple genomic positions by, e.g., NGS panels [24,25]. However, most available assays are either cost intensive, not adapted to single cell whole genome amplified DNA, or cover only single or not all relevant genomic positions for therapy selection in breast cancer. Here, we developed and provided the protocol for a multiplex PCR-based, Ampli1 WGA-adapted NGS panel that covers mutation hotspots in the *PIK3CA*, the *ESR1*, the *AKT1*, and the *ERBB2* genes that are most relevant for a liquid biopsy-based targeted therapy in breast cancer.

The assay was validated with spiked cell line cells and finally applied to CTCs from metastatic breast cancer patients. Mutations that provide the basis for potential targeted therapy were detected in 10 out of 13 patients. Although one CTC from patient 4 was found to harbor the *ERBB2* A775T mutation, it was not considered as clinically targetable since this mutation has not been characterized in breast cancer, yet. For the treatment of hormone receptor-positive, HER2-negative advanced breast cancers harboring *PIK3CA* variants such as N345K, E545K, H1047L, or H1047R, alpelisib plus fulvestrant is indicated [26]. Furthermore, HER2-positive tumors of patients with this variant exhibited resistance to trastuzumab [27]. The detected *ESR1* L536P mutation leads to a conformational change of the protein and to a ligand-independent ER α activation, thereby conferring resistance to aromatase inhibition [13]. However, treatment with second-generation selective ER α degraders such as fulvestrant is recommended [28]. The *AKT1* E17K mutation hyperactivates the mTOR pathway that could be targeted by mTOR inhibitors, such as everolimus, or AKT-inhibitors, such as ipatasertib and capivasertib [29,30]. Mutations such as S310F, L755S, or V777L in the *ERBB2* gene offer to be treated with the tyrosine kinase inhibitor neratinib [16].

The frequency of *PIK3CA* and *AKT1* mutations in our patient cohort exceeded the frequencies published in literature. *AKT1* E17K mutation frequencies between 1.4% and 8.2% have been described in breast cancer tissue [31] and mutations of the *PIK3CA* gene are found in 25%–40% of all breast cancers [32,33]. One reason for that is that such mutations can be acquired during the metastasis process leading to a discrepancy of the mutation status of the primary tumor and a liquid biopsy [34]. Furthermore, mutations in the *PIK3CA* and the *AKT1* gene lead to an increased proliferation of the tumor combined with reduced apoptosis [10,15] and might thereby cause a higher probability of CTCs detected in general and of CTCs with a high genome integrity in particular.

Most of the mutations we detected were located in well-described hotspot regions. Some mutations, however, are novel and have not been reported yet, such as the *PIK3CA* M318T, *ERBB2* L313P, or the *ERBB2* A775T mutations. These mutations were only detected in single WGA products and due to prior WGA, a false positive result cannot be totally excluded. To further investigate these mutations, tools to predict the effect on the protein structure have been applied [35–37]. Although they are not fully consistent, the results indicate that these mutations might affect the function of the protein (Table S3).

Heterogeneity was observed within CTCs from almost all patients. Such heterogeneity indicates that different subclones exist within the tumor and that the patient might therefore benefit from the combination of treatments targeting different proteins. However, it cannot be excluded that the observed assumed heterogeneity may at least in part be caused by false negativity due to allelic dropout during WGA. Thus, the absence of mutations does not necessarily mean that these mutations were not present in the initial cells. One example for allelic dropout is the T47-D reference cell 3: in this cell, only the mutated allele was amplified during WGA. This effect of allelic dropout is limiting all approaches for the analysis of DNA from single cells. The probability of allelic dropout increases with reduced DNA integrity. To minimize it, we focused on the analysis of WGA products that showed at least three bands in the quality control PCR.

Most studies on the clinical utility on mutations have been performed on ctDNA. However, the clinical utility of ctDNA and CTCs has not been compared so far and the analysis of CTCs and ctDNA might be complementary [34].

Altogether, we have developed a time and cost effective multiplex PCR-based assay for the analysis on a MiSeq system. Our assay covers the most relevant positions indicative for targeted therapies in breast cancer and can thereby improve therapy decision and help to improve the treatment of cancer patients eventually.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2076-3417/10/10/3364/s1>, Table S1: Primer concentrations used in first PCR, Table S2: PCR protocols, Figure S1: Validation of first PCR, Figure S2: Distribution of reads to the analyzed fragments, Table S3: Prediction of effect of detected amino acid substitutions.

Author Contributions: A.F., T.F., and H.N. conceived the project and provided project leadership. A.F. and H.N. wrote the manuscript. M.R. and L.Y. contributed to single cell isolation. B.J. and N.K. contributed to sample collection. E.H. and D.N. contributed to data analysis. All authors read and approved the final manuscript.

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References

- Perakis, S.; Speicher, M.R. Emerging concepts in liquid biopsies. *BMC Med.* **2017**, *15*, 75. [CrossRef] [PubMed]
- Turajlic, S.; Sottoriva, A.; Graham, T.; Swanton, C. Resolving genetic heterogeneity in cancer. *Nat. Rev. Genet.* **2019**, *20*, 404–416. [CrossRef] [PubMed]
- Palmirotta, R.; Lovero, D.; Cafforio, P.; Felici, C.; Mannavola, F.; Pellè, E.; Quaresmini, D.; Tucci, M. Liquid biopsy of cancer: A multimodal diagnostic tool in clinical oncology. *Ther. Adv. Med. Oncol.* **2018**, *10*, 1758835918794630. [CrossRef] [PubMed]
- Pantel, K.; Alix-Panabières, C. Circulating tumour cells in cancer patients: Challenges and perspectives. *Trends Mol. Med.* **2010**, *16*, 398–406. [CrossRef]
- Barradas, A.M.C.; Terstappen, L.W.M.M. Towards the biological understanding of CTC: Capture technologies, definitions and potential to create metastasis. *Cancers* **2013**, *5*, 1619–1642. [CrossRef]
- Yu, M.; Stott, S.; Toner, M.; Maheswaran, S.; Haber, D.A. Circulating tumor cells: Approaches to isolation and characterization. *J. Cell Biol.* **2011**, *192*, 373–382. [CrossRef]
- Allard, W.J.; Matera, J.; Miller, M.C.; Repollet, M.; Connelly, M.C.; Rao, C.; Tibbe, A.G.J.; Uhr, J.W.; Terstappen, L.W.M.M. Tumor Cells Circulate in the Peripheral Blood of All Major Carcinomas but not in Healthy Subjects or Patients with Nonmalignant Diseases. *Clin. Cancer Res.* **2005**, *10*, 6897–6904. [CrossRef]
- Wan, J.C.M.; Massie, C.; Garcia-Corbacho, J.; Mouliere, F.; Brenton, J.D.; Caldas, C.; Pacey, S.; Baird, R.; Rosenfeld, N. Liquid biopsies come of age: Towards implementation of circulating tumour DNA. *Nat. Rev. Cancer* **2017**, *17*, 223–238. [CrossRef]
- FDA Oncology Update. Available online: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6684051/> (accessed on 1 March 2020).
- Janku, F.; Yap, T.A.; Meric-Bernstam, F. Targeting the PI3K pathway in cancer: Are we making headway? *Nat. Rev. Clin. Oncol.* **2018**, *15*, 273–291. [CrossRef]
- Zhao, L.; Vogt, P.K. Helical domain and kinase domain mutations in p110 α of phosphatidylinositol 3-kinase induce gain of function by different mechanisms. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 2652–2657. [CrossRef]
- Arpino, G.; Wiechmann, L.; Osborne, C.K.; Schiff, R. Crosstalk between the Estrogen Receptor and the HER Tyrosine Kinase Receptor Family: Molecular Mechanism and Clinical Implications for Endocrine Therapy Resistance. *Endocr. Rev.* **2008**, *29*, 217–233. [CrossRef] [PubMed]
- Toy, W.; Shen, Y.; Won, H.; Green, B.; Sakr, R.A.; Will, M.; Gala, K.; Fanning, S.; King, T.a.; Hudis, C.; et al. ESR1 ligand binding domain mutations in hormone-resistant breast cancer. *Nat. Genet.* **2013**, *45*, 1439–1445. [CrossRef] [PubMed]

14. Franken, A.; Honisch, E.; Reinhardt, F.; Meier-Stiegen, F.; Yang, L.; Jaschinski, S.; Esposito, I.; Alberter, B.; Polzer, B.; Huebner, H.; et al. Detection of ESR1 Mutations in Single Circulating Tumor Cells on Estrogen Deprivation Therapy but Not in Primary Tumors from Metastatic Luminal Breast Cancer Patients. *J. Mol. Diagn.* **2019**, *22*, 111–121. [\[CrossRef\]](#)
15. Carpten, J.D.; Faber, A.L.; Horn, C.; Donoho, G.P.; Briggs, S.L.; Robbins, C.M.; Hostetter, G.; Boguslawski, S.; Moses, T.Y.; Savage, S.; et al. A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. *Nature* **2007**, *448*, 439–444. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Bose, R.; Kavuri, S.M.; Searleman, A.C.; Shen, W.; Shen, D.; Koboldt, D.C.; Monsey, J.; Goel, N.; Aronson, A.B.; Li, S.; et al. Activating HER2 Mutations in HER2 Gene Amplification Negative Breast Cancer. *Cancer Discov.* **2013**, *3*, 224–237. [\[CrossRef\]](#) [\[PubMed\]](#)
17. Neumann, M.H.D.; Schneck, H.; Decker, Y.; Schömer, S.; Franken, A.; Endris, V.; Pfarr, N.; Weichert, W.; Niederacher, D.; Fehm, T.; et al. Isolation and characterization of circulating tumor cells using a novel workflow combining the CellSearch® system and the CellCelector™. *Biotechnol. Prog.* **2017**, *33*, 125–132. [\[CrossRef\]](#)
18. Afgan, E.; Baker, D.; van den Beek, M.; Blankenberg, D.; Bouvier, D.; Čech, M.; Chilton, J.; Clements, D.; Coraor, N.; Eberhard, C.; et al. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. *Nucleic Acids Res.* **2016**, *44*, W3–W10. [\[CrossRef\]](#)
19. Li, H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *arXiv* **2013**, arXiv:1303.3997.
20. Thorvaldsdóttir, H.; Robinson, J.T.; Mesirov, J.P. Integrative Genomics Viewer (IGV): High-performance genomics data visualization and exploration. *Brief. Bioinform.* **2013**, *14*, 178–192. [\[CrossRef\]](#)
21. Franken, A.; Driemel, C.; Behrens, B.; Meier-stiegen, F.; Endris, V.; Stenzinger, A.; Niederacher, D.; Fischer, J.C.; Stoecklein, N.H.; Ruckhaeberle, E.; et al. Label-Free Enrichment and Molecular Characterization of Viable Circulating Tumor Cells from Diagnostic Leukapheresis Products. *Clin. Chem.* **2019**, *65*, 549–558. [\[CrossRef\]](#)
22. Lampignano, R.; Yang, L.; Neumann, M.H.D.; Franken, A.; Fehm, T.; Niederacher, D.; Neubauer, H. A novel workflow to enrich and isolate patient-matched EpCAMhigh and EpCAMlow/negative CTCs enables the comparative characterization of the PIK3CA status in metastatic breast cancer. *Int. J. Mol. Sci.* **2017**, *18*, 1885. [\[CrossRef\]](#)
23. Paolillo, C.; Mu, Z.; Rossi, G.; Schiewer, M.J.; Nguyen, T.; Austin, L.; Capoluongo, E.; Knudsen, K.; Cristofanilli, M.; Fortina, P. Detection of activating estrogen receptor gene (ESR1) mutations in single circulating tumor cells. *Clin. Cancer Res.* **2017**, *23*, 6086–6093. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Shaw, J.A.; Guttery, D.S.; Hills, A.; Fernandez-Garcia, D.; Page, K.; Rosales, B.M.; Goddard, K.S.; Hastings, R.K.; Luo, J.; Ogle, O.; et al. Mutation analysis of cell-free DNA and single circulating tumor cells in metastatic breast cancer patients with high circulating tumor cell counts. *Clin. Cancer Res.* **2017**, *23*, 88–96. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Onidani, K.; Shoji, H.; Kakizaki, T.; Yoshimoto, S.; Okaya, S.; Miura, N.; Sekikawa, S.; Furuta, K.; Lim, C.T.; Shibahara, T.; et al. Monitoring of cancer patients via next-generation sequencing of patient-derived circulating tumor cells and tumor DNA. *Cancer Sci.* **2019**, *110*, 2590–2599. [\[CrossRef\]](#) [\[PubMed\]](#)
26. André, F.; Ciruelos, E.; Rubovszky, G.; Campone, M.; Loibl, S.; Rugo, H.S.; Iwata, H.; Conte, P.; Mayer, I.A.; Kaufman, B.; et al. Alpelisib for PIK3CA-Mutated, Hormone Receptor-Positive Advanced Breast Cancer. *N. Engl. J. Med.* **2019**, *380*, 1929–1940. [\[CrossRef\]](#)
27. Chandarlapaty, S.; Sakr, R.A.; Giri, D.; Patil, S.; Heguy, A.; Morrow, M.; Modi, S.; Norton, L.; Rosen, N.; Hudis, C.; et al. Frequent Mutational Activation of the PI3K-AKT Pathway in Trastuzumab-Resistant Breast Cancer. *Clin. Cancer Res.* **2012**, *18*, 6784–6791. [\[CrossRef\]](#)
28. Fribbens, C.; O’Leary, B.; Kilburn, L.; Hrebien, S.; Garcia-Murillas, I.; Beaney, M.; Cristofanilli, M.; Andre, F.; Loi, S.; Loibl, S.; et al. Plasma ESR1 Mutations and the Treatment of Estrogen Receptor-Positive Advanced Breast Cancer. *J. Clin. Oncol.* **2016**, *34*, 2961–2968. [\[CrossRef\]](#)
29. Hyman, D.M.; Smyth, L.M.; Donoghue, M.T.A.; Westin, S.N.; Bedard, P.L.; Emma, J.; Bando, H.; El-khoueiry, A.B.; Mita, A.; Schellens, J.H.M.; et al. AKT Inhibition in Solid Tumors with AKT1 Mutations. *J. Clin. Oncol.* **2019**, *35*, 2251–2262. [\[CrossRef\]](#)
30. Zardavas, D.; Fumagalli, D.; Loi, S. Phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin pathway inhibition: A breakthrough in the management of luminal (ER+/HER2-) breast cancers? *Curr. Opin. Oncol.* **2012**, *24*, 623–634. [\[CrossRef\]](#)

