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Prognostisch und prädiktiv relevante Biomarker in Leber- und Lungenmetastasen des Kolorektalen Karzinom

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Friederike Claudia Beate Wrana

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München, 18.04.2024 — Friederike Claudia Beate Wrana

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Inhaltsverzeichnis

1. Abkürzungsverzeichnis

KRK	-	Kolorektale Karzinom
CRC	-	Colorectal Caner
UICC	-	Union Internationale Contre le Cancer
MSI	-	Mikrosateliten-Instabilität
dMMR	-	Mismatch-Reparatur-Defizienz
EGF-R	-	Epidermal-Growth-Factor-Receptor
VEGF-R	-	Vascular Endothelian Growth Factor Receptor
Erα	-	Östrogen-Rezeptor alpha
PR	-	Progesteron-Rezeptor
Her2/neu	-	Human Epidermal Growth Factor Receptor 2
HGF-R	-	Hepatocyte Growth Factor Receptor
FDA	-	Food and Drug Administration
Muc1	-	Mucin 1
HA	-	Hyaloronsäure
MAPK/ERK	-	Mitogen-Activated Protein Kinase/ extracellular-signal regulated kinases
PI3K/Akt	-	Phosphoinositid-3-Kinase/Serin/Threonin-Kinase
IGF-1R	-	Insulin Like Growth Factor 1 Rezeptor
Hsp90	-	Heat shock protein 90
PD-1	-	Programmed Cell Death Protein 1
PD-L1	-	Programmed Cell Death Ligand 1
TNM-Klassifikation	-	Tumor/Nodus/Metastasen Klassifikation
PFS	-	Progressionsfreie Überleben/progression free survival

2. Veröffentlichungen zum Erhalt des Doktortitels

- *Paper 1*

Schlueter, F.; Doetzer, K.; Pruefer, M.; Bazhin, A.V.; Werner, J.; Mayer, B.

Integrating Routine Clinical Factors to Stratify Colorectal Cancer Patients with Liver and Lung Metastases for Immune Therapy.

Journal of Cancer Science and Clinical Therapeutics **2021**, *5*, 049-062. DOI: 10.26502/jcsct.5079100

- *Paper 2*

Wrana, F.; Dötzer, K.; Prüfer, M.; Werner, J.; Mayer, B.

High Dual Expression of the Biomarkers CD44v6/α2β1 and CD44v6/PD-L1 Indicate Early Recurrence after Colorectal Hepatic Metastasectomy.

Cancers (Basel) **2022**. 14(8). DOI: 10.3390/cancers14081939

3. Einleitung

3.1 Epidemiologie des fernmetastasierten kolorektalen Karzinoms

Das Kolorektale Karzinom (KRK) ist sowohl in Deutschland als auch weltweit eines der häufigsten malignen Tumore. Es handelt sich in Deutschland um den zweithäufigsten Tumor mit jährlich ca. 61.000 Neuerkrankungen [1]. Weltweit ist das KRK die dritthäufigste Tumorerkrankung [2]. Zum Zeitpunkt der Diagnose besteht bei bis zu 25% der Patient:innen ein fortgeschrittenes Stadium und sogenannte synchrone Metastasen haben sich gebildet [3,4]. Zudem zeigen sich im Laufe der Zeit bei bis zu weiteren 25% der Patient:innen metachrone Metastasen [4,5]. Bei synchronen Metastasen handelt es sich um Metastasen, die bereits zum Zeitpunkt der Diagnose des Primärtumors bestehen. Metachrone Metastasen bezeichnet man hingegen diese, welche sich frühestens 6 Monate nach Erstdiagnose gebildet haben [6]. Beim Primärtumor unterscheidet man beim KRK zwischen Kolon- und Rektumkarzinom. Abhängig vom Sitz des Primärtumors, zeigen sich die Fernmetastasen in unterschiedlichen Organen. Die häufigste Lokalisation ist die Leber, hier finden sich 70-80% aller Kolorektalen Metastasen [3]. Die Lunge ist mit 10% das zweithäufigste Organ für Fernmetastasen [7]. Weitere Lokalisationen für eine Metastasierung sind neben den Lymphknoten das Peritoneum, das Gehirn und Knochen, selten weitere Organe[5,8].

Die Prognose hängt beim KRK stark von dem ‚Union Internationale Contre le Cancer' (UICC) Stadium zum Zeitpunkt der Diagnose ab. Die 5-Jahres Überlebensrate für Patient:innen mit resektablen synchronen Metastasen (Stadium IV) ist deutlich geringer als in niedrigeren UICC-Stadien. Primär operable Lebermetastasen zeigen lediglich eine 5-Jahres Überlebensrate von 50% und nicht resektable Metastasen von unter 10% [5,9,10]. Patient:innen mit operablen metachronen Lebermetastasen weisen eine bessere Prognose als Patient:innen mit synchronen Metastasen auf, die 5-Jahres-Überlebensrate liegt trotzdem nur bei ca. 65% [11]. Weitere Prognosefaktoren neben des Residualstatus, also das Fehlen, bzw. Vorhandensein von Resttumor nach der chirurgischen Entfernung, und des UICC-Stadiums, sind sogenannte ‚risk-scores', wie z.B. der FONG, GAME oder CERR-Score [12-14]. Alle drei Scores dienen als Prognosefaktoren und nehmen als Entscheidungshilfen eine immer größere Bedeutung ein. Beim FONG-Score handelt es sich um eine Entscheidungshilfe in Bezug auf die Resektabilität. Beim GAME-Score sowie CERR-Score werden auch noch die genetischen Faktoren KRAS-Status (GAME-Score) sowie NRAS-Status und BRAF-Status (CERR-Score) berücksichtigt. Beide Scores können bei der Festlegung optimaler klinischer Behandlungsstrategien unterstützend sein. In der deutschen S3-Leitlinie wird bisher jedoch nur der FONG-Score verwendet [15].
Patient:innen mit operablen metachronen Lungenmetastasen weisen ein 5-Jahres Überleben von ca. 70% [16], Patient:innen mit operablen synchronen Lungenmetastasen nur von ca. 32-68% auf[17]. Im Gegensatz zu Lebermetastasen, sind für Lungenmetastasen keine Scores zur weiteren Prognoseabschätzung publiziert.

3.2 Therapieoptionen des fernmetastasierten kolorektalen Karzinoms

In der folgenden Zusammenfassung der Therapieoptionen werden die nationalen und internationalen Leitlinien in Bezug auf die Behandlung des metastasierten KRK betrachtet.

In der deutschen S3-Leitlinie erfolgt die Therapieauswahl primär anhand des UICC-Stadiums zum Zeitpunkt der Erstdiagnose. Bei 25% der Patient:innen mit synchronen Lebermetastasen ist initial eine kurative Behandlung möglich [18,19]. Auch isolierte Lungenmetastasen haben ein kurativ es Potential. Hierfür entscheidend ist die Möglichkeit der Resektion. Sowohl bei Leber- als auch Lungenmetastasen gilt: Ist eine Resektion möglich, wird von Fall zu Fall entschieden, ob neben dem chirurgischen Eingriff eine perioperative oder adjuvante Chemotherapie nötig ist [20]. Die vollständige Resektion der Metastasen (Residualstatus R0) scheint eine vielversprechende Therapie im Hinblick auf die Prognose zu sein [3,21], diese stellt derzeit die beste Chance für ein langfristiges Überleben dar [22,23]. Eine zusätzliche Chemotherapie verlängert zwar die progressionsfreie Zeit, jedoch nicht das Gesamtüberleben [24,25]. Im Gegensatz dazu ist bei Patient:innen mit resektablen Lungenmetastasen der Einfluss einer perioperativen Chemotherapie nicht eindeutig [26,27]. Es zeigt sich nur ein Benefit in Bezug auf das progressionsfreie Intervall für kleine Untergruppen und nicht für eine unselektierte Chemotherapie. Insgesamt erleiden trotz kurativer Metastasektomie mehr als die Hälfte der Patient:innen, sowohl bei Leber- als auch Lungenmetastasen, ein Rezidiv [28,29].
Im Falle von potenziell resektablen Metastasen ist es das Ziel durch eine vorausgehende Chemotherapie eine Verkleinerung und somit mögliche Resektabilität der Metastase zu erreichen. Diese Vorgehensweise erfolgt sowohl bei synchronen als auch metachronen Metastasen. Insgesamt können somit 5-25% der initial nicht resektablen Metastasen sekundär reseziert werden [30,31]. Generell wird aktuell nicht zwischen Leber- und Lungenmetastasen in der Auswahl der systemischen Therapie unterschieden. Es wurde jedoch ein unterschiedliches Therapieansprechen von Metastasen in verschiedenen Lokalisationen gezeigt [32]. Dieser Befund unterstreicht die Notwendigkeit, zukünftig bei der Therapieentscheidung zwischen Leber- und Lungenmetastasen zu differenzieren.

Bei primär nicht resektablen Leber- und Lungenmetastasen steht die palliative Behandlung im Vordergrund und es wird in der Regel eine Chemotherapie begonnen [33,34]. Die Wahl der Therapie wird interdisziplinär getroffen und ist abhängig von mehreren Faktoren. Zunächst wird der Allgemeinzustand der Patient:innen betrachtet, hierfür entscheidend sind das biologische Alter, Komorbiditäten und die generelle Fitness der Patient:innen [15]. Ebenfalls relevant ist die lokale Tumorausdehnung. Dies bezieht sich auf die Größe des Tumors, sowie die Anzahl der im jeweiligen Organ betroffenen Segmente. Im Anschluss wird ein molekularbiologisches Profil erstellt, welches die zur Therapieentscheidung aktuell relevanten Biomarker bestimmt, hierunter fallen der MSI/dMMR Status, BRAF Status und RAS Status. Im Falle eines KRAS Wildtyps wird die Lokalisation des Primärtumors mit in die Therapieauswahl einbezogen. Dies ist darin begründet, dass Patient:innen mit linksseitigem Primärtumor und vorliegendem KRAS-Wildtyp am meisten von einer anti-EGFR Therapie im Hinblick auf das Überleben profitieren [35,36].

Neben der deutschen S3-Leitlinie haben auch die europäische [37] und US-amerikanische Leitlinie [38] primär das Ziel, durch eine vorausgeschaltete Chemotherapie eine maximale Größenreduktion und somit eine Resektabilität der Metastasen zu erreichen. Ebenso verfolgen die zwei Leitlinien, wie die deutsche S3-Leitlinie, eine palliative Therapie im Falle von nicht resektablen Metastasen. Ein wichtiger Unterschied der europäischen zur deutschen

S3-Leitlinie besteht darin, auch Patient:innen mit primär resektablen isolierten Leber- oder Lungenmetastasen immer mit einer systemischen Chemotherapie zu versorgen [37].

International zugelassen sind neben den seit Jahren gängigen Oxaliplatin- bzw. Irinotecan basierten Therapien FOLFOX und FOLFIRI auch zielgerichtete Antikörper. Hierzu zählen der VEGF-Inhibitor Bevazizumab [39], Epithel-Growth-Factor-Receptor (EGF-R) -Inhibitoren Cetuximab [40] oder Panitumumab [41] oder weitere Therapeutika wie das Fusionsprotein Aflibercept [42], Tyrosinkinase-Inhibitor Regorafenib [43], PD-1-Inhibitor Pembrolizumab [44] oder den BRAF-Inhibitor Encorafenib [45] auch in Kombination mit Cetuximab [46]. Diese werden teilweise in der Erstlinien-, aber auch Zweitlinientherapie eingesetzt und dabei in der Regel mit FOLFOX oder FOLFIRI kombiniert. Welcher der zielgerichteten Antikörper in Frage kommt, zeigten die bereits oben erwähnten molekularbiologischen Untersuchungen. Neben den Kombinationen FOLFOX und FOLFIRI wird auch die Chemotherapie-Triplette FOLFOXIRI verwendet. Diese kommt hauptsächlich beim Auftreten von RAS-mutierten Tumoren oder einer BRAF V-600 Mutation in Kombination mit Antikörpern, wie Cetuximab oder Bevacizumab, zum Einsatz. Hierdurch soll eine bessere Prognose erzielt werden [47]. Voraussetzung ist jedoch ein guter Allgemeinzustand [15], da mit diesen Kombinationen die meisten Nebenwirkungen zu erwarten sind.

Es ist bekannt, dass die Wahl der Erstlinientherapie sehr entscheidend ist, da hier die Erfolgsquote im Vergleich zur Zweit- oder Drittlinientherapie und die Anzahl der möglichen zu behandelnden Patient:innen deutlich höher liegt [48,49]. Man vermutet, dass dies an der veränderten Tumorbiologie sowie Resistenzbildungen durch jede Therapie liegt. Generell können durch die zielgerichteten Therapien, basierend auf molekularbiologischen Untersuchungen, nur geringe Subgruppen des Patientenkollektivs abgedeckt werden [50-52]. Somit ist es derzeit nicht möglich die breite Masse der Patient:innen mit metastasieren KRK gezielt zu therapieren. Umso wichtiger ist es, anhand von zukünftigen Studien, weitere Therapieoptionen zu identifizieren, um eine noch größere Anzahl an Patient:innen zu erfassen.

3.3 Biomarkerexpression des fernmetastasierten kolorektalen Karzinoms

Wie bereits erwähnt, werden in der Therapie des metastasierten KRK, Chemotherapien mit Antikörpern und Tyrosinkinase-Inhibitoren kombiniert, welche gezielt gegen definierte Biomarker des Tumors gerichtet sind. Die Voraussetzung für eine zielgerichtete Therapie ist der Expressionsnachweis des Biomarkers durch eine molekularpathologische Untersuchung. Bei Biomarkern handelt es sich um definierte messbare Parameter, welche im Blut oder Gewebe erfasst werden und als Indikator dienen und unterschiedliche Aussagen zu bestimmten Prozessen oder Krankheitszuständen geben können. Biomarker müssen objektiv messbar sein. Biomarker lassen sich in folgende Kategorien einteilen: prognostische, prädiktive und diagnostische Biomarker, sowie als Verlaufsparameter bei Krankheiten, Reaktionsbiomarker in Verbindung mit Medikamenten, Sicherheitsfaktoren und Anfälligkeits-/Risikobiomarker [53]. Der Hauptfokus liegt im Folgenden auf den prognostischen Biomarkern. Prognostische Biomarker können einen wahrscheinlichen Krankheitsverlauf vorhersehen. Sie sind besonders wichtig für die Vorhersage des Risikos eines Ereignisses, wie z.B. Fortschreiten der Krankheit oder das Eintreten des Todes. Sie sind in der Medizin ein häufig verwendeter

Parameter. Prognostische Biomarker gilt es vor allem von prädiktiven Biomarker zu unterscheiden [54,55].