31. Troxell, M.L. PIK3CA/AKT1 Mutations in Breast Carcinoma: A Comprehensive Review of Experimental and Clinical Studies. *J. Clin. Exp. Pathol.* **2012**. [[CrossRef](#)]
32. Mukohara, T. Pi3k mutations in breast cancer: Prognostic and therapeutic implications. *Breast Cancer Targets Ther.* **2015**, *7*, 111–123. [[CrossRef](#)]
33. Samuels, Y.; Waldman, T. Oncogenic mutations of PIK3CA in human cancers. *Curr. Top. Microbiol. Immunol.* **2010**, *346*, 21–41.
34. Tzanikou, E.; Markou, A.; Politaki, E.; Koutsopoulos, A.; Psyrris, A.; Mavroudis, D.; Georgoulas, V.; Lianidou, E. PIK3CA hotspot mutations in circulating tumor cells and paired circulating tumor DNA in breast cancer: A direct comparison study. *Mol. Oncol.* **2019**, *13*, 2515–2530. [[CrossRef](#)] [[PubMed](#)]
35. Adzhubei, I.; Jordan, D.M.; Sunyaev, S.R. Predicting functional effect of human missense mutations using PolyPhen-2. *Curr. Protoc. Hum. Genet.* **2013**, *76*, 7.20.1–7.20.41. [[CrossRef](#)] [[PubMed](#)]
36. Tavtigian, S.V.; Deffenbaugh, A.M.; Yin, L.; Judkins, T.; Scholl, T.; Samollow, P.B.; De Silva, D.; Zharkikh, A.; Thomas, A. Comprehensive statistical study of 452 BRCA1 missense substitutions with classification of eight recurrent substitutions as neutral. *J. Med. Genet.* **2006**, *43*, 295–305. [[CrossRef](#)] [[PubMed](#)]
37. Choi, Y.; Chan, A.P. PROVEAN web server: A tool to predict the functional effect of amino acid substitutions and indels. *Bioinformatics* **2015**, *31*, 2745–2747. [[CrossRef](#)]



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4 Ergebnisse und Ausblick

Die Anzahl detektierter CTCs ist beim MK und anderen Tumortypen ein unabhängiger prognostischer Marker für das progressionsfreie Überleben und das Gesamtüberleben in der neoadjuvanten, adjuvanten und metastasierten Situation. Ihr prädiktiver Nutzen konnte allerdings noch nicht abschließend nachgewiesen werden und wird derzeit in zahlreichen Studien untersucht. Dabei könnten die Anzahl der CTCs zu Beginn einer neuen Behandlung, die Dynamik der CTC-Zahl während einer neuen Therapie, oder bestimmte Marker der CTCs für eine Therapieentscheidung genutzt werden.

Das Ziel dieser Dissertation war es, einen Beitrag zum Nachweis des klinischen Nutzens von CTCs zu leisten. Hierfür wurden Methoden zur Charakterisierung von CTCs mit dem Ziel der Selektion geeigneter zielgerichteter Therapien entwickelt und schließlich in die klinische Anwendung übertragen.

Zunächst wurde das Vorkommen von *ESR1* Mutationen untersucht, die einen Resistenzmechanismus gegenüber einer endokrinen Therapie beim luminalen MK darstellen können.

Insbesondere lebende CTCs tragen ein Maximum an Informationen über den Ort ihrer Herkunft. Daher wurde im zweiten Teil dieser Dissertation ein Workflow entwickelt, um lebende CTCs *in vitro* kultivieren und dadurch weitere Informationen für mögliche klinische Entscheidungen auf der Basis von CTCs gewinnen zu können.

Im dritten Teil wurde mit Hilfe multiparametrischer *Liquid Biopsies* der Verlauf der Erkrankung einer Index-Patientin mit metastasiertem lumbalem MK beobachtet, vorhandene Resistenzmechanismen erkannt, potentielle Zielstrukturen für eine personalisierte Behandlung identifiziert und schließlich eine *Liquid Biopsy*-basierte Therapie angewendet.

Abschließend wurde ein Multiplex-PCR-basiertes NGS-*Panel* entwickelt, das die schnelle und kostengünstige Analyse von Mutationen in CTCs ermöglicht, die zur Identifikation geeigneter, zugelassener zielgerichteter Therapien genutzt werden können.

4.1 Der Nachweis von *ESR1*-Mutationen in CTCs von metastasierten luminalen Mammakarzinompatientinnen

Mutationen im für die LBD kodierenden Teil des *ESR1*-Gens können eine Resistenz gegenüber östrogendepprivierender Therapie vermitteln. Patientinnen mit *ESR1*-Mutationen könnten daher von einem Wechsel auf eine andere Therapieform profitieren. Da *ESR1*-Mutationen nur selten bereits im Primärtumor nachweisbar sind und regelmäßige Biopsien von Metastasen nur schwerlich durchführbar sind, könnte daher der Nachweis von *ESR1*-Mutationen unter Verwendung einer *Liquid Biopsy* eine wichtige Rolle spielen.

Im Rahmen dieser Dissertation wurden CTCs von 46 metastasierten MK-Patientinnen mit luminalem Primärtumor untersucht. Die CTCs wurden mittels CellSearch angereichert, durch Mikromanipulation isoliert und ihre genomische DNA wurde amplifiziert. Zur Sequenzierung des *ESR1*-Gens wurde ein NGS-Assay entwickelt, der, soweit technisch möglich, die gesamte kodierende Region des *ESR1*-Gens abdeckt.

ESR1 Mutationen wurden in CTCs von 12 Patientinnen detektiert; und zwar nur in solchen, die zuvor mit östrogendepprivierender Therapie behandelt worden sind. In sieben Fällen befanden sich die detektierten Mutationen in *Hotspot*-Regionen innerhalb des Teils des Gens, der für die LBD kodiert. Außerdem wurden sechs bislang nicht bekannte Mutationen gefunden. Die detektierten Mutationen konnten nicht in Biopsien des Primärtumors oder von Metastasen nachgewiesen werden, die vor der CTC-Analyse entnommen wurden. Im Gegensatz zur Analyse dieser Biopsien zeigt also die Analyse der CTCs, dass die Patientinnen vermutlich eine Resistenz gegenüber weiteren östrogendepprivierenden Therapien zeigen würden. In zwei verfügbaren Biopsien von Metastasen, die erst nach der CTC-Analyse detektiert wurden, waren die in den CTCs gefundenen Mutationen in den Gewebebiopsien allerdings ebenfalls vorhanden. Diese Beobachtung lässt darauf schließen, dass die analysierten CTCs möglicherweise bereits von diesen Metastasen abstammen, die klinisch erst neun bzw. zwölf Monate nach der CTC-Analyse detektiert wurden oder dass die analysierten CTCs zu der Tumorzellpopulation gehörten, die später diese Metastasen gründete. Unabhängig davon zeigen diese Ergebnisse, dass mit Hilfe einer CTC-Analyse ein frühzeitiger Nachweis von *ESR1*-Mutationen möglich ist.

Die Detektion von *ESR1*-Mutationen aus *Liquid Biopsies* kann dazu beitragen, Patienten zu identifizieren, die eine ungünstige Prognose unter östrogendepprivierender Therapie haben. Dieses Kollektiv könnte somit von einem Wechsel auf eine andere Therapie profitieren

(74,75). In der klinischen Phase III-Studie PADA-1 (NCT03079011) wird derzeit der prädiktive Nutzen von *ESR1*-Mutationen untersucht. Als Analyt wird dabei die ctDNA von Hormonrezeptor-positiven und HER2-negativen metastasierten MK-Patientinnen genutzt. In dieser Studie wird untersucht, ob die eingeschlossenen Patientinnen bei der Detektion einer *ESR1*-Mutation von einem Wechsel von einem AI zu Fulvestrant profitieren. Unabhängig davon erhalten die Patientinnen zusätzlich Palbociclib (184).

Analog zu der PADA-1-Studie erfolgt die Analyse von *ESR1*-Mutationen in der Regel mit Hilfe von ctDNA. Ein derartiges Analyseverfahren setzt voraus, dass die untersuchten Genloci *a priori* bekannt sein müssen. Die Ergebnisse dieser Dissertation und anderer Studien (71,185) deuten darauf hin, dass auch Mutationen außerhalb der typischen Hotspot-Positionen zu Resistenzen gegenüber östrogendepprivierender Therapie beitragen könnten.

Zur weiteren Verifizierung der hier gefundenen Mutationen außerhalb der typischen *Hotspots*, sollten diese im nächsten Schritt funktionell charakterisiert werden. Hierbei sollte untersucht werden, ob sie einen proliferativen Effekt in Abwesenheit von Östrogen im Vergleich zum wildtypischen ER α vermitteln und eine Resistenz gegenüber östrogendepprivierender Therapie verursachen können. Dies könnte mittels ektopischer Expression des mutierten ER α -Proteins untersucht werden und im Zellkulturmodell mit Zellen eines ER α -abhängigen Zelllinien-Modells erfolgen. Um Effekte des endogenen ER α -Proteins auszuschließen, würde es sich anbieten, das endogene *ESR1*-Gen zuvor mittels CRISPR/Cas-Knockout zu entfernen.

4.2 Die Entwicklung einer Methode zur Kultivierung von CTCs aus DLA-Produkten

Die Analyse lebender CTCs ermöglicht den Gewinn eines Maximums an Informationen über ihren Herkunftsort. Des Weiteren erlaubt die Kultivierung von CTCs weiterführende Studien, die zum generellen Verständnis des Metastasierungsprozesses beitragen können. Allerdings stellt die Kultivierung von CTCs noch immer eine große Herausforderung dar, was in erster Linie darauf zurück zu führen ist, dass CTCs extrem selten sind und die Anzahl von Patienten mit hohen CTC-Zahlen sehr gering ist. Durch die Gewinnung von MNCs aus mehreren Litern Blut stellt die DLA eine Möglichkeit dar, eine größere Menge CTCs zu erhalten.

Im Rahmen dieser Dissertation sollte dieser Vorteil der DLA für die Kultivierung von CTCs genutzt werden. Dazu wurden die CTCs mittels Mikrofiltration aus DLA-Produkt und Blut

von neun Patientinnen angereichert und *in vitro* kultiviert. Anschließend wurden die kultivierten CTCs mit den Ausgangs-CTCs bezüglich chromosomaler Aberrationen, Mutationen in ausgewählten Tumor-assoziierten Genen sowie der Expression von ER α und HER2 verglichen. Außerdem wurde untersucht, inwieweit sich CTCs aus kryokonservierten DLA-Produkten für weitere Untersuchungen eignen.

Es konnte gezeigt werden, dass CTCs mit dem Parsortix-System sowohl aus Blut als auch aus DLA-Produkten isoliert werden können und dabei lebensfähig bleiben. Für eine erfolgreiche Kultivierung war es wichtig, dass die Probe möglichst viele CTCs enthielt, die nicht apoptotisch sein durften. Bei einer Probe, bei der keine CTCs aus einer Blutprobe kultiviert werden konnten, war die Kultivierung von CTCs aus dem DLA-Produkt erfolgreich. Der Vergleich von Ausgangs-CTCs und kultivierten CTCs ergab keine Unterschiede bezüglich der analysierten Parameter. Außerdem konnte gezeigt werden, dass es auch nach mehrjähriger Lagerung von kryokonservierten DLA-Produkten nur zu minimalen CTC-Verlusten kommt und die Qualität und Viabilität der CTCs beim Einfrieren und Auftauen erhalten bleibt.