Beim metastasierten KRK haben sich bereits prädiktive und prognostische Biomarker als wichtige Marker für die Therapiewahl etabliert. Bereits 2004 wurden die ersten Therapeutika bzw. monoklonale Antikörper gegen EGF-R (Cetuximab) und Vascular Endothelial Growth Factor (VEGF) (Bevacizumab) für das KRK zugelassen, gefolgt von Panitumumab (2006) gerichtet gegen EGF-R und Aflibercept, Regorafenib (2012) und Ramucirumab (2015) für VEGF [56].

Im Rahmen dieser Dissertation wurden Biomarker anderer Tumorentitäten, welche dort eine therapieentscheidende Rolle spielen analysiert. Zur Diagnostik beim Mammakarzinom wird leitliniengerecht die Expression des Proliferationsmarker Ki67, die Rezeptoren Her2/neu, Östrogenrezeptor alpha (ERa) und Progesteronrezeptor (PR) erfasst und bewertet [57]. Ki67 färbt die Zellen an, welche einen Teilungsprozess durchlaufen. Somit wird Ki67 anhand des Proliferationsindex zur Feststellung der Proliferationsrate herangezogen. Her2/neu kann mit anderen Tyrosinkinaserezeptoren Heterodimere bilden, welche über Zwischenschritte für eine Aktivierung verschiedener Signalwege führen, welche wiederum eine Zellproliferation verstärken und eine Apoptose hemmen. Für Her2/neu sind die monoklonalen Antikörper Trastuzumab und Pertuzumab zur Therapie des Mammakarzinoms zugelassen [58,59]. Beim metastasierten KRK wurde bereits eine Effektivität von Trastuzumab in Kombination mit Lapatinib bei einer Her2-Amplifikation nachgewiesen [60], jedoch ist diese Therapie aktuell noch nicht zugelassen. In der europäischen und US-amerikanischen Leitlinie wird diese Kombination nicht als Therapieansatz erwähnt [37,38]. Allerdings erhielt die Kombination Trastuzumab mit Tucatinib im Januar 2023 die Zulassung zur Behandlung von Her2/neu positiven RAS-Wildtyp-Patienten des nicht resektablen oder metastasierten kolorektalen Karzinoms [61].

Für weitere Tumorentitäten spielt der Wachstumsfaktor-Rezeptor, Hepatocyte-Growth-Factor-Receptor (HGF-R), eine therapeutische Rolle. HGF-R ist ebenfalls eine Rezeptortyrosinkinase. Durch Bindung von HGF kommt es zur Dimerisierung von zwei HGF-Rezeptoren, welche verschiedene Signalwege aktivieren, die verantwortlich für die Proliferation und Migration der Zellen sind. Für das nicht kleinzellige Bronchialkarzinom wurde der Inhibitor Crizotinib [62] und für Schilddrüsentumore der Inhibitor Cabozantinib [63] von der FDA zugelassen. Bei beiden handelt es sich um Multi-Tyrosinkinaseinhibitoren. Crizotinib inhibiert neben HGF-R auch die Anaplastische-Lymphom-Kinase (ALK). Cabozantinib inhibiert zusätzlich zu HGF-R unter anderem auch VEGF-Rezeptoren.

Mucin-1 (MUC-1) ist ein Zelladhäsionsmolekül. Die Expression von MUC-1 fördert die Angiogenese bei Krebs und somit in gewissem Maße auch die Migration und Invasion des Tumors [64]. MUC-1, spielt vor allem in der Brustkrebsdiagnostik, aber auch in anderen Tumorentitäten wie das Ovarialkarzinom, aufgrund des CA 15-3 Serumspiegels als Marker für die Tumoraktivität, eine Rolle. Die lösliche Form von MUC-1 wird durch CA 15-3 nachgewiesen. Dadurch kann dieser Marker zur Prognoseabschätzung oder bei Beurteilung des Therapieerfolgs, bzw. als Verlaufskontrolle genutzt werden [65,66]. Zusätzlich spielt MUC-1

eine Rolle in der Entwicklung von Krebsimpfstoffen. Diese sind eine vielversprechende Strategie zur Verhinderung der lokalen Tumorprogression und der Metastasierung [64].

Neben den bereits in anderen Entitäten etablierten bzw. zugelassenen Biomarkern, wurden im Rahmen dieser Dissertation noch andere Antigene untersucht, die im Folgenden vorgestellt werden. Diese könnten als Basis weiterer Therapieoptionen dienen und somit zukunftsweisend sein.

Bei CD44v6 handelt es sich um die Variante 6 des Zelladhäsionsmolekül CD44. Es steht in Wechselwirkung mit Hyaluronsäure (HA), Osteopontin oder Kollagen oder als Korezeptor für verschiedene Zytokine. HA interagiert mit CD44v6 und führt über Zwischenschritte zur Aktivierung des MAPK/ERK- und PI3K/Akt-Signalweg. Diese bewirken Proliferation, Apoptose und gleichzeitig auch zu einer verstärkten Produktion von weiterem HA, welche diese Prozesse ankurbelt [67]. Es gibt aktuell noch keine spezifisch für CD44v6 zugelassenen Therapeutika, jedoch laufen bereits klinische Studien, die mögliche zukünftige Therapieoptionen prüfen. Hierunter fällt auch der Wirkstoff Bivatuzumab (NCT02254005 (aufgerufen am 16.01.2023)) beim Mammakarzinom. Aber auch für das KRK gibt es aktuelle Studien. Zum einen die Studie NCT03009214 mit dem Peptid AMC303 [68], sowie die Studie NCT04427449 mit CAR T-Zellen (aufgerufen am 16.01.2023)) [69]. Zu beiden Studien liegen noch keine Ergebnisse vor.

Integrin α2β1 ist ein weiteres Zelladhäsionsmolekül, das bei der Tumorprogression eine Rolle spielt. In Interaktion mit der extrazellulären Matrix, vor allem Kollagen Typ 1, führt es zur Proliferation, Invasion und Metastasierung in die Leber [70]. Hier wurden mit dem Therapeutikum E7820 Studien durchgeführt, auch zum KRK (NCT01347645 (aufgerufen am 16.01.2023)). Zu dieser Studie liegen die ersten Ergebnisse vor, welche zeigen, dass das progressionsfreie Intervall in der Kontrollgruppe mit FOLFIRI größer ist, als in der Studiengruppe mit E7820 in Kombination mit Irinotecan.