Die gesteigerte Zahl an CTCs aus DLA-Produkten vergrößert also auch die Möglichkeiten der funktionalen Analyse von CTCs. Damit hat die DLA das Potenzial, zu einem besseren Verständnis der Biologie von CTCs beizutragen und die Anzahl der Patienten zu steigern, die von einem *ex vivo* durchgeführten Therapeutika-Test profitieren könnten. Im dritten Teil dieser Dissertation konnte der hier entwickelte Workflow genutzt werden, um die Wirksamkeit ausgewählter Therapeutika *in vitro* zu testen.

Ein wichtiger Baustein ist dabei die Kryokonservierung von DLA-Produkten, die die Lagerung und den Transport von Patientenmaterial erlaubt und damit ein weites Feld an Möglichkeiten öffnet. Dadurch können nun vergleichende Analysen an größeren Mengen von lebenden CTCs an verschiedenen für die jeweilige Methode spezialisierten Standorten durchgeführt werden. Außerdem ermöglicht die mehrjährige Lagerung die Sammlung von Probenkohorten über verschiedene Standorte hinweg, sodass spezifische, die Biologie von CTCs sowie deren klinischen Nutzen adressierende Fragestellungen beantwortet werden können.

4.3 Können CTC-basierte Mutationsanalysen dazu beitragen, die Behandlung einer metastasierten Mammakarzinompatientin zu optimieren?

Im dritten Teil dieser Dissertation wurden CTCs von fünf Patientinnen mit metastasiertem MK, die zuvor mittels CellSearch angereichert wurden, im Rahmen einer *Proof of Concept*-Studie für den klinischen Nutzen von CTCs mittels WES analysiert. Mutationen, die Zielstrukturen von durch die FDA zugelassenen Medikamenten darstellen, wurden im *PIK3CA*-, im *ESR1*- und im *AKT1*-Gen detektiert.

Schließlich wurden am Beispiel einer der Patientinnen über einen Zeitraum von drei Jahren multiparametrische *Liquid Biopsies* durchgeführt. Dabei sollte weiterhin untersucht werden, ob sich mittels *Liquid Biopsy*-Untersuchungen die Entwicklung des Tumors in der Reaktion auf verschiedene Therapien longitudinal nachvollziehen lässt und sich potenzielle Zielstrukturen für eine Therapie finden lassen.

Dazu wurden während der in diesem Zeitraum angewendeten Behandlungsschemata regelmäßig Blutproben entnommen und die Anzahl der CTCs mittels CellSearch-Analyse bestimmt. Durch WES und *Array Comparative Genome Hybridization* wurden Mutationen und chromosomale Aberrationen untersucht. Zum Vergleich wurden ebenfalls Mutationen auf ctDNA-Ebene betrachtet. Weiterhin wurde das Transkriptom des Primärtumors sowie das der CTCs, die mittels DLA erhalten wurden, analysiert.

Die longitudinale Analyse der CTC-Zahl ergab eine Korrelation mit der klinischen Situation der Patientin. Die Detektion dynamischer Mutationen, einschließlich zweier *ESR1*-Mutationen, ermöglichte es, die Evolution des Tumors nachzuvollziehen, Resistenzmechanismen, die im Laufe der Therapie entstanden sind, zu erklären und die *AKT1*-Mutation E17K als mutmaßlicher Treiber der Metastasierung zu identifizieren. Diese Mutation führt zu einer Assoziation des Proteins mit der Plasmamembran und infolgedessen zu einer konstitutiven Aktivierung der Serin-Threonin-Kinase AKT1 (186). Daraus ergab sich, dass die Inhibition des AKT1-Signalwegs ein potenzielles Target für eine Therapie darstellt. Mögliche Therapeutika wurden anschließend *in vitro* unter Verwendung des im zweiten Teilprojekt entwickelten *Workflows* getestet. Basierend auf der multiparametrischen *Liquid Biopsy*-Analyse wurde der mTOR-Inhibitor Everolimus als geeignetes Therapeutikum identifiziert. Unter Everolimus reduzierte sich die mittels CellSearch-Analyse ermittelte CTC-Zahl um 97,3% und es konnte eine über einige Monate stabile Erkrankung beobachtet werden,

während unter der zuvor standardmäßig gegebenen Chemotherapie eine massive Verschlechterung des klinischen Zustands der Patientin festzustellen war. Die identifizierte *AKT1* E17K Mutation war im Primärtumor nicht nachweisbar.

Die Ergebnisse dieser *Proof of Concept*-Studie unterstreichen das Potenzial einer longitudinalen in der personalisierten Medizin. Dabei erlaubt insbesondere eine multiparametrische Analyse detaillierte Aussagen über erworbene Resistenzen und vorhandene Zielstrukturen für eine zielgerichtete und personalisierte Therapie.

Alternativ zum mTOR-Inhibitor Everolimus würde sich, falls im Rahmen von *Liquid Biopsy*-Analysen aktivierende Mutationen im *AKT1*-Gen identifiziert werden, auch eine Therapie mit AKT1-Inhibitoren wie Capivasertib anbieten, die zurzeit in klinischen Studien getestet werden. Die Wirksamkeit von Capivasertib konnte in der BEECH (NCT01625286)-Studie am ER-positiven metastasierten MK in Kombination mit Paclitaxel und in der FAKTION (NCT01992952)-Studie ebenfalls am ER-positiven metastasierten MK in Kombination mit Fulvestrant gezeigt werden (187,188). Insbesondere Patienten mit *AKT1*-mutiertem Tumor könnten dabei von einer Behandlung mit Capivasertib profitieren (189). Auch beim triple-negativen MK konnte ein verlängertes progressionsfreies Überleben bei einer Kombinationstherapie von Paclitaxel und Capivasertib beobachtet werden (190).

Neben der bereits aufgeführten PADA-1-Studie wird derzeit in der fünf-armigen Phase II-Studie plasmaMATCH (NCT03182634) der klinische Nutzen einer *Liquid Biopsy*-basierten Mutationsanalyse untersucht. In dieser Studie werden Mutationen in mehreren Genen analysiert. Je nach detektierter Mutation werden die Patienten in dieser Studie mit unterschiedlichen Therapeutika behandelt. Wie in der PADA-1-Studie wird in diesen klinischen Studien ctDNA als Analyt verwendet (191). In der klinischen Phase III-Studie SOLAR-1 (NCT02437318) wurde bereits der klinische Nutzen von mittels *Liquid Biopsy* detektierten *PIK3CA*-Mutationen in der ctDNA analysiert. Patientinnen mit Mutationen im *PIK3CA*-Gen erhielten zusätzlich zu Fulvestrant den PI3K-Inhibitor Alpelisib und zeigten ein verlängertes progressionsfreies Überleben (192).

Auch am Standort Düsseldorf ist mit der CTCpredict Studie derzeit eine explorative klinische Studie in Planung. Die Studie basiert auf der hier gezeigten *Proof-of-Concept*-Studie und soll an einer größeren Kohorte von Patientinnen mit metastasiertem MK den klinischen Nutzen der CTC-basierten Mutationsanalyse von Genen des PI3K/Akt1/mTOR-Signalwegs untersuchen.

4.4 Entwicklung eines *Next Generation Sequencing*-Panels zur Identifizierung von Mutationen zur Therapiefindung auf CTC-Ebene

Die im dritten Teil dieser Dissertation vorgestellte Analyse von Mutationen von CTCs mittels WES stellt eine Methode dar, die mit einem erheblichen Zeit- und Kostenaufwand verbunden ist. Daher sollte im vierten Teil dieser Dissertation ein Multiplex-PCR-basiertes NGS-*Panel* entwickelt werden, das die wichtigsten Mutationen für eine zielgerichtete Therapie beim MK abdeckt und auf einem MiSeq-System sequenziert werden kann. Als Zielstrukturen für Therapeutika, die von der FDA zugelassen sind, eignen sich beim metastasierten MK neben Mutationen in den bereits diskutierten Genen *PIK3CA*, *ESR1* und *AKT1* Mutationen im Gen *ERBB2*. Die Rezeptor-Tyrosinkinase ERBB2 (HER2) aktiviert die RAS/MAPK-, PI3K/AKT- und JAK/STAT-Signalwege und steigert dadurch die Zellproliferation und das Zellüberleben. Mutationen in der extrazellulären Domäne und der Kinase-Domäne führen zu einer Aktivierung des Proteins (193). Mutationen im *ERBB2*-Gen wie solche, die zu den Aminosäuresubstitutionen S310F, L755S oder V777L führen, ermöglichen eine zielgerichtete Therapie mit dem Tyrosinkinase-Inhibitor Neratinib (193). Wie bereits im ersten und dritten Teil gezeigt, sind Mutationen in den mit diesem Assay analysierten Genen häufig im Primärtumor nicht nachweisbar oder werden erst mit Fortschreiten der Erkrankung erworben.

Mittels des hier entwickelten Assays, der zunächst mit Hilfe von Zelllinienzellen mit bekannten Mutationen validiert wurde, konnten Mutationen in CTCs aus 10 von 13 Patientinnen mit metastasiertem Brustkrebs detektiert werden, die sich als Zielstruktur für eine zielgerichtete Therapie eignen. In einigen Fällen konnten Mutationen nur in einem Teil der analysierten CTCs beobachtet werden. Diese Heterogenität kann zu einem gewissen Anteil technisch bedingt sein, da *Allelic Dropout* während der WGA auftreten kann. Sie spiegelt aber auch die Heterogenität der verschiedenen metastatischen Läsionen, sowie die Heterogenität innerhalb einer Metastase wider. Dies könnte darauf hindeuten, dass die Kombination verschiedener Therapien erfolgsversprechend wäre.

Mit dem hier etablierten Multiplex-PCR-basierten NGS-*Panel* steht damit am Standort Düsseldorf ein Assay zur Verfügung, der in der Klinik für die Identifikation therapierelevanter Mutationen in CTCs von metastasierten Brustkrebspatientinnen eingesetzt werden kann. Detektierte Mutationen können mit zugelassenen zielgerichteten Therapien adressiert werden können.

Insgesamt stellen die in diese Dissertation etablierten Methoden den Ausgangspunkt für die Verwendung von Informationen, die durch die molekulare Charakterisierung von CTCs erhalten wurden, im Rahmen klinischer Fragestellungen am Standort Düsseldorf dar. Die entwickelten Methoden und Arbeitsabläufe ermöglichen die molekulare Charakterisierung von CTCs auf genomischer, aber auch auf funktionaler und auf weiteren Ebenen. Innerhalb dieser Dissertation konnte der mögliche klinische Nutzen in einer *Proof of Concept*-Studie mittels DNA-Analyse und anschließender longitudinaler und multiparametrischer *Liquid Biopsy*-Analyse gezeigt werden, die in dieser Form bislang einzigartig ist.

Die Ergebnisse unterstreichen das Potenzial von CTC-basierten *Liquid Biopsy*-Analysen zur Therapieoptimierung bei metastasierten MK-Patientinnen. Mit den entwickelten Methoden wird diese Dissertation letztendlich dazu beitragen, dass Patientinnen mit metastasiertem MK mit spezifischeren und effektiveren Therapien behandelt werden können.

5 Zusammenfassung

Brustkrebs ist die weltweit häufigste Krebserkrankung bei Frauen. Zur Behandlung hat sich im Laufe der letzten Jahrzehnte die zielgerichtete Therapie als das bevorzugte Vorgehen etabliert. Der prädiktive Nutzen der Analyse des Primärtumors ist für die metastasierte Situation jedoch limitiert und auch die regelmäßige Entnahme von Biopsien der Metastasen stellt einige Herausforderungen dar. Eine Lösung dieses Problems könnte in der Analyse von Tumormaterial im Blut der Patientinnen liegen, was als *Liquid Biopsy* bezeichnet wird. Einen der in Frage kommenden Analyten stellen dabei zirkulierende Tumorzellen (CTCs) dar. CTCs sind sowohl beim Mammakarzinom (MK) als auch bei Tumoren anderer Entitäten ein unabhängiger prognostischer Faktor. Ihr klinischer Nutzen, der bislang noch nicht abschließend gezeigt werden konnte, wird derzeit in zahlreichen Studien untersucht und wurde auch in dieser Dissertation adressiert. Dazu sollten Methoden zur molekularen Charakterisierung von CTCs mit dem Ziel der Identifikation geeigneter zielgerichteter Therapien entwickelt und schließlich in die klinische Anwendung übertragen werden.

Im ersten Teil wurde ein *Next Generation Sequencing* (NGS)-basierter Assay zur Detektion von Mutationen im *ESR1*-Gen auf Einzel-CTC-Ebene entwickelt. Entsprechende Mutationen wurden nur bei Patientinnen nach Behandlung mit östrogendeprivierender Therapie detektiert, gegenüber der sie Resistenzen vermitteln. In Gewebe-Biopsien, die vor der CTC-Analyse entnommen wurden, konnten diese Mutationen nicht gefunden werden. Allerdings wiesen zwei von zwei Biopsien der Metastasen, die nach der CTC-Analyse detektiert wurden, dieselben Mutationen auf. Diese Ergebnisse zeigen, dass mittels CTC-Analyse ein frühzeitiger Nachweis therapierelevanter *ESR1*-Mutationen möglich ist und Heterogenität auf Einzelzellebene dargestellt werden kann.

Zusätzlich zur Mutationsanalyse könnte insbesondere die Untersuchung von lebenden CTCs in der Zukunft relevant werden, da sich aus ihnen ein Maximum an Informationen, einschließlich der Möglichkeit der *in vitro*-Therapie-Testung, gewinnen lässt. Da die Kultivierung von CTCs unter anderem wegen deren Seltenheit im Blut mit erheblichen Schwierigkeiten verbunden ist, wurde mit Hilfe des Parsortix-Systems eine Methode entwickelt, um eine größere Menge lebender CTCs aus diagnostischen Leukapherese (DLA)-Produkten anzureichern. Aus drei von zwölf DLA-Produkten konnten erfolgreich CTCs kultiviert werden. Dabei war das DLA-Produkt dem peripheren Blut als Ausgangsmaterial für die CTC-Kultivierung überlegen.

Anschließend wurde in einer *Proof-of-Concept*-Studie eine longitudinale und multiparametrische *Liquid Biopsy*-Analyse zur Identifikation einer geeigneten Therapie für eine Index-Patientin mit metastasiertem MK eingesetzt. Dabei kristallisierte sich die *AKT1* E17K-Mutation als vermutlicher Treiber der Metastasierung und als ein mögliches Ziel für eine zielgerichtete Therapie heraus. Auf dieser Basis wurde Everolimus als geeignetes Therapeutikum ausgewählt. Während der CTC-basierten Kombinationstherapie aus Everolimus, Tamoxifen und Denosumab kam es zu einer Reduktion der CTC-Zahl um 97,3%. Die Tumorlast der Patientin zeigte sich unter dieser Therapie über Monate stabil.

Darauf aufbauend wurde schließlich ein Multiplex-PCR-basiertes NGS-*Panel* entwickelt, das die für die zielgerichtete Therapie beim MK relevantesten genomischen Positionen in den Genen *PIK3CA*, *ESR1*, *AKT1* und *ERBB2* abdeckt, in denen Mutationen häufig erst im Laufe des Fortschreitens der Erkrankung erworben werden. Der Assay wurde mit Hilfe von Zelllinienzellen validiert und an CTCs von metastasierten MK-Patientinnen getestet. Er ermöglicht die Identifikation von Mutationen, die sich mit zugelassenen zielgerichteten Therapien ansteuern lassen, und kann unmittelbar in der klinischen Diagnostik eingesetzt werden.

Insgesamt unterstreichen diese Ergebnisse das prädiktive Potenzial der Analyse von *Liquid Biopsies* und CTCs. Es wurde gezeigt, dass solche Analysen ermöglichen, Strukturen, wie z.B. Mutationen im *ESR1*-Gen und anderen Genen, zu identifizieren, die im Primärtumor nicht nachweisbar wären und die durch zielgerichtete Therapien adressiert werden können. Dabei könnten insbesondere longitudinale und multiparametrische *Liquid Biopsy*-Analysen erfolgsversprechend sein. Die hier gezeigte *Proof-of-Concept*-Studie, die in dieser Form bislang einmalig ist, zeigt den klinischen Nutzen einer solchen Analyse – einschließlich eines Tests in Frage kommender Therapeutika an kultivierten CTCs.

Die Ergebnisse dieser Dissertation werden dazu beitragen, das CTC-Feld voranzubringen, die molekulare Charakterisierung von CTCs zur Therapie-Findung einzusetzen und schließlich die Behandlung der Patientinnen zu optimieren.

6 Summary

Breast cancer (BC) is the leading type of cancer for women worldwide. Over the last decades, targeted therapy has become the preferred approach to treat BC. However, the predictive utility of the primary tumor is limited once the tumor has metastasized and obtaining serial biopsies of metastatic lesions is challenging. Thus, the idea of deriving information about the tumor from liquid biopsies by analyzing e.g. circulating tumor cells (CTCs) is an appealing concept. CTCs are an independent prognostic indicator of progression free and overall survival in breast cancer and other cancer types. Their predictive utility, however, is still under investigation and was also addressed in this study. To that aim, methods to characterize CTCs for the identification of a suitable targeted therapy were developed and applied in the clinical situation.

In the first part, single CTCs were analyzed with a next generation sequencing (NGS) approach for the *ESR1* coding region. *ESR1* mutations were detected exclusively in patients treated with estrogen deprivation therapy to which they confer resistance. The mutations were absent in tissue biopsies of the primary tumor and were detected only in metastases obtained after CTC characterization. These results show that a CTC analysis enables the early detection of *ESR1* mutations relevant for therapy and demonstrate heterogeneity on single cell level.

In addition to mutation analysis, the analysis of viable CTCs could be of particular interest since that promises to obtain a maximum of information including performing *in vitro* drug screening assays. However, culturing CTCs is a mayor challenge mainly because of their rareness in blood. This was addressed by combining the diagnostic leukapheresis (DLA) and a microfluidic enrichment to obtain large numbers of viable CTCs. CTCs could be cultured from 3 out of 12 patients and it was shown that DLA product was superior to peripheral blood as the CTC source for cultivation.

Furthermore, a longitudinal and multiparametric proof-of-concept study of a single index patient suffering from metastasized BC over a time period of three years with the aim to understand the clinical utility of CTCs was performed. An observed *AKT1* E17K mutation, which was not detected in the primary tumor, was hypothesized to be the driver of the metastatic process. The findings led to selection of everolimus combined with tamoxifen and denosumab to target the AKT1 pathway. CTC numbers dropped by 97.3% during this CTC-based treatment. The observations on CTC numbers associated with the imaging of the metastatic burden.

Finally, a multiplex PCR-based NGS panel covering genomic positions in the *PIK3CA*, the *ESR1*, the *AKT1*, and the *ERBB2* genes most relevant for selection of a targeted therapy to treat metastatic BC was established. Such mutations are often not present in the primary tumor but acquired during the course of the disease. The assay was validated using cell line cells and tested with CTCs from metastatic breast cancer patients. It can be readily applied in the clinical practice to detect mutations that can be targeted with approved therapies.

All together, these results underline the predictive potential of liquid biopsies and CTCs. Such analyses enable the detection of targets for targeted therapy – such as mutations in the *ESR1* and other genes – that cannot be detected in the primary tumor or are acquired during the course of the disease. Longitudinal and multiparametric liquid biopsies could be of particular interest: The proof of concept-study shown here highlights the clinical utility of such an analysis – including *in vitro* drug testing on cultured CTCs.

The findings of this thesis will advance the CTC field, will support the usage of a molecular characterization of CTCs for selection of targeted therapies and will finally optimize the treatment of patients.

7 Literaturverzeichnis

1. Weinberg RA. The Biology of Cancer. 2013.
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin.* 2018;68:394–424.
3. Hanahan D, Weinberg RA. The Hallmarks of Cancer. *Cell.* 2000;100:57–70.
4. Hunter T. Oncoprotein Networks. *Cell.* 1997;88:333–46.
5. Weinberg RA. The Retinoblastoma Protein and Cell Cycle Control. *Cell.* 1995;81:323–30.
6. Butt AJ, Firth SM, Baxter RC. The IGF axis and programmed cell death. *Immunol Cell Biol.* 1999;77:256–62.
7. Hayflick L. Mortality and Immortality at the cellular level. *Biochemistry.* 1997;62:1180–90.
8. Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu C-P, Morin GB, et al. Extension of Life-Span by Introduction of Telomerase into Normal Human Cells. *Science.* 1998;279:349–52.
9. Veikkola T, Alitalo K. VEGFs, receptors and angiogenesis. *Semin Cancer Biol.* 1999;9:211–20.
10. Christofori G, Semb H. The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. *Trends Biochem Sci.* 1999;24:73–6.
11. Warburg O. On respiratory impairment in cancer cells. *Science.* 1956;124:269–70.
12. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. *Cell.* 2011;144:646–74.
13. Jackson SP, Bartek J. The DNA-damage response in human biology and disease. *Nature.* 2009;461:1071–8.
14. Stratton MR, Campbell PJ, Futreal PA. The cancer genome. *Nature.* 2009;458:719–24.

15. Grivennikov SI, Greten FR, Karin M. Immunity, Inflammation, and Cancer. *Cell*. 2010;140:883–99.
16. Chaffer CL, Weinberg RA. A Perspective on Cancer Cell Metastasis. *Science*. 2011;331:1559–64.
17. Lambert A, Pattabraman D, Weinberg RA. Emerging Biological Principles of Metastasis. *Cell*. 2017;168:670–91.
18. Kang Y, Pantel K. Tumor Cell Dissemination: Emerging Biological Insights from Animal Models and Cancer Patients. *Cancer Cell*. 2013;23:573–81.
19. Bednarz-Knoll N, Alix-panabières C, Pantel K. Plasticity of disseminating cancer cells in patients with epithelial malignancies. *Cancer Metastasis Rev*. 2012;31:673–87.
20. Grosse-Wilde A, Fouquier d’Hérouel A, McIntosh E, Ertaylan G, Skupin A, Kuester RE, et al. Stemness of the hybrid Epithelial / Mesenchymal State in Breast Cancer and Its Association with Poor Survival. *PLoS One*. 2015;10.
21. Li W, Kang Y. Probing the Fifty Shades of EMT in Metastasis. *Trends in Cancer*. 2016;2:65–7.
22. Thiery JP, Acloque H, Huang RYJ, Nieto MA. Epithelial-Mesenchymal Transitions in Development and Disease. *Cell*. 2009;139:871–90.
23. Tam WL, Weinberg RA. The epigenetics of epithelial-mesenchymal plasticity in cancer. *Nat Med*. 2013;19:1438–49.
24. Kishi S, Bayliss PE, Hanai J. A prospective epigenetic paradigm between cellular senescence and epithelial-mesenchymal transition in organismal development and aging. *Transl Res*. 2014;165:241–9.
25. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest*. 2009;119:1420–8.
26. Nieto MA, Huang RY, Jackson RA, Thiery JP. EMT: 2016. *Cell*. 2016;166:21–45.
27. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer*. 2002;2:442–54.
28. Jordan NV, Johnson GL, Abell AN. Tracking the intermediate stages of epithelial-

- mesenchymal transition in epithelial stem cells and cancer. *Cell Cycle*. 2011;10:2865–73.
29. Mani SA, Guo W, Liao M, Eaton EN, Ayyanan A, Zhou AY, et al. The Epithelial-Mesenchymal Transition Generates Cells with Properties of Stem Cells. *Cell*. 2008;133:704–15.
 30. Morel A-P, Lièvre M, Thomas C, Hinkal G, Ansieau S, Puisieux A. Generation of Breast Cancer Stem Cells through Epithelial-Mesenchymal Transition. *PLoS One*. 2008;3.
 31. Gupta PB, Onder TT, Jiang G, Tao K, Kuperwasser C, Weinberg RA, et al. Identification of Selective Inhibitors of Cancer Stem Cells by High-Throughput Screening. *Cell*. 2009;138:645–59.
 32. Kurrey N, Jalgaonkar S, Joglekar A, Ghanate A, Chaskar P, Doiphode R, et al. Snail and Slug Mediate Radioresistance and Chemoresistance by Antagonizing p53-Mediated Apoptosis and Acquiring a Stem-Like Phenotype in Ovarian Cancer Cells. *Stem Cells*. 2009;27:2059–68.
 33. Ghadimi BM, Sackett DL, Difilippantonio MJ, Schro E, Neumann T, Jauho A, et al. Centrosome Amplification and Instability Occurs Exclusively in Aneuploid, But Not in Diploid Colorectal Cancer Cell Lines, and Correlates With Numerical Chromosomal Aberrations. *Genes Chromosomes Cancer*. 2000;27:183–90.
 34. Godinho SA, Picone R, Burute M, Dagher R, Su Y, Leung CT, et al. Oncogene-like induction of cellular invasion from centrosome amplification. *Nature*. 2014;510:167–71.
 35. Friedl P, Locker J, Sahai E, Segall JE. Classifying collective cancer cell invasion. *Nat Cell Biol*. 2012;14:777–83.
 36. de Boer M, van Dijck JAAM, Bult P, Borm GF, Tjan-Heijnen VCG. Breast Cancer Prognosis and Occult Lymph Node Metastases, Isolated Tumor Cells, and Micrometastases. *J Natl Cancer Inst*. 2010;102:410–25.
 37. Yu M, Bardia A, Wittner BS, Stott SL, Smas ME, Ting DT, et al. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. *Science*. 2013;339:580–4.