Durch Aktivierung von Insulin-like Growth Factor 1 Rezeptor (IGF-1R) an der Plasmamembran werden Schlüsselprozesse vermittelt wie Zellwachstum, Zellproliferation, Differenzierung, Apoptose und Angiogenese [71]. Für IGF-1R existieren noch keine zugelassenen Inhibitoren, jedoch nimmt die Bedeutung dieses Markers für gewisse Entitäten, wie Mamma- und das nicht-kleinzelliges Lungenkarzinom, zu. Aktuell befinden sich mehrere Inhibitoren in klinischen Studien. Hierunter fällt Dalotuzumab, welcher bereits am metastasierten KRK in einer Studie zum Einsatz kam (NCT00614393 (aufgerufen am 02.02.2023)). Bei dieser Studie wurde Dalotuzumab zusammen mit Irinotecan oder Cetuximab verabreicht. Diese Kombination wurde von den Patienten gut toleriert, jedoch erhöht sich dadurch nicht das Überleben. Trotzdem geht man davon aus, dass IGF-1R-Liganden vielversprechende Biomarker für das unterschiedliche Ansprechen auf Anti-EGFR- und Anti-IGF-1R-Therapien sind [72]. Weitere Inhibitoren sind Linsitinib oder Ceritinib, ALK Tyrosinkinase-Hemmer, welche auch IGF-1R blockieren.

Beim Heat shock protein 90 (Hsp90) handelt es sich um ein Chaperon. Hsp90 aktiviert unter anderem MMP-2, welches maßgeblich an der Invasion und Migration von Tumorzellen beteiligt ist [73]. Die Inhibitoren für Hsp90 sind noch für keine Entität zugelassen, jedoch gibt es bereits einige abgeschlossene Studien zum Prostatakarzinom (NCT01685268 (aufgerufen am 02.02.2023)), aber auch zum kolorektale Karzinom mit den Hsp90-Inhibitoren Ganetespib

(NCT01111838 (aufgerufen am 02.02.20223)) oder Pimitespib [74]. In der Studie zu Ganetespib zeigte sich jedoch keine nennenswerte Antitumor-Wirksamkeit [75].

Zusätzlich zu den charakteristischen molekularen Eigenschaften der Tumorzellen spielt das organspezifische Mikromilieu bei der Metastasierung eine entscheidende Rolle. Die disseminierenden Tumorzellen interagieren unterschiedlich im Zielorgan der Metastasierung und setzen sich in den sogenannten Metastasennischen ab [76,77]. Es ist bekannt, dass sich Leber- und Lungenmetastasen in der Zusammensetzung der extrazellulären Matrix, der Gefäße und des Immunzellinfiltrats aber auch im Sekretom-Profil unterscheiden [77]. Im folgenden Abschnitt werden das Immunzellinfiltrat und seine therapeutische Bedeutung vorgestellt.

3.4 Immunzellinfiltrat des fernmetastasierten kolorektalen Karzinoms

Die Immuntherapie stellt einen weiteren Pfeiler in der Krebstherapie dar. Die Immuntherapie hat zum Ziel, Immunzellen zu aktivieren und dadurch die Tumorzellen am Wachstum zu hindern [78,79].

Im Rahmen dieser Dissertation wurde der Fokus auf den Rezeptor ‚programmed cell death protein 1' (PD-1), dessen Ligand ‚programmed death-ligand 1' (PD-L1), sowie die T-Zellmarker CD3 und CD8 gesetzt. Die Charakterisierung des Immunstatus hat bei mehreren Tumorentitäten zur Zulassung von Immuntherapeutika geführt. Dies gilt mit Pembrolizumab, einem PD-1-Antikörper, auch für das metastasierte KRK [44]. Dieser monoklonale Antikörper kommt bei Patient:innen mit metastasierten KRK zum Einsatz, welche entweder eine hochfrequente Mikrosatelliten-Instabilität (MSI-high) oder ein Mismatch-Reparatur-Defizit (dMMR) aufweisen. Des Weiteren sind mit dem gegen PD-1 gerichteten Antikörper Nivolumab (2017) und dem CTLA-4-Inhibitor Ipilimumab (2017) und deren Kombination zwei weitere Checkpoint-Inhibitoren für das metastasierte KRK zugelassen [56,80].

Bisher ist bekannt, dass eine erhöhte Expression von PD-L1 aber auch PD-1 mit einer günstigeren Prognose beim primären KRK sowie bei Lebermetastasen in Verbindung steht [81,82]. Sowohl das progressionsfreie Intervall, sowie das Gesamtüberleben ist verlängert [83]. Bei Lungenmetastasen zeigt sich ein zeitlicher Zusammenhang in Bezug auf die PD-L1 Expression zwischen dem Primärtumor und den korrespondierenden Lungenmetastasen. Je größer das zeitliche Intervall zwischen den Läsionen, umso größer ist die PD-L1 Expression in den Lungenmetastasen [84].

Es ist ebenso bekannt, dass ein hohes CD3-Infiltrat sowie ein erhöhtes CD8-Infiltrat mit einer günstigen Prognose beim primären KRK im Zusammenhang stehen [85-87]. Der von Galon et al. entwickelte Immunoscore bezieht sich auf die Dichte von CD3 und CD8 positiven T-Zellen in Kombination. Ein hoher Immunoscore ist mit einer günstigen Prognose assoziiert [88], daher wird darüber diskutiert, den Immunoscore in die TNM-Klassifikation als weiteren Faktor für UICC Stadium III Tumore zu implementieren [89]. Die Bestimmung der Immunzellmarker wird auch bei Leber- und Lungenmetastasen immer wichtiger. So ist auch hier inzwischen bekannt, dass ein hohes Immunzellinfiltrat mit einer besseren Prognose einhergeht [90-92]. Des

Weiteren ist ein hohes T-Zell-Infiltrat mit einem guten Ansprechen auf Chemotherapie verbunden [93].

4. Zielsetzung der Dissertation

Wie oben bereits beschrieben haben Patient:innen mit metastasiertem kolorektalem Karzinom eine schlechte Prognose. Die aktuell zugelassenen Therapieoptionen bringen nicht den erhofften Erfolg auf Heilung bzw. Langzeitüberleben. Die Rückfallrate, vor allem in den ersten zwei Jahren nach Diagnose, ist noch immer sehr hoch, trotz radikaler chirurgischer Resektion und adjuvanter oder perioperativer Chemotherapie [15]. Der Bedarf an neuen prognostischen und prädiktiven Biomarkern ist weiterhin sehr hoch.

Ziel dieser Dissertation ist es, mithilfe von immunhistochemischer Analysen kolorektaler Leber- und Lungenmetastasen, zukünftig relevante prognostische und prädiktive Biomarker zu finden. Hierfür wurde das Tumorgewebe sowie das direkt angrenzende benigne Gewebe untersucht. Anschließend das Expressionsmuster mit klinisch-pathologischen Daten korreliert.

In der ersten Veröffentlichung lag der Fokus auf dem Immunzellinfiltrat, insbesondere den Immunzellmarker CD3, CD8, PD-1 und PD-L1. Untersucht wurden hier auch die Unterschiede zwischen den Leber- und Lungenmetastasen aber auch innerhalb dieser Metastasen in den unterschiedlichen Regionen des Tumors. In der zweiten Veröffentlichung lag der Fokus auf der Expression progressionsrelevanter Biomarker auf den Tumorzellen.