38. Aceto N, Bardia A, Miyamoto DT, Donaldson MC, Wittner BS, Spencer JA, et al. Circulating Tumor Cell Clusters Are Oligoclonal Precursors of Breast Cancer Metastasis. *Cell*. 2014;158:1110–22.
39. Szczerba BM, Castro-Giner F, Vetter M, Krol I, Gkoutela S, Landin J, et al. Neutrophils escort circulating tumour cells to enable cell cycle progression. *Nature*. 2019;566:553–7.
40. Baccelli I, Schneeweiss A, Riethdorf S, Stenzinger A, Schillert A, Vogel V, et al. Identification of a population of blood circulating tumor cells from breast cancer patients that initiates metastasis in a xenograft assay. *Nat Biotechnol*. 2013;31:539–44.
41. Headley MB, Bins A, Nip A, Edward W, Looney MR, Gerard A, et al. Visualization of immediate immune responses to pioneer metastatic cells in the lung. *Nature*. 2016;531:513–7.
42. Kopp H-G, Placke T, Salih HR. Platelet-Derived Transforming Growth Factor- β Down-Regulates NKG2D Thereby Inhibiting Natural Killer Cell Antitumor Reactivity. *Cancer Res*. 2009;69:7775–83.
43. Nieswandt B, Hafner M, Echtenacher B, Männel DN. Lysis of Tumor Cells by Natural Killer Cells in Mice Is Impeded by Platelets. *Cancer Res*. 1999;59:1295–300.
44. Palumbo JS, Talmage KE, Massari J V, La Jeunesse CM, Flick MJ, Kombrinck KW, et al. Platelets and fibrin(ogen) increase metastatic potential by impeding natural killer cell-mediated elimination of tumor cells. *Blood*. 2005;105:178–85.
45. Coffelt SB, Kersten K, Doornebal CW, Weiden J, Vrijland K, Hau C-S, et al. IL-17-producing $\gamma\delta$ T cells and neutrophils conspire to promote breast cancer metastasis. *Nature*. 2015;522:345–8.
46. Spiegel A, Brooks MW, Houshyar S, Reinhardt F, Ardolino M, Fessler E, et al. Neutrophils Suppress Intraluminal NK Cell-Mediated Tumor Cell Clearance and Enhance Extravasation of Disseminated Carcinoma Cells. *Cancer Discov*. 2016;6:630–49.
47. Labelle M, Begum S, Hynes RO. Direct Signaling between Platelets and Cancer Cells Induces an Epithelial-Mesenchymal-Like Transition and Promotes Metastasis. *Cancer Cell*. 2011;20:576–90.

48. Cools-Lartigue J, Spicer J, McDonald B, Gowing S, Chow S, Giannias B, et al. Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis. Find the latest version: Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis. *J Clin Invest*. 2013;123:3446–58.
49. Qian B-Z, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, et al. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature*. 2011;475:222.
50. Condeelis J, Pollard JW. Macrophages: Obligate Partners for Tumor Cell Migration, Invasion, and Metastasis. *Cell*. 2006;124:263–6.
51. Reymond N, D'Água BB, Ridley AJ. Crossing the endothelial barrier during metastasis. *Nat Rev Cancer*. 2013;13:858.
52. Gupta GP, Massagué J. Cancer Metastasis: Building a Framework. *Cell*. 2006;127:679–95.
53. Mundy GR. Metastasis to bone: causes, consequences and therapeutic opportunities. *Nat Rev Cancer*. 2002;2:584–93.
54. Luzzi KJ, Macdonald IC, Schmidt EE, Kerkvliet N, Morris VL, Chambers AF, et al. Multistep Nature of Metastatic Inefficiency Dormancy of Solitary Cells after Successful Extravasation and Limited Survival of Early Micrometastases. *Am J Pathol*. 1998;153:865–73.
55. Hassiotou F, Geddes D. Anatomy of the Human Mammary Gland: Current Status of Knowledge. *Clin Anat*. 2013;26:29–48.
56. Tao Z, Shi A, Lu C. Breast Cancer: Epidemiology and Etiology. *Cell Biochem Biophys*. 2015;72:333–8.
57. Bertos NR, Park M. Breast cancer — one term, many entities? *J Clin Invest*. 2011;121:3789–96.
58. Sims AH, Howell A, Howell SJ, Clarke RB. Origins of breast cancer subtypes and therapeutic implications. *Nat Clin Pract Oncol*. 2007;4:516–25.
59. Liedtke C, Jackisch C, Thill M, Thomssen C, Müller V, Janni W. AGO Recommendations for the Diagnosis and Treatment of Patients with Early Breast Cancer: Update 2018. *Breast Care*. 2018;13:196–208.

60. Thill M, Liedtke C, Müller V, Janni W, Schmidt M. AGO Recommendations for the Diagnosis and Treatment of Patients with Advanced and Metastatic Breast Cancer: Update 2018. *Breast Care*. 2018;13:209–15.
61. Masood S. Estrogen and Progesterone Receptors in Cytology: A Comprehensive Review. *Diagn Cytopathol*. 1992;8:475–91.
62. Musgrove EA, Sutherland RL. Biological determinants of endocrine resistance in breast cancer. *Nat Rev Cancer*. 2009;9:631–43.
63. Robinson DR, Wu YM, Vats P, Su F, Lonigro RJ, Cao X, et al. Activating ESR1 mutations in hormone-resistant metastatic breast cancer. *Nat Genet*. 2013;45:1446–51.
64. Toy W, Shen Y, Won H, Green B, Sakr R a, Will M, et al. ESR1 ligand binding domain mutations in hormone-resistant breast cancer. *Nat Genet*. 2013;45:1439–45.
65. Arpino G, Wiechmann L, Osborne CK, Schiff R. Crosstalk between the Estrogen Receptor and the HER Tyrosine Kinase Receptor Family: Molecular Mechanism and Clinical Implications for Endocrine Therapy Resistance. *Endocr Rev*. 2008;29:217–33.
66. Kumar R, Thompson EB. The structure of the nuclear hormone receptors. *Steroids*. 1999;64:310–9.
67. Pike ACW. Lessons learnt from structural studies of the oestrogen receptor. *Best Pract Res Clin Endocrinol Metab*. 2006;20:1–14.
68. Skafar DF. Formation of a Powerful Capping Motif Corresponding to Start of “Helix 12” in Agonist-Bound Estrogen Receptor- α Contributes to Increased Constitutive Activity of the Protein. *Cell Biochem Biophys*. 2000;33:53–62.
69. Merenbakh-Lamin K, Ben-Baruch N, Yeheskel A, Dvir A, Soussan-Gutman L, Jeselsohn R, et al. D538G Mutation in Estrogen Receptor- α : A Novel Mechanism for Acquired Endocrine Resistance in Breast Cancer. *Cancer Res*. 2013;73:6856–65.
70. Katzenellenbogen JA, Mayne Christopher G, Katzenellenbogen BS, Greene GL, Chandarlapaty S. Structural Underpinnings of Estrogen Receptor Mutations in Endocrine Therapy Resistance. *Nat Rev Cancer*. 2018;18:377–88.
71. Jeselsohn R, Buchwalter G, Angelis C De, Brown M, Schiff R. ESR1 mutations as a mechanism for acquired endocrine resistance in breast cancer. *Nat Rev Clin Oncol*.

2016;12:573–83.

72. Schiavon G, Hrebien S, Garcia-Murillas I, Cutts RJ, Pearson A, Tarazona N, et al. Analysis of ESR1 mutation in circulating tumor DNA demonstrates evolution during therapy for metastatic breast cancer. *Sci Transl Med*. 2015;7.
73. Chandarlapaty S, Chen D, He W, Sung P, Samoila A, You D, et al. Prevalence of ESR1 Mutations in Cell-Free DNA and Outcomes in Metastatic Breast Cancer: A Secondary Analysis of the BOLERO-2 Clinical Trial. *JAMA Oncol*. 2017;2:1310–5.
74. Fribbens C, O’Leary B, Kilburn L, Hrebien S, Garcia-Murillas I, Beaney M, et al. Plasma ESR1 Mutations and the Treatment of Estrogen Receptor-Positive Advanced Breast Cancer. *J Clin Oncol*. 2016;34:2961–8.
75. Turner N, Swift C, Kilburn L, Garcia-Murillas I, Johnston S, Budzar A, et al. Abstract PD2-04: Baseline circulating ESR1 mutation analysis in the randomised phase III Efect study of fulvestrant versus exemestane in advanced hormone receptor positive breast cancer. *Cancer Res*. 2019.
76. Weir HM, Bradbury RH, Rabow AA, Buttar D, Callis RJ, Curwen JO, et al. AZD9496: An oral estrogen receptor inhibitor that blocks the growth of ER-positive and ESR1-mutant breast tumors in preclinical models. *Cancer Res*. 2016;76:3307–18.
77. Yu M, Bardia A, Aceto N, Bersani F, Madden MW, Donaldson MC, et al. Ex vivo culture of circulating breast tumor cells for individualized testing of drug susceptibility. *Science*. 2014;345:216–20.
78. Wang Y, Lonard DM, Yu Y, Chow DC, Palzkill TG, Wang J, et al. Bufalin is a potent small-molecule inhibitor of the steroid receptor coactivators SRC-3 and SRC-1. *Cancer Res*. 2014;74:1506–17.
79. Perakis S, Speicher MR. Emerging concepts in liquid biopsies. *BMC Med*. 2017;15.
80. Reinhardt F, Franken A, Fehm T, Neubauer H. Navigation through inter- and intratumoral heterogeneity of endocrine resistance mechanisms in breast cancer: A potential role for Liquid Biopsies? *Tumor Biol*. 2017;39.
81. Palmirotta R, Lovero D, Cafforio P, Felici C, Mannavola F, Pellè E, et al. Liquid biopsy of cancer: a multimodal diagnostic tool in clinical oncology. *Ther Adv Med Oncol*. 2018;10.

82. Pantel K, Alix-Panabières C. Circulating tumour cells in cancer patients: Challenges and perspectives. *Trends Mol Med*. 2010;16:398–406.
83. Barradas AMC, Terstappen LWMM. Towards the biological understanding of CTC: Capture technologies, definitions and potential to create metastasis. *Cancers*. 2013;5:1619–42.
84. Heitzer E, Haque IS, Roberts CES, Speicher MR. Current and future perspectives of liquid biopsies in genomics-driven oncology. *Nat Rev Genet*. 2019;20.
85. Hayes DF. ScienceDirect Biomarker validation and testing. *Mol Oncol*. 2015;9:950–6.
86. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Ph R, et al. Circulating Tumor Cells, Disease Progression, and Survival in Metastatic Breast Cancer. *N Engl J Med*. 2004;351:781–91.
87. Weber B, Meldgaard P, Hager H, Wu L, Wei W, Tsai J, et al. Detection of EGFR mutations in plasma and biopsies from non-small cell lung cancer patients by allele-specific PCR assays. *BMC Cancer*. 2014;14.
88. Grützmann R, Molnar B, Pilarsky C, Habermann JK, Schlag PM, Saeger HD, et al. Sensitive Detection of Colorectal Cancer in Peripheral Blood by Septin 9 DNA Methylation Assay. *PLoS One*. 2008;3.
89. FDA Oncology Update. *Am Heal Drugs Benefits*. 2019;12:198–200.
90. Ashworth TR. A Case of Cancer in Which Cells Similar to Those in the Tumors Were Seen in the Blood after Death. *Australas Med J*. 1869;14:146–9.
91. Alix-Panabières C, Schwarzenbach H, Pantel K. Circulating Tumor Cells and Circulating Tumor DNA. *Annu Rev Med*. 2012;63:199–215.
92. Rink M, Chun FKH, Minner S, Friedrich M, Mauermann O, Heinzer H, et al. Detection of circulating tumour cells in peripheral blood of patients with advanced non-metastatic bladder cancer. *BJU Int*. 2011;107:1668–75.
93. Rack B, Schindlbeck C, Jückstock J, Andergassen U, Hepp P, Zwingers T, et al. Circulating Tumor Cells Predict Survival in Early Average-to-High Risk Breast Cancer Patients. *J Natl Cancer Inst*. 2014;106.
94. Alix-Panabières C, Pantel K. Technologies for detection of circulating tumor cells :

- facts and vision. *Lab Chip*. 2014;14:57–62.
95. Maertens Y, Humberg V, Erlmeier F, Steffens S, Steinestel J, Bögemann M, et al. Comparison of isolation platforms for detection of circulating renal cell carcinoma cells. *Oncotarget*. 2017;8:87710–7.
 96. Tewes M, Aktas B, Welt A, Mueller S, Hauch S, Kimmig R, et al. Molecular profiling and predictive value of circulating tumor cells in patients with metastatic breast cancer : an option for monitoring response to breast cancer related therapies. *Breast Cancer Res Treat*. 2009;115:581–90.
 97. Miltenyi S, Muller W, Weichel W, Radbruch A. High Gradient Magnetic Cell Separation With MACS. *Cytom Part A*. 1990;238:231–8.
 98. Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, et al. Tumor Cells Circulate in the Peripheral Blood of All Major Carcinomas but not in Healthy Subjects or Patients With Nonmalignant Diseases. *Clin Cancer Res*. 2005;10:6897–904.
 99. Harb W, Fan A, Tran T, Danila DC, Keys D, Schwartz M, et al. Mutational Analysis of Circulating Tumor Cells Using a Novel Microfluidic Collection. *Transl Oncol*. 2013;6:528–38.
 100. Saucedo-Zeni N, Mewes S, Niestroj R, Gasiorowski L, Murawa D, Nowaczyk P, et al. A novel method for the in vivo isolation of circulating tumor cells from peripheral blood of cancer patients using a functionalized and structured medical wire. *Int J Oncol*. 2012;41:1241–50.
 101. Desitter I, Guerrouahen BS, Benali-furet N, Wechsler J, Jänne PA, Kuang Y, et al. A New Device for Rapid Isolation by Size and Characterization of Rare Circulating Tumor Cells. *Anticancer Res*. 2011;31:427–41.
 102. Vona G, Sabile A, Louha M, Sitruk V, Romana S, Schütze K, et al. Isolation by size of epithelial tumor cells : a new method for the immunomorphological and molecular characterization of circulating tumor cells. *Am J Pathol*. 2000;156:57–63.
 103. Kolostova K, Spicka J, Matkowski R, Bobek V. Isolation, primary culture, morphological and molecular characterization of circulating tumor cells in gynecological cancers. *Am J Transl Res*. 2015;7:1203–13.
 104. Xu L, Mao X, Imrali A, Syed F, Mutsvangwa K, Berney D, et al. Optimization and

- evaluation of a novel size based circulating tumor cell isolation system. *PLoS One*. 2015;10:1–23.
105. Warkiani ME, Khoo BL, Tan DS-W, Bhagat AAS, Lim W-T, Yap YS, et al. An ultra-high-throughput spiral microfluidic biochip for the enrichment of circulating tumor cells. *Analyst*. 2014;139:3245–55.
 106. Lu J, Fan T, Zhao Q, Zeng W, Zaslavsky E, Chen JJ, et al. Isolation of circulating epithelial and tumor progenitor cells with an invasive phenotype from breast cancer patients. *Int J Cancer*. 2010;126:669–83.
 107. Kagan M, Howard D, Bendele T, Mayes J, Silvia J, Repollet M, et al. A sample preparation and analysis system for identification of circulating tumor cells. *J Clin Ligand Assay*. 2002;25:104–10.
 108. Thege FI, Lannin TB, Saha TN, Tsai S, Kochman ML, Hollingsworth MA, et al. Microfluidic immunocapture of circulating pancreatic cells using parallel EpCAM and MUC1 capture: characterization, optimization and downstream analysis. *Lab Chip*. 2014;1775–84.
 109. Nagrath S, Sequist L V, Maheswaran S, Bell DW, Irimia D, Ulkus L, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. *Nature*. 2007;450:1235–9.
 110. Maheswaran S, Sequist L V, Nagrath S, Ulkus L, Brannigan B, Collura C V., et al. Detection of Mutations in EGFR in Circulating Lung-Cancer Cells. *N Engl J Med*. 2008;359:366–77.
 111. Armstrong AJ, Marengo MS, Oltean S, Kemeny G, Bitting RL, Turnbull JD, et al. Circulating Tumor Cells from Patients with Advanced Prostate and Breast Cancer Display Both Epithelial and Mesenchymal Markers. *Mol Cancer Res*. 2011;9:997–1007.
 112. Gorges TM, Tinhofer I, Drosch M, Röse L, Zollner TM, Krahn T, et al. Circulating tumour cells escape from EpCAM-based detection due to epithelial-to-mesenchymal transition. *BMC Cancer*. 2012;12.
 113. Kasimir-Bauer S, Hoffmann O, Wallwiener D, Kimmig R, Fehm T. Expression of stem cell and epithelial-mesenchymal transition markers in primary breast cancer patients

- with circulating tumor cells. *Breast Cancer Res. BioMed Central Ltd*; 2012;14.
114. Spizzo G, Fong D, Wurm M, Ensinger C, Obrist P, Hofer C, et al. EpCAM expression in primary tumour tissues and metastases : an immunohistochemical analysis. *J Clin Pathol*. 2011;64:415–20.
 115. Hao S, Wan Y, Xia Y, Zou X, Zheng S. Size-based separation methods of circulating tumor cells. *Adv Drug Deliv Rev [Internet]. Elsevier B.V.*; 2018;125:3–20. Available from: <https://doi.org/10.1016/j.addr.2018.01.002>
 116. Coumans FAW, van Dalum G, Beck M, Terstappen LWMM. Filter Characteristics Influencing Circulating Tumor Cell Enrichment from Whole Blood. *PLoS One*. 2013;8.
 117. Hvichia GE, Parveen Z, Wagner C, Janning M, Quidde J, Stein A, et al. A novel microfluidic platform for size and deformability based separation and the subsequent molecular characterization of viable circulating tumor cells. *Int J Cancer*. 2016;138:2894–904.
 118. Ozkumur E, Shah AM, Ciciliano JC, Emmink BL, David T, Brachtel E, et al. Inertial Focusing for Tumor Antigen–Dependent and –Independent Sorting of Rare Circulating Tumor Cells. *Sci Transl Med*. 2013;5.
 119. Strati A, Kasimir-Bauer S, Markou A, Parisi C, Lianidou ES. Comparison of three molecular assays for the detection and molecular characterization of circulating tumor cells in breast cancer. *Breast Cancer Res. BioMed Central Ltd*; 2013;15.
 120. Stathopoulou A, Gizi A, Perraki M, Apostolaki S, Malamos N, Mavroudis D, et al. Real-Time Quantification of CK-19 mRNA-Positive Cells in Peripheral Blood of Breast Cancer Patients Using the Lightcycler System. *Clin Cancer Res*. 2003;9:5145–51.
 121. Alix-Panabières C, Vendrell J, Pellé O, Rebillard X, Riethdorf S, Müller V, et al. Detection and Characterization of Putative Metastatic Precursor Cells in Cancer Patients. *Clin Chem*. 2007;53:537–9.
 122. Tibbe AGJ, Miller MC, Terstappen LWMM. Statistical Considerations for Enumeration of Circulating Tumor Cells. *Cytom Part A*. 2007;71:154–62.
 123. Coumans FAW, Siesling S, Terstappen LWMM. Detection of cancer before distant metastasis. *BMC Cancer*. 2013;13:283.

124. Griwatz C, Brandt B, Assmann G, Zanker KS. An immunological enrichment method for epithelial cells from peripheral blood. *J Immunol Methods*. 1995;183:251–65.
125. Stoecklein NH, Fischer JC, Niederacher D, Terstappen LWMM. Challenges for CTC-based liquid biopsies: Low CTC frequency and diagnostic leukapheresis as a potential solution. *Expert Rev Mol Diagn*. 2016;16:147–64.
126. Choi S, Rajan S, Trivedi M V. The Incidence of Tumor Cell Contamination of Peripheral Blood Stem Cells : A Meta-Analysis to Evaluate the Impact of Mobilization Regimens and the Influence on Outcomes in Breast Cancer Patients. *Acta Haematol*. 2014;131:133–40.
127. Müller AMS, Kohrt HEK, Cha S, Laport G, Klein J, Guardino AE, et al. Long-term outcome of patients with metastatic breast cancer treated with high-dose chemotherapy and transplantation of purified autologous hematopoietic stem cells. *Biol Blood Marrow Transplant*. 2012;18:125–33.
128. Fischer JC, Niederacher D, Topp SA, Honisch E, Schumacher S, Schmitz N, et al. Diagnostic leukapheresis enables reliable detection of circulating tumor cells of nonmetastatic cancer patients. *Proc Natl Acad Sci*. 2013;110:16580–5.
129. Burgstaler EA. Blood Component Collection by Apheresis. *J Clin Apher*. 2006;21:142–51.
130. Fehm TN, Meier-Stiegen F, Driemel C, Jäger B, Reinhardt F, Naskou J, et al. Diagnostic Leukapheresis for CTC Analysis in Breast Cancer Patients: CTC Frequency, Clinical Experiences and Recommendations for Standardized Reporting. *Cytom Part A*. 2018;93:1213–9.
131. Bidard FC, Peeters DJ, Fehm T, Nolé F, Gisbert-Criado R, Mavroudis D, et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: A pooled analysis of individual patient data. *Lancet Oncol*. 2014;15:406–14.
132. Huang X, Gao P, Song Y, Sun J, Chen X, Zhao J, et al. Meta-analysis of the prognostic value of circulating tumor cells detected with the CellSearch System in colorectal cancer. *BMC Cancer*. 2015;15.
133. Scher HI, Jia X, de Bono JS, Fleisher M, Pienta KJ, Raghavan D, et al. Circulating Tumor Cell Number as a Prognostic Marker in Progressive Castration-Resistant

- Prostate Cancer: Use in Clinical Practice and Clinical Trials. *Lancet Oncol.* 2009;10:233–9.
134. Krebs MG, Sloane R, Priest L, Lancashire L, Hou J, Greystoke A, et al. Evaluation and Prognostic Significance of Circulating Tumor Cells in Patients With Non-Small-Cell Lung Cancer. *J Clin Oncol.* 2011;29:1556–63.
 135. Hou J, Krebs MG, Lancashire L, Sloane R, Backen A, Swain RK, et al. Clinical Significance and Molecular Characteristics of Circulating Tumor Cells and Circulating Tumor Microemboli in Patients With Small-Cell Lung Cancer. *J Clin Oncol.* 2012;30:525–32.
 136. Janni WJ, Rack B, Terstappen LWMM, Pierga J-Y, Taran F-A, Fehm T, et al. Pooled Analysis of the Prognostic Relevance of Circulating Tumor Cells in Primary Breast Cancer. *Clin Cancer Res.* 2016;22:2583–93.
 137. Bidard F-C, Michiels S, Riethdorf S, Mueller V, Esserman LJ, Lucci A, et al. Circulating Tumor Cells in Breast Cancer Patients Treated by Neoadjuvant Chemotherapy: A Meta-analysis. *J Natl Cancer Inst.* 2018;110:560–7.
 138. Bidard FC, Huguet F, Louvet C, Mineur L, Bouché O, Chibaudel B, et al. Circulating tumor cells in locally advanced pancreatic adenocarcinoma: the ancillary CirCe 07 study to the LAP 07 trial. *Ann Oncol.* 2013;24:2057–61.
 139. van Dalum G, Stam G, Scholten LFA, Mastboom WJB. Importance of circulating tumor cells in newly diagnosed colorectal cancer. *Int J Oncol.* 2015;46:1361–8.
 140. Tol J, Koopman M, Miller MC, Tibbe A, Cats A, Creemers GJM, et al. Circulating tumour cells early predict progression-free and overall survival in advanced colorectal cancer patients treated with chemotherapy and targeted agents. *Ann Oncol.* 2010;21:1006–12.
 141. Cohen SJ, Punt CJA, Iannotti N, Saidman BH, Sabbath KD, Gabrail NY, et al. Relationship of Circulating Tumor Cells to Tumor Response, Progression-Free Survival, and Overall Survival in Patients With Metastatic Colorectal Cancer. *J Clin Oncol.* 2008;26:3213–21.
 142. Sastre J, Maestro ML, Gómez-Espana A, Rivera F, Valladares M, Massuti B, et al. Circulating tumor cell count is a prognostic factor in metastatic colorectal cancer

- patients receiving first-line chemotherapy plus bevacizumab: a Spanish Cooperative Group for the Treatment of Digestive Tumors study. *Oncologist*. 2012;17:947–55.
143. de Bono JS, Scher HI, Montgomery RB, Parker C, Miller MC, Tissing H, et al. Clinical Circulating Tumor Cells Predict Survival Benefit from Treatment in Metastatic Castration-Resistant Prostate Cancer. *Clin Cancer Res*. 2008;14:6302–9.
 144. Pestrin M, Salvianti F, Galardi F, Luca F De, Turner N, Malorni L, et al. Heterogeneity of PIK3CA mutational status at the single cell level in circulating tumor cells from metastatic breast cancer patients. *Mol Oncol*. 2015;9:749–57.
 145. Mayer JA, Pham T, Wong KL, Scoggin J, Sales E V, Clarin T, et al. FISH-based determination of HER2 status in circulating tumor cells isolated with the microfluidic CEETM platform. *Cancer Genet*. Elsevier Inc.; 2011;204:589–95.
 146. Kanwar N, Hu P, Bedard P, Clemons M, McCreedy D, Done SJ. Identification of genomic signatures in circulating tumor cells from breast cancer. *Int J Cancer*. 2015;137:332–44.
 147. Chimonidou M, Kallergi G, Georgoulas V, Welch DR, Lianidou ES. Breast Cancer Metastasis Suppressor-1 promoter methylation in primary breast tumors and corresponding Circulating Tumor Cells. *Mol Cancer Res*. 2013;11.
 148. Mostert B, Sieuwerts AM, Kraan J, Bolt-de Vries J, Van Der Spoel P, Van Galen A, et al. Gene expression profiles in circulating tumor cells to predict prognosis in metastatic breast cancer patients. 2015;510–6.
 149. Khoo BL, Warkiani ME, Tan DS, Bhagat AAS, Irwin D, Lau DP, et al. Clinical Validation of an Ultra High-Throughput Spiral Microfluidics for the Detection and Enrichment of Viable Circulating Tumor Cells. *PLoS One*. 2014;9.
 150. Antonarakis ES, Lu C, Wang H, Lubber B, Nakazawa M, Roeser JC, et al. AR-V7 and Resistance to Enzalutamide and Abiraterone in Prostate Cancer. *N Engl J Med*. 2014;371:1028–38.
 151. Cabel L, Proud'hon C, Gortais H, Loirat D, Coussy F, Yves J, et al. Circulating tumor cells: clinical validity and utility. *Int J Clin Oncol*. Springer Japan; 2017;22:421–30.
 152. Babayan A, Hannemann J, Spötter J, Müller V, Pantel K, Joosse SA. Heterogeneity of Estrogen Receptor Expression in Circulating Tumor Cells from Metastatic Breast

Cancer Patients. PLoS One. 2013;8:1–11.