5. Beitrag Eigenanteil

Für beide Veröffentlichungen führte Friederike Wrana die Herstellung der Gefrierschnitte, die immunhistochemische Färbung, die Fotodokumentation der Ergebnisse sowie deren Auswertung und statistischen Aufarbeitung durch.

Die Auswertung der Präparate für die erste Veröffentlichung erfolgte anhand des QTiS-Algorithmus an dessen Entwicklung Friederike Wrana zuvor mitbeteiligt war [94]. Die statistische Analyse beinhaltete die Korrelationen der Immunzellmarker an der Invasionsfront, im Stroma und intratumoral mit den klinisch-pathologischen Parametern.

Die Auswertung der Präparate für die zweite Veröffentlichung erfolgte durch eine semi-quantitative Bewertung der positiv gefärbten Tumorzellen. Die statistische Analyse beinhaltete die uni- und multivariate Analyse der Biomarkerexpression mit den klinisch-pathologischen Parameter.

Das endgültige Manuskript beider Veröffentlichungen, inklusive aller Abbildungen und Tabellen, wurden von Friederike Wrana unter Beaufsichtigung von Barbara Mayer erstellt.

Für beide Veröffentlichungen dieser Dissertation wurden die klinisch-pathologischen Daten der analysierten Proben doppelt kodiert von der Biobank der Abteilung für Allgemein-, Viszeral- und Transplantationschirurgie der Ludwig-Maximilians-Universität (LMU) zur Verfügung gestellt. Die Biobank steht unter der Verwaltung der Stiftung für ‚Human Tissue and Cell Research' (HTCR). Die Rahmenbedingungen der Stiftung HTCR, zu denen auch die Einholung einer schriftlichen Einverständniserklärung aller Spender gehört, wurden von der

Ethikkommission der Medizinischen Fakultät der LMU (Genehmigungsnummer 025-12) sowie von der Bayerischen Landesärztekammer (Genehmigungsnummer 11142) genehmigt.

6. Zusammenfassung (deutsch)

Bereits 25% aller Patient:innen befinden sich zum Zeitpunkt der Diagnose eines kolorektalen Karzinoms im metastasierten Stadium. Bei bis zu weiteren 25% der Patient:innen entwickeln sich im Laufe der Erkrankung Metastasen. Trotz einiger vielversprechender Therapieoptionen ist die Prognose im vorangeschrittenen Tumorstadium sehr schlecht. Der Bedarf an weiteren prädiktiven und prognostischen Markern zur Therapieauswahl ist sehr hoch. In der Veröffentlichung ‚Integrating Routine Clinical Factors to Stratify Colorectal Cancer Patients with Liver and Lung Metastases for Immune Therapy' wurden Unterschiede in Bezug auf das Immunzellinfiltrat zwischen Leber- und Lungenmetastasen in verschiedenen Regionen des Tumorgewebes quantitativ untersucht. Das Ausmaß des Immuninfiltrats wurde mit den klinisch-pathologischen Parameter korreliert. Es zeigte sich eine immunologische Heterogenität zwischen Leber- und Lungenmetastasen. Für beide Organe zeigte sich, dass das untersuchte CD3, CD8 und PD-1-Immunzellinfiltrat an der Invasionsfront (IM) am höchsten war, gefolgt vom stromalen Areal (S). Das geringste Immunzellinfiltrat zeigte sich intratumoral (IT). Im Vergleich zur Leber wies die Lunge signifikant höhere Immunzellinfiltrate in folgenden Bereichen auf: CD3 IT (p = .005), CD3 S (p = .021), CD8 IT (p = .001), PD-1 S (p = .001) und PD-1 IT (p = .01). Im Gegensatz dazu lag bei den Lebermetastasen bei CD3 IM ein höheres Immunzellinfiltrat vor (p = .037). Neben den Unterschieden zwischen Leber- und Lungenmetastasen zeigten sich Zusammenhänge zwischen gewissen klinisch-pathologischen Parametern und dem Immunzellinfiltrat. CD3 IM und CD8 IM waren bei Patient:innen mit Lebermetastasen erhöht, welche im Anschluss an die Entfernung des Primärtumors mit einer adjuvanten Chemotherapie behandelt wurden (CD3 IM p = .011; CD8 IM p = .02). Ebenso zeigte sich bei Patienten mit Lebermetastasen mit KRAS Wildtyp ein erhöhtes CD8 IT Infiltrat (p = .038). Das CD8 IT Infiltrat in der Leber war ebenso erhöht, wenn der Tumor nur auf eine Hälfte (p = .019) und/oder auf maximal zwei Lebersegmente (p = .038) beschränkt war. Auch in Bezug auf die Lungenmetastasen gab es signifikante Zusammenhänge zwischen klinisch-pathologischen Parametern und dem Immunzellinfiltrat. Rechtsseitige Lungenmetastasen zeigten ein erhöhtes stromales CD8 Infiltrat (p = .041) und auch ein erhöhtes PD-1 Infiltrat an der Invasionsfront (p =.041) im Vergleich zu linksseitigen Lungenmetastasen. Ein erhöhtes PD-1 IM Infiltrat zeigte sich zudem bei Patient:innen mit Lungenmetastasen, welche eine neoadjuvante Chemotherapie unmittelbar vor der Metastasenresektion erhalten hatten (p = .041). Zuletzt wurde die PD-L1 Expression beider Metastasengruppen sowohl mit den Immunzellmarker als auch mit den klinisch-pathologischen Parametern korreliert. Es zeigten sich signifikante Zusammenhänge für Lebermetastasen zwischen einer erhöhten PD-L1 Expression und erhöhtem Immunzellinfiltrat (CD3 IM p = .05; CD3 IT p = .002; CD8 IM p = .002; CD8 IT p = .024; PD-1 IM p = .025; PD-1 S p = .014; PD-1 IT p = .017). Ebenso bestand ein Zusammenhang zwischen hoher PD-L1 Expression und dem KRAS Wildtyp (p = .03). Weitere Korrelationen zeigten sich zwischen einer hohen PD-L1 Expression und der Tumorausdehnung innerhalb der Leber (Begrenzung auf eine Hälfte, p = .014; einzelne Metastase, p = .02; Begrenzung auf maximal zwei Segmente, p = 0.018). Es zeigten jedoch die analysierten Proben

eine erhöhte PD-L1 Expression, welche nicht zuvor mit einer neoadjuvanten Therapie behandelt wurden (p = .025).

Neben der Betrachtung des Immunzellinfiltrats wurden in der Veröffentlichung ‚High Dual Expression of the Biomarkers CD44v6/α2ß1 and CD44v6/PD-L1 Indicate Early Recurrence After Colorectal Hepatic Metastasectomy' unterschiedliche, jedoch aus anderen Entitäten bekannte, Biomarker/Tumormarker analysiert. Auch hier wurden die Leber- und Lungenmetastasen in Bezug auf die Expression der Biomarker verglichen und es zeigten sich signifikante Unterschiede für die Biomarker IGF-1R (p = .013), EGF-R (p = .004), CD44v6 (p = .019), α2β1 (p = .001) und PD-L1 (p = .005). Für alle 5 Marker zeigten die Lungenmetastasen eine signifikante erhöhte Expression. Des Weiteren wurde die Biomarkerexpression der Lebermetastasen sowie die klinisch-pathologischen Parameter mit dem Überleben korreliert. Eine hohe Expression von CD44v6 (p = .016) sowie synchrone Metastasen (p= .004) wurden als unabhängige Prognosefaktoren in Bezug auf das progressionsfreie Überleben (PFS) erkannt. In beiden Fällen ist das progressionsfreie Intervall signifikant verkürzt.