153. Fehm T, Müller V, Aktas B, Janni W, Schneeweiss A, Stickeler E, et al. HER2 status of circulating tumor cells in patients with metastatic breast cancer: a prospective, multicenter trial. *Breast Cancer Res Treat.* 2010;124:403–12.
154. Nadal R, Fernandez A, Sanchez-Rovira P, Salido M, Rodríguez M, García-Puche JL, et al. Biomarkers characterization of circulating tumour cells in breast cancer patients. *Breast Cancer Res.* 2012;14.
155. Somlo G, Lau SK, Frankel P, Hsieh H Ben, Liu X, Yang L, et al. Multiple biomarker expression on circulating tumor cells in comparison to tumor tissues from primary and metastatic sites in patients with locally advanced/inflammatory, and stage IV breast cancer, using a novel detection technology. *Breast Cancer Res Treat.* 2011;128:155–63.
156. Pestrin M, Bessi S, Puglisi F, Minisini AM, Masci G, Battelli N, et al. Final results of a multicenter phase II clinical trial evaluating the activity of single-agent lapatinib in patients with HER2- negative metastatic breast cancer and HER2-positive circulating tumor cells. A proof-of-concept study. *Breast Cancer Res Treat.* 2012;134:283–9.
157. Georgoulas V, Bozionelou V, Agelaki S, Perraki M, Apostolaki S, Kallergi G, et al. Trastuzumab decreases the incidence of clinical relapses in patients with early breast cancer presenting circulating tumor cells: results of a randomized phase II study. *Ann Oncol.* 2012;23:1744–50.
158. Hainsworth JD, Murphy PB, Alemar JR, Daniel BR, Young RR, Yardley DA. Use of a multiplexed immunoassay (PRO Onc assay) to detect HER2 abnormalities in circulating tumor cells of women with HER2-negative metastatic breast cancer : lack of response to HER2-targeted therapy. *Breast Cancer Res Treat.* 2016;160:41–9.
159. Bidard F-C, Cottu P, Dubot C, Venat-Bouvet L, Lortholary A, Bourgeois H, et al. Anti-HER2 therapy efficacy in HER2-negative metastatic breast cancer with HER2-amplified circulating tumor cells: results of the CirCe T-DM1 trial (117P). *Ann Oncol.* 2017;28:v22–42.
160. Ignatiadis M, Litière S, Rothe F, Riethdorf S, Proudhon C, Fehm T, et al. Trastuzumab versus observation for HER2 nonamplified early breast cancer with circulating tumor cells (EORTC 90091-10093, BIG 1-12 , Treat CTC): a randomized phase II trial. *Ann*

Oncol. 2018;29:1777–83.

161. Stebbing J, Payne R, Reise J, Frampton AE, Avery M, Woodley L, et al. The Efficacy of Lapatinib in Metastatic Breast Cancer with HER2 Non-Amplified Primary Tumors and EGFR Positive Circulating Tumor Cells: A Proof-Of-Concept Study. *PLoS One*. 2013;8.
162. Smerage JB, Barlow WE, Hortobagyi GN, Winer EP, Leyland-jones B, Srkalovic G, et al. Circulating Tumor Cells and Response to Chemotherapy in Metastatic Breast Cancer: SWOG S0500. *J Clin Oncol*. 2014;32:3483–90.
163. Bidard F-C, Pierga J-Y. Clinical Utility of Circulating Tumor Cells in Metastatic Breast Cancer. *J Clin Oncol*. 2015;33.
164. Bidard. Clinical utility of circulating tumor cell count as a tool to chose between first line hormone therapy and chemotherapy for ER+ HER2- metastatic breast cancer: Results of the phase III STIC CTC trial. *SABCS*. 2018;
165. Helissey C, Berger F, Cottu P, Diéras V, Mignot L, Servois V, et al. Circulating tumor cell thresholds and survival scores in advanced metastatic breast cancer: The observational step of the CirCe01 phase III trial. *Cancer Lett*. 2015;360:213–8.
166. Schramm A, Friedl TWP, Schochter F, Scholz C, Gregorio N De, Huober J, et al. Therapeutic intervention based on circulating tumor cell phenotype in metastatic breast cancer: concept of the DETECT study program. *Arch Gynecol Obstet*. 2016;293:271–81.
167. De Gregorio A, Friedl TWP, Huober J, Scholz C, De Gregorio N, Rack B, et al. Discordance in Human Epidermal Growth Factor Receptor 2 (HER2) Phenotype Between Primary Tumor and Circulating Tumor Cells in Women With HER2-Negative Metastatic Breast Cancer. *JCO Precis Oncol*. 2017;2:1–12.
168. Alix-Panabières C, Bartkowiak K, Pantel K. Functional studies on circulating and disseminated tumor cells in carcinoma patients. *Mol Oncol*. 2016;10:443–9.
169. Pantel K, Alix-Panabieres C. Functional Studies on Viable Circulating Tumor Cells. *Clin Chem*. 2016;62:328–34.
170. Smerage JB, Budd GT, Doyle G V, Brown M, Paoletti C, Muniz M, et al. Monitoring apoptosis and Bcl-2 on circulating tumor cells in patients with metastatic breast cancer.

Mol Oncol. 2013;7:680–92.

171. Deutsch TM, Riethdorf S, Nees J, Hartkopf AD, Schönfish B, Domschke C, et al. Impact of apoptotic circulating tumor cells (aCTC) in metastatic breast cancer. *Breast Cancer Res Treat.* 2016;160:277–90.
172. Jansson S, Bendahl P-O, Larsson A-M, Aaltonen KE, Rydén L. Prognostic impact of circulating tumor cell apoptosis and clusters in serial blood samples from patients with metastatic breast cancer in a prospective observational cohort. *BMC Cancer.* 2016;16:433.
173. Denève E, Riethdorf S, Ramos J, Nocca D, Coffy A, Daurès J, et al. Capture of Viable Circulating Tumor Cells in the Liver of Colorectal Cancer Patients. *Clin Chem.* 2013;59:1384–92.
174. Bobek V, Gurlich R, Eliasova P, Kolostova K. Circulating tumor cells in pancreatic cancer patients : Enrichment and cultivation. *World J Gastroenterol.* 2014;20:17163–70.
175. Bobek V, Matkowski R, Gürlich R, Grabowski K, Szelachowska J, Lischke R, et al. Cultivation of circulating tumor cells in esophageal cancer. *Folia Histochem Cytobiol.* 2014;52:171–7.
176. Kolostova K, Matkowski R, Gürlich R, Grabowski K, Soter K, Lischke R, et al. Detection and cultivation of circulating tumor cells in gastric cancer. *Cytotechnology.* 2016;68:1095–102.
177. Zhang L, Ridgway LD, Wetzel M a, Ngo J, Yin W, Kumar D, et al. The identification and characterization of breast cancer CTCs competent for brain metastasis. *Sci Transl Med.* 2013;5.
178. Cayrefourcq L, Mazard T, Joosse S, Solassol J, Ramos J, Assenat E, et al. Establishment and characterization of a cell line from human Circulating colon cancer cells. *Cancer Res.* 2015;75:892–901.
179. Gao D, Vela I, Sboner A, Iaquinta PJ, Wouter R, Arora VK, et al. Organoid cultures derived from patients with advanced prostate cancer. *Cell.* 2014;159:176–87.
180. Zhang Z, Shiratsuchi H, Lin J, Chen G, Reddy RM, Azizi E, et al. Expansion of CTCs from early stage lung cancer patients using a microfluidic co-culture model.

Oncotarget. 2014;5:12383–97.

181. Rossi E, Rugge M, Facchinetti A, Pizzi M, Nardo G, Barbieri V, et al. Retaining the long-survive capacity of Circulating Tumor Cells (CTCs) followed by xeno-transplantation: not only from metastatic cancer of the breast but also of prostate cancer patients. *Oncoscience*. 2014;1:49–56.
182. Hodgkinson CL, Morrow CJ, Li Y, Metcalf RL, Rothwell DG, Trapani F, et al. Tumorigenicity and genetic profiling of circulating tumor cells in small-cell lung cancer. *Nat Med*. 2014;20:897–903.
183. Maheswaran S, Haber DA. Ex vivo culture of CTCs: An emerging resource to guide cancer therapy. *Cancer Res*. 2015;75:2411–5.
184. Bidard FC, Sabatier R, Frederique B, Pistilli B, Dalenc F, De La Motte Rouge T, et al. PADA-1: A randomized, open label, multicentric phase III trial to evaluate the safety and efficacy of palbociclib in combination with hormone therapy driven by circulating DNA ESR1 mutation monitoring in ER-positive, HER2-negative metastatic breast cancer. *J Clin Oncol*. 2018;36:TPS1105-TPS1105.
185. Toy W, Weir H, Razavi P, Lawson M, Goeppert AU, Mazzola AM, et al. Activating ESR1 mutations differentially affect the efficacy of ER antagonists. *Cancer Discov*. 2017;7:277–87.
186. Rudolph M, Anzeneder T, Schulz A, Beckmann G, Byrne AT, Jeffers M, et al. AKT1E17K mutation profiling in breast cancer: Prevalence, concurrent oncogenic alterations, and blood-based detection. *BMC Cancer*. 2016;16.
187. Turner NC, Alarcón E, Armstrong AC, Philco M, López Chuken YA, Sablin M-P, et al. BEECH: a dose-finding run-in followed by a randomised phase II study assessing the efficacy of AKT inhibitor capivasertib combined with paclitaxel in patients with estrogen receptor-positive advanced or metastatic breast cancer. *Ann Oncol*. 2019;30:774–80.
188. Jones RH, Carucci M, Casbard AC, Butler R, Alchami F, Bale CJ, et al. Capivasertib (AZD5363) plus fulvestrant versus placebo plus fulvestrant after relapse or progression on an aromatase inhibitor in metastatic ER-positive breast cancer (FAKTION): A randomized, double-blind, placebo-controlled, phase II trial. *J Clin Oncol*.

2019;37:1005.

189. Hyman DM, Smyth LM, Donoghue MTA, Westin SN, Bedard PL, Emma J, et al. AKT Inhibition in Solid Tumors With AKT1 Mutations. *J Clin Oncol*. 2019;35:2251–62.
190. Schmid P, Abraham J, Chan S, Wheatley D, Brunt M, Nemsadze G, et al. AZD5363 plus paclitaxel versus placebo plus paclitaxel as first-line therapy for metastatic triple-negative breast cancer (PAKT): A randomised, double-blind, placebo-controlled, phase II trial. *J Clin Oncol*. 2018;36:1007.
191. Turner N, Bye H, Kernaghan S, Proszek P, Fribbens C, Moretti L, et al. The plasmaMATCH trial: A multiple parallel cohort, open-label, multi-centre phase II clinical trial of ctDNA screening to direct targeted therapies in patients with advanced breast cancer. *Cancer Res*. 2018;78:Supplement.
192. André F, Ciruelos E, Rubovszky G, Campone M, Loibl S, Rugo HS, et al. Alpelisib for PIK3CA-Mutated, Hormone Receptor-Positive Advanced Breast Cancer. *N Engl J Med*. 2019;380:1929–40.
193. Bose R, Kavuri SM, Searleman AC, Shen W, Shen D, Koboldt DC, et al. Activating HER2 Mutations in HER2 Gene Amplification Negative Breast Cancer. *Cancer Discov*. 2013;3:224–37.