Die prognostische Relevanz des Markers CD44v6 wurde bestätigt durch die Ergebnisse der multivariaten Analyse der dualen Expression von CD44v6 mit weiteren Biomarkern. So zeigte sich, dass eine hohe Expression von CD44v6/α2β1 (p = .002) und CD44v6/PD-L1 (p = .017) ebenfalls unabhängige Prognosefaktoren für das PFS sind.

Auf der Suche nach weiteren Therapieansätzen und möglichen Stratifizierungsmarker kristallisierten sich in beiden Publikationen Unterschiede zwischen Leber- und Lungenmetastasen heraus. Zudem wurden weitere Tumorantigene und Immunmarker identifiziert auf deren Grundlage zukünftig neue Therapieansätze zur Behandlung des metastasierten KRK entwickelt werden könnten.

7. Zusammenfassung (englisch)

At the time of diagnosis, 25% of all patients have already developed metastases. In up to another 25% of patients, metastases develop in the course of the disease. Despite some promising therapeutic options, the prognosis at advanced stages is very poor. The need for further predictive and prognostic stratification markers is very high. Within the publication 'Integrating Routine Clinical Factors to Stratify Colorectal Cancer Patients with Liver and Lung Metastases for Immune Therapy', differences in relation to the immune infiltrate between liver and lung metastases were investigates as well as the associated clinicopathologic parameters in different areas of tumor tissue. Immunological heterogeneity between liver and lung metastases was found. For both organs, considering all three markers (CD3, CD8, and PD-1), the immune cell infiltrate was highest at the invasion front (IM), followed by the stromal area (S). The lowest immune cell infiltrate is shown intratumoral (IT). In comparison between distant metastatic organs, the lung showed significantly higher immune cell infiltrates in the following areas: CD3 IT (p = .005), CD3 S (p = .021), CD8 IT (p = .001), PD-1 S (p = .001), and PD-1 IT (p = .01). Conversely, a higher immune cell infiltrate is present in liver metastases with CD3 IM compared to lung metastases (p = .037). In addition to the differences between liver and lung metastases, certain clinicopathological parameters showed correlations with the immune cell infiltrate. CD3 IM and CD8 IM were increased in patients with liver metastases who were treated with adjuvant chemotherapy after surgery of the primary tumor (CD3 IM p

= .011; CD8 IM *p* = .02). Patients with liver metastases with KRAS wild type showed an increased CD8 IT infiltrate (*p* = .038). Further significant correlations are also seen in liver metastases with an increased CD8 IT infiltrate. The immune infiltrate is increased in patients when the tumor is limited to only one half (*p* = .019) and/or to a maximum of two liver segments (p = .038). There were also significant associations between clinicopathological parameters and the immune cell infiltrate of lung metastases. Right-sided lung metastases showed an increased stromal CD8 infiltrate (*p* = .041) and also an increased PD-1 infiltrate at the invasion margin (*p* = .041) compared to left-sided lung metastases. Similarly, an increased PD-1 IM infiltrate was found in patients with lung metastases who received neoadjuvant chemotherapy immediately before metastasectomy (*p* =.041). Lastly, the PD-L1 expression of both metastatic groups were correlated with immune cell markers as well as with clinicopathological parameters. There were significant correlations for liver metastases between increased PD-L1 expression and increased immune cell infiltrate (CD3 IM *p* = .05; CD3 IT *p* = .002; CD8 IM *p* = .002; CD8 IT *p* = .024; PD-1 IM *p*= .025; PD-1 S *p* = .014; PD-1 IT *p* = .017). Similarly, there was an association between high PD-L1 expression and the KRAS wild type (*p* = .03). Further significances existed between high PD-L1 expression and tumor extension within the liver (limitation to one half, *p* = .014; single metastasis, *p* = .02; limitation to a maximum of two segments, *p* = .018). Finally, the analyzed samples showed an increased PD-L1 expression which was not previously treated with neoadjuvant therapy (*p* = .025). In addition to the immune cell infiltrate, different biomarkers / tumor markers, which are known from other entities, were analyzed in the publication 'High Dual Expression of the Biomarkers CD44v6/α2ß1 and CD44v6/PD-L1 Indicate Early Recurrence After Colorectal Hepatic Metastasectomy'.

Again, liver and lung metastases were compared in relation to biomarker expression, showing significant differences for the biomarkers IGF-1R (*p* = .013), EGF-R (*p* = .004), CD44v6 (*p* = .019), α2β1 (*p* = .001), and PD-L1 (*p* = .005). For all five markers, the lung metastases showed a significantly increased expression. Furthermore, liver metastasis expression and clinicopathologic parameters were correlated with survival. A high expression of CD44v6 (*p* = .016) and synchronous metastases (*p* = .004) were found to be independent prognostic factors in terms of progression-free survival (PFS). In both cases, the progression-free interval is significantly shortened. The prognostic relevance of the marker CD44v6 is confirmed by the results of multivariate analysis of dual expression of CD44v6 with other biomarkers. Thus, high expressions of CD44v6/α2β1 (*p* = .002) and CD44v6/PD-L1 (*p* = .017) are also shown to be independent prognostic factors for PFS.

In the search for further therapeutic approaches and potential stratification markers, differences between liver and lung metastases were found in both publications; moreover, other clinicopathological parameters and immune markers also emerged as potential approaches for therapies to treat metastatic CRC.

8. Vollständige Artikel zum Erhalt des Doktortitels

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Research Article

Integrating Routine Clinical Factors to Stratify Colorectal Cancer Patients with Liver and Lung Metastases for Immune Therapy

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Abstract

Background: Treatment with checkpoint inhibitors is approved for a small subgroup of mCRC patients with microsatellite instable high (MSI-H) or deficient microsatellite mismatch repair (dMMR) tumors indicating the strong need for additional stratification markers for immune therapy.

Patients and Methods: The immunophenotype (CD3, CD8, PD-1, PD-L1) was immunohistochemically analysed at the invasion margin (IM), the stromal (S) and intratumoral (IT) areas of 53 liver metastases (LM) and 15 lung metastases (LuM), correlated with clinical pathological parameters and statistically evaluated by the Fisher`s exact-Test (two-tailed).

Results: In LM adjuvant chemotherapy resulted in a high $CD3^+$ (p=0.011) and $CD8^+$ (p=0.02) infiltrate at the IM. Neoadjuvant chemotherapy of LM correlated with a reduced PD-L1 expression (p=0.025). LM originating from KRAS wildtype tumors demonstrated a high fraction of $CD8^+$ IT cells (p=0.038) and a strong PD-L1 expression

(p=0.03). Locally restricted LM were characterized by a high $CD8^+$ IT infiltrate (unilobular, p=0.019; maximum of 2 segments affected, p=0.038) and were found strongly PD-L1 positive (solitary LM, p=0.02; unilobular, p=0.014; ≤2 liver segments affected, p=0.018). LuM were characterized by a stronger infiltrate of $CD3^+$IT (p=0.005), $CD8^+$S (p=0.021), $CD8^+$IT (p=0.001), PD-1^+IM (p=0.007), PD-1^+S (p=0.001) and PD-1^+IT (p=0.01) compared to LM. In LuM neoadjuvant chemotherapy was accompanied by a high PD-1^+IM cell density (p=0.041). Right-sided lesions showed a high infiltrate of $CD8^+$S cells and PD-1^+IM cells (p=0.041).

Conclusion: The findings suggest previous chemotherapy, RAS status, tumor burden and sidedness as stratification markers of mCRC patients for immunotherapy, precising treatment management of distant metastases.

Keywords: Stratification parameters; Distant metastases; Immunological factors; Risk assessment; Precision immunotherapy

1. Introduction

Up to 25% [1] of the colorectal cancer (CRC) patients initially are diagnosed with synchronous liver metastasis and less frequently with lung metastasis [2-5]. In addition, despite radical surgery accompanied by drug treatment, locally advanced CRC frequently develops metachronous metastasis mostly within the first three years after first-line treatment [6, 7]. The obvious need for more efficient treatment strategies to fight metastatic CRC is reflected by an increasing number of drugs and combination therapies available. For example, recent approval was obtained for TAS-102 [8], ramucirumab [9], aflibercept [10] or regorafenib [11, 12]. Furthermore, three immunotherapy options, namely pembrolizumab [13], nivolumab and the combination nivolumab plus ipilimumab [14] are approved for metastatic colorectal cancer patients.

However, treatment with checkpoint inhibitors is restricted to the small fraction of MSI-high/dMMR selected CRC patients [15]. Currently an increasing number of predictive biomarkers independent of the MMR status for immune therapy are being discussed, such as tumor mutation burden (TMB), specific gene expression signatures, PD-L1 expression, the microbiome and immunophenotyping [16]. Nevertheless, some patients who fulfil these selection criteria do not respond to immunotherapy. Conversely, there are patients without any of these criteria revealing a good response to checkpoint inhibitors [17]. This discrepancy underscores the need of predictive factors allowing precise selection of cancer patients for immunotherapy.

In metastatic CRC most reports investigated the prognostic value of the immune microenvironment in distant metastases revealing a positive association between high densities of various immune cell phenotypes and good prognosis in both liver [18-23] and lung metastases [24, 25]. Moreover, high T-cell density in liver metastases is linked to successful chemotherapy [26]. In addition, high PD-1 expression by tumor-infiltrating lymphocytes in lung metastasis represents a rationale for the treatment of lung metastasis with checkpoint inhibitors [27]. In contrast, routine parameters to stratify patients with CRC metastases for immune therapy are rare. So far, patients receiving neoadjuvant chemotherapy [18, 20, 21, 28, 29] or presenting RAS wildtype status 30 have been identified to show a high immune infiltrate in liver metastases. Corresponding data for lung metastases is currently missing. Therefore, the aim of the present study was to identify correlations of routine clinical parameters with the

immune phenotype in distant metastases. This might help to select the most appropriate CRC patients with liver or lung metastases for immunotherapy.

2. Patients and Methods

2.1 Patient cohort

The patient cohort consists of 68 patients with metastatic colorectal cancer, receiving metastasectomy at the Department of General, Visceral, and Transplantation Surgery, Ludwig-University Hospital, LMU Munich, Munich, Germany. From each patient a liver metastasis (LM, n=53) or a lung metastasis (LuM, n=15) was analysed. Double-coded tissues and the corresponding data used in this study were provided by the Biobank of the Department of General, Visceral and Transplant Surgery in Ludwig-Maximilians-University (LMU). This Biobank operates under the administration of the Human Tissue and Cell Research (HTCR) Foundation. The framework of HTCR Foundation, which includes obtaining written informed consent from all donors, has been approved by the ethics commission of the Faculty of Medicine at the LMU (approval number 025-12) as well as the Bavarian State Medical Association (approval number 11142) in Germany.

2.2 Immunohistochemistry

Fresh tumor samples including adjacent benign reference tissue were collected according to biobanking standards. The tumor samples were immediately snap frozen in liquid nitrogen. Serial cryo-sections (5 µm) were performed using a cryotome (Leica CM 1950, Wetzler, Germany) and air dried over night at room temperature. Sections were fixed with acetone and stained immunohistochemically using the standard avidin-biotin-peroxidase complex method [31, 32]. Briefly, unspecific Fc -receptors were blocked with 10% AB-serum-DPBS (Bio-Rad; Hercules, California, USA; 805135), pH 7.4, for 20 minutes. Endogenous biotin was barred using the Avidin-/Biotin-blocking Kit (Vector Laboratories; Burlingame, California, USA; SP-2001) for 15 minutes. The primary monoclonal antibodies (mab) were incubated for one hour. The anti-CD3-mab (clone UCHT1; mouse IgG1; working concentration (wc) 1.25 µg/ml; Becton Dickenson; Franklin Lakes, New Jersey, USA; 550368) was detected with the secondary biotinylated antibody (wc 0.75 µg/ml; Jackson ImmunoResearch; West Grove, Pennsylvania, USA; 315-065-048) for 30 minutes followed by a peroxidase-conjugated streptavidin (wc 1 µg/ml; Jackson ImmunoResearch; 016-030-084) for another 30 minutes. Anti-CD8-mab (clone C8/144B; mouse IgG1; wc 3.0 µg/ml; Dako; Hamburg, Germany; M7103), anti-PD-1-mab (clone MIH4; mouse IgG1; wc 10.0 µg/ml; Affymetrix; Santa Clara, California, USA; 14-9969) and anti-PD-L1-mab (clone MIH1; mouse IgG1; wc 10.0 µg/ml; Affymetrix; 14-5983) were identified with the amplification Kit ZytoChemPlus (Zytomed Systems; Bargteheide, Germany; HRP060) according to the instructions of the manufacturer. For visualization of the antigen-antibody reaction all slides were developed in 3-amino-9-ethylcarbazol (AEC; Sigma-Aldrich; Taufkirchen, Germany; A5754) -Peroxid-solution for eight minutes. Counterstaining was performed with Mayer`s hemalum solution (Merck; Darmstadt, Germany; 109249). All incubation steps were performed in a humid chamber at room temperature. For the IgG1 isotype control the purified immunoglobulin MOPC-21 (clone MOPC-21; mouse IgG1; wc 10.0 µg/ml; Sigma-Aldrich, Germany; M5284) and for the positive control anti-CD45-mab (clone 2B11+PD7/26; mouse IgG1; wc 4.5 µg/ml; Becton Dickenson; M0701) were used. For identification of cancer cells anti-EpCAM-mab (clone BerEP4; mouse IgG1; wc 5.0 µg/ml; Dako; Hamburg, Germany; M0804) and anti-pancytokeratin-mab (clone KL1; mouse IgG1; wc 0.32 µg/ml; Zytomed Systems; MSK113) were stained.

Additionally, a standardized HE-staining was performed to estimate the percentage of cancer cells and the portion of necrosis.