8 Abkürzungsverzeichnis

AI	Aromataseinhibitor
ALDH1	Aldehyddehydrogenase 1
AKT1	RAC-alpha Serin/Threonin-Proteinkinase
AR	Androgenrezeptor
CD8	<i>Cluster of Differentiation 8</i>
CD34	<i>Cluster of Differentiation 34</i>
CD44	<i>Cluster of Differentiation 44</i>
CD45	Rezeptor-Typ Tyrosin-Proteinphosphatase C (<i>Cluster of Differentiation 45</i>)
CD47	<i>Cluster of Differentiation 47</i>
CD66	<i>Cluster of Differentiation 66</i>
CDX	<i>Cell-Line Derived Xenograft</i>
cfDNA	zellfreie DNA
CTC	zirkulierende Tumorzelle
ctDNA	zirkulierende Tumor-DNA
CRISPR/Cas	<i>Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-Associated</i>
CRC	colorektales Karzinom (<i>Colorectal Cancer</i>)
CRPC	Kastrations-resistentes Prostatakarzinom (<i>Castration Resistant Prostate Cancer</i>)
DLA	diagnostische Leukapherese
DNA	Desoxyribonukleinsäure
EDT	Östrogen-deprivierende Therapie
EGFR	<i>Epidermal Growth Factor Receptor</i>
EMT	Epithelial-Mesenchymale-Transition
EpCAM	epitheliale Zell-Adhäsions-Molekül
ER	Östrogenrezeptor

ET	endokrine Therapie
et al.	<i>et alii/aliae</i>
FACS	Fluoreszenz-aktivierte Zellsortierung
FDA	<i>U. S. Food and Drug Administration</i>
GNRH	Gonadotropin-freisetzendes Hormon (<i>Gonadotropin Releasing-Hormone</i>)
GNRH-A	<i>Gonadotropin Releasing-Hormone-Analagon</i>
HER2	ERBB2, HER2/neu, <i>Human Epidermal Growth Factor Receptor 2</i>
HPSE	Heparanase
HSP90	<i>Heat Shock Protein 90</i>
IL-11	Interleukin 11
ICF	Immunzytofluoreszenz
JAK	Januskinase
l, ml	(Milli-) Liter
LA	Leukapherese
LBD	Liganden-Binde-Domäne
MAPK	<i>Mitogen-Activated Protein Kinase</i>
MK	Mammakarzinom
MMP	Matrix-Metalloprotease
MNC	mononukleäre Zelle
MET	Tyrosin-Proteinkinase Met
mRNA	<i>Messenger Ribonukleinsäure</i>
mTOR	<i>Mechanistic Target of Rapamycin</i>
NGS	<i>Next Generation Sequencing</i>
NK-Zelle	natürliche Killerzelle
NOD/SCID	Maus-Modell mit einer <i>Severe Combined Immunodeficiency</i> und einem <i>Non-Obese Diabetic</i> -Typ

NSCLC	nicht-kleinzelliges Lungenkarzinom (<i>Non Small Cell Lung Cancer</i>)
NTD	N-terminalen Domäne
Pa	Pascal
PARP	Poly-ADP-Ribose-Polymerase
PBMC	Mononukleäre Zelle des peripheren Blutes (<i>Peripheral Blood Mononuclear Cell</i>)
PCR	Polymerase-Kettenreaktion (<i>Polymerase Chain Reaction</i>)
PI3K	Phosphoinositid-3-Kinase
PR	Progesteronrezeptor
PTHrP	<i>Parathyroid Hormone-Related Protein</i>
RANKL	<i>Receptor Activator of NF-κB Ligand</i>
RAS	<i>Rat Sarcoma</i>
RNA	Ribonukleinsäure
SCLC	kleinzelliges Lungenkarzinom (<i>Small Cell Lung Cancer</i>)
STAT	<i>Signal Transducers and Activators of Transcription</i>
STR	<i>Short Tandem Repeat</i>
TGF-β	<i>Transforming Growth Factor β</i>
VEGF	vaskulärer endothelialer Wachstumsfaktor
WES	<i>Whole Exome Sequencing</i>
WGA	<i>Whole Genome Amplification</i>
WNT	Ligand des Wnt-Signalwegs (<i>Wingless/Int-1</i>)
μm	Mikrometer

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10 Erklärung

Hiermit erkläre ich, André Franken, dass ich die vorliegende Dissertation selbstständig verfasst und bei keiner anderen Universität bzw. Fakultät in der vorgelegten oder einer ähnlichen Form eingereicht habe. Für die Anfertigung der Dissertation habe ich keine anderen als die angegebenen Hilfsmittel verwendet. Die Stellen, die anderen Arbeiten dem Wortlaut oder dem Sinn nach entnommen sind, wurden unter Angabe der dazugehörigen Quelle kenntlich gemacht.

Düsseldorf, den 12. Mai 2020

11 Appendix

Publikationen (Erstautorenschaft)

- **Franken A**, Behrens B, Reinhardt F, Yang Y, Rivandi M, Marass F, Jäger B, Krawczyk N, Cieslik J-P, Honisch E, Asperger H, Jeannot E, Proudhon C, Beerenwinkel N, Schölermann N, Esposito I, Dietzel F, Stoecklein NH, Niederacher D, Fehm T, Neubauer H. Multiparametric circulating tumor cell analysis to select targeted therapies for breast cancer patients. Eingereicht.
- **Franken A**, Rivandi M, Yang L, Jäger B, Krawczyk N, Honisch E, Niederacher D, Fehm T, Neubauer H. A multiplex PCR-Based NGS-Panel to Identify Mutations for Targeted Therapy in Breast Cancer Circulating Tumor Cells. Appl Sci. 2020 May;10(10).
- **Franken A**, Honisch E, Reinhardt F, Meier-Stiegen F, Yang L, Jaschinski S, Esposito I, Alberter B, Polzer B, Huebner H, Fasching PA, Pancholi S, Martin LA, Ruckhaeberle E, Schochter F, Tzschaschel M, Hartkopf AD, Mueller V, Niederacher D, Fehm T, Neubauer H. Detection of ESR1 Mutations in Single Circulating Tumor Cells on Estrogen Deprivation Therapy but Not in Primary Tumors from Metastatic Luminal Breast Cancer Patients. J Mol Diagn. 2020 Jan;22(1).
- **Franken A**, Driemel C, Behrens B, Meier-Stiegen F, Endris V, Stenzinger A, Niederacher D, Fischer JC, Stoecklein NH, Ruckhaeberle E, Fehm T, Neubauer H. Label-Free Enrichment and Molecular Characterization of Viable Circulating Tumor Cells from Diagnostic Leukapheresis Products. Clin Chem. 2019 Apr;65(4).

Publikationen (Koautorenschaft)

- Chen C, Yang L, Rivandi M, **Franken A**, Fehm T, Neubauer H. Bioinformatic identification of a breast tissue-specific gene expression profile. Proteomics Clin Appl. 2020 Nov;14(6)

- Reinhardt F, **Franken A**, Meier-Stiegen F, Driemel C, Stoecklein NH, Fischer JC, Niederacher D, Ruckhaeberle E, Fehm T, Neubauer H. Diagnostic Leukapheresis Enables Reliable Transcriptomic Profiling of Single Circulating Tumor Cells to Characterize Inter-Cellular Heterogeneity in Terms of Endocrine Resistance. *Cancers*. 2019 Jun;11(7).
- Kersting D, Fasbender S, Pilch R, Kurth J, **Franken A**, Ludescher M, Naskou J, Hallenberger A, von Gall C, Mohr CJ, Lukowski R, Raba K, Jaschinski S, Esposito I, Fischer JC, Fehm T, Niederacher D, Neubauer H, Heinzel T. From in vitro to ex vivo: Subcellular localization and uptake of graphene quantum dots into solid tumors. *Nanotechnology*. 2019 Sep;30(39).
- Fehm TN, Meier-Stiegen F, Driemel C, Jäger B, Reinhardt F, Naskou J, **Franken A**, Neubauer H, Neves RPL, van Dalum G, Ruckhäberle E, Niederacher D, Rox JM, Fischer JC, Stoecklein NH. Diagnostic leukapheresis for CTC analysis in breast cancer patients: CTC frequency, clinical experiences and recommendations for standardized reporting. *Cytometry A*. 2018 Dec;93(12).
- Reinhardt F, **Franken A**, Fehm T, Neubauer H. Navigation through inter- and intratumoral heterogeneity of endocrine resistance mechanisms in breast cancer: A potential role for Liquid Biopsies? *Tumour Biol*. 2017 Nov;39(11). Review.
- Lampignano R, Yang L, Neumann MHD, **Franken A**, Fehm T, Niederacher D, Neubauer H. A Novel Workflow to Enrich and Isolate Patient-Matched EpCAMhigh and EpCAMlow/negative CTCs Enables the Comparative Characterization of the PIK3CA Status in Metastatic Breast Cancer. *Int J Mol Sci*. 2017 Aug;18(9).
- Neumann MH, Schneck H, Decker Y, Schömer S, **Franken A**, Endris V, Pfarr N, Weichert W, Niederacher D, Fehm T, Neubauer H. Isolation and characterization of circulating tumor cells using a novel workflow combining the CellSearch® system and the CellCelector™. *Biotechnol Prog*. 2017 Jan;33(1).

Vorträge

- *Selected Abstract*, 39. Jahrestagung der Deutschen Gesellschaft für Senologie e.V. – Berlin, Deutschland (2019): Analysis of ESR1 mutations in single circulating tumor cells from metastatic luminal breast cancer patients upon estrogen deprivation therapy. (Franken et al. Senol. - Zeitschrift für Mammadiagnostik und -therapie. 2019;16(2))
- *Selected Abstract*, 2nd Translational Oncology Symposium – Essen, Deutschland (2019): Circulating Tumor Cells from Cryo-Conserved Leukapheresis Product can be Used for In Vitro Culture.
- *Invited Speaker*, IV Symposio Liquida Biopsia: the way of precision oncology – Santiago de Compostela, Spanien (2019): CTC Enrichment from Diagnostic Leukapheresis Product and CTC Culture.
- *Invited Speaker*, 38. Jahrestagung der Deutschen Gesellschaft für Senologie e.V. – Stuttgart, Deutschland (2018): Kultivierung von CTCs/DTCs zur weiteren Diagnostik - Fiktion oder Realität?
- *Invited Speaker*, 4th Thomas Ashworth CTC & Liquid Biopsy Symposium – Sydney, Australien (2017): Cultivation and Molecular Analysis of CTCs from Breast Cancer Patients upon Diagnostic Leukapheresis.

Weitere ausgewählte Kurzpublikationen und Posterpräsentationen auf nationalen und internationalen Kongressen ²

- San Antonio Breast Cancer Symposium – San Antonio, Vereinigte Staaten (2019): The clinical utility of mutations detected on circulating tumor cells: A proof of concept study based on a metastasized breast cancer index patient. (Franken et al. Cancer Research 2019;80(4;Supplement))

² Der Übersicht halber ist an dieser Stelle lediglich eine Auswahl der Posterpräsentationen aufgeführt. Zusätzlich wurden zahlreiche weitere Poster als Erst- und Koautor auf nationalen und internationalen Kongressen präsentiert.

- 4th International Symposium on Advances in Circulating Tumor Cells “Liquid Biopsy: Latest Advances and Future Challenges” – Korfu, Griechenland (2019): A Proof of Concept Study for the Clinical Use of Liquid Biopsies: What Can We Learn from the Longitudinal Analysis of an Index Patient?
- 20th International AEK Cancer Congress – Heidelberg, Deutschland (2019): Diagnostic Leukapheresis Increases the Number of Circulating Tumor Cells for in Vitro Cultivation.
- 62. Kongress der Deutschen Gesellschaft für Gynäkologie und Geburtshilfe – Berlin, Deutschland (2018): Cryo conservation preserves viability of circulating tumor cells from leukapheresis product for in vitro culture.
(Franken et al. Geburtshilfe Frauenheilkd 2018;78(10))
- 38. Jahrestagung der Deutschen Gesellschaft für Senologie e.V. – Stuttgart, Deutschland (2018): Circulating tumor cells from cryo-conserved leukapheresis product can be used for in vitro culture
(Franken et al. Senol. - Zeitschrift für Mammadiagnostik und -therapie. 2018;15(2))
- 11th ISMRC International Symposium on Minimal Residual Cancer: Liquid Biopsy in Cancer Diagnostics and Treatment – Montpellier, Frankreich (2018): Cryopreserved CTCs gained from DLA can be used for in vitro culture.
- 3rd International Symposium on Advances in Circulating Tumor Cells “Liquid Biopsy in Clinical Practice” – Rhodos, Griechenland (2017): Parsortix System Enables Isolation of Viable CTCs from Leukapheresis Product with Subsequent Culture
- 37. Jahrestagung der Deutschen Gesellschaft für Senologie e.V. – Berlin, Deutschland (2017): Isolation of viable CTCs from leukapheresis product via Parsortix system enables subsequent culture
(Franken et al. Senol. - Zeitschrift für Mammadiagnostik und -therapie. 2018;14(2))

- 19th International AEK Cancer Congress – Heidelberg, Deutschland (2017): Isolation of Viable CTCs from Leukapheresis Product via Parsortix System for Subsequent Culture.
- 61. Kongress der Deutschen Gesellschaft für Gynäkologie und Geburtshilfe – Stuttgart, Deutschland (2018): NGS basierte Multi-Gen-Panel Analyse zirkulierender Tumorzellen bei Mammakarzinom-Patientinnen.
(Franken et al. Geburtshilfe Frauenheilkd 2016;76(10))
- 10th ISMRC International Symposium on Minimal Residual Cancer: Liquid Biopsy in Cancer Diagnostics and Treatment – Hamburg, Deutschland (2016): *In Vitro* Expansion of Circulating Tumor Cells of Metastatic Gynecological Cancer Patients.

Preise

Vortragspreis, 39. Jahrestagung der Deutschen Gesellschaft für Senologie e.V. – Berlin, Deutschland (2019)

Posterpreis, 10. Wissenschaftliches Symposium der Kommission TraFo der AGO – Düsseldorf, Deutschland (2018)

Posterpreis, 7. Wissenschaftliches Symposium der Kommission TraFo der AGO – Düsseldorf, Deutschland (2015)

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