2.3 Quantitative analysis of immune cells

The immune cell markers CD3, CD8 and PD-1 were quantitatively analyzed as previously described [31, 33-35]. Briefly, for each marker, three hot spot regions were chosen in three topographically different areas, namely the invasive margin (IM), the stromal (S) and intratumoral (IT) areas. The selection of the hot spot region focused on the highest density of $CD3^+$ cells identified at low magnification (50x). The IM was defined as the junction between the benign reference tissue and the tumor area according to pathological consensus guidelines [36]. Stromal leukocytes were determined as positive cells, which are in the connective tissue, without any contact to tumor cells and outside of the invasive margin. Intratumoral leukocytes were defined as positive cells which stay in close contact with tumor cells. Examples for evaluation are given in Figure 1 A-D. Three pictures were taken of each region with 200x magnification. $CD3^+$ cells and $CD8^+$ cells were examined in the same hot spot regions. For PD-1, hot spots were detected in other tumor regions.

Cell counting was done with the open source Program ImageJ. First, background was subtracted. Afterwards a colour deconvolution was performed manually by choosing three representative areas, namely background, haematoxylin and AEC-staining. An automatic threshold transferred the AEC-layer-picture into a binary picture. The watershed method was used to separate cell clusters. The absolute number of cells was counted adjusting the ´Analyze-Particle-Tool` with the values 700 to infinity for size and 0.2 to 1.0 for circularity. For the evaluation of the intratumoral cells, the area to be evaluated within the picture was additionally defined exactly with the ROI (Region of Interest)- Manager.

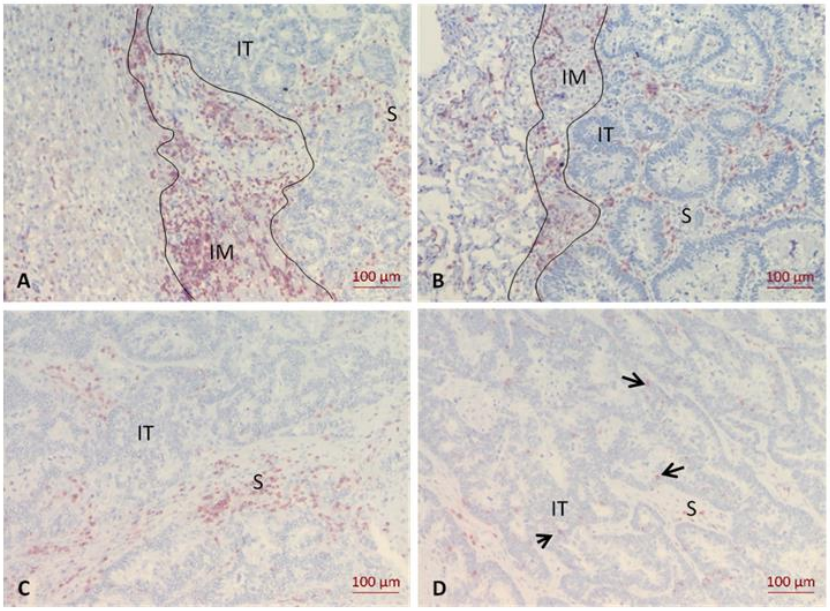

Figure 1: Immunohistochemistry of the $CD3^+$ infiltrate in liver and lung metastases. A, $CD3^+$ expression at the invasion margin (IM) of liver metastasis; B, $CD3^+$ expression at the IM of lung metastasis; C, $CD3^+$ expression in the stromal area (S) of liver metastasis; D, $CD3^+$ expression in the intratumoral area (IT) of liver metastasis. Arrows indicate individual positive cells.

2.4 Semiquantitative analyse of PD-L1

PD-L1 was expressed on tumor cells, fibroblasts and leukocytes with different intensities [37]. Therefore, the percentage of PD-L1 positive cells had to be evaluated semi-quantitatively in relation to the total amount of epithelial cells positive for EpCAM or cytokeratin and intratumoral CD45 positive immune cells. The cut off for PD-L1-positivity was ≥1% of tumor cells and intratumoral immune cells according to previous studies [38, 39].

2.5 Statistical analysis

All statistical analyses were performed with IBM SPSS v. 23 and GraphPad Prism 5. The average cell count of the three hot spots for each topographic area was calculated and then multiplied with a factor to cells/mm² for each slide and each region. Separately for each marker and each topographic area of counting, the counts of $CD3^+$, $CD8^+$ and $PD\text{-}1^+$ cells were classified in low and high by the cut-off related to the mean. Normal distribution of cell counts was determined using the Kolmogorov-Smirnov-test. Because variables were not normally distributed, the Mann-Whitney-U-Test was used to compare LM and LuM. Correlation of clinical-pathological parameters and lymphocyte counts was performed with the two-tailed Fisher's exact test. Adjuvant chemotherapy was defined as treatment of the primary tumor after its removal. Neoadjuvant chemotherapy was defined as treatment of the metastasis before surgical resection. A p-value of ≤ 0.05 was considered significant.

3. Results

3.1 Patient characteristics

A total of 68 advanced colorectal cancer patients diagnosed with liver metastasis (n=53) or lung metastasis (n=15) were included in this study. Most of the patients were initially diagnosed in an advanced UICC stage. Thus, the majority of patients received adjuvant oxaliplatin-based first line chemotherapy. Patients with a KRAS wildtype tumor more frequently developed LM (27 of 38, 71.05%) while a mutated KRAS status was often associated with LuM (6 of 9, 66.67%). Patient characteristics are summarized in detail in Table 1.

Parameters	**Location of Metastasis**					
	All Metastases		**Liver Metastases**		**Lung Metastases**	
	n	%	n	%	n	%
Patient related						
Sex						
Male	46	67.65	34	64.15	12	80
Female	22	32.35	19	35.85	3	20
Age (Years)						
Mean	63		64		59	
Median	63		64		62	
Range	30-89		30-89		37-74	
Primary Tumor Related						
Adjuvant Chemotherapy						
Yes	42	61.76	32	60.38	10	66.67
No	26	38.24	21	39.62	5	33.33

KRAS status						
Wildtype	30	63.83	27	71.05	3	33.33
Mutated	17	36.17	11	28.95	6	66.67
Missing	21		15		6	
Metastasis Related						
Grading						
G1/G2	50	79.37	39	81.25	11	73.33
G3	13	20.63	9	18.75	4	26.67
Missing	5		5		0	
Number of Metastases						
1	26	38.24	19	35.85	7	46.67
> 1	42	61.76	34	64.15	8	53.33
Diameter of the Largest Metastasis (cm)						
Mean	3.84		4.29		2.25	
Median	3.25		3.5		1.8	
Range	0.9-21.7		1.3-21.7		0.9-3.3	
Type of Metastasis						
Synchronous	35	51.47	35	66.04	0	0
Metachronous	33	48.53	18	33.6	15	100
R-Status						
R0	51	75	39	73.58	12	80
R1/R2	17	25	14	26.42	3	20
Distinction of Metastasis						
Unilobular	28	41.18	23	43.4	5	33.33
Bi-/Multilobular	40	58.82	30	56.6	10	66.67
Anatomical Site						
Left Sided	14	20.59	7	13.21	7	46.67
Right Sided	23	33.82	15	28.3	8	53.33
Both Sided	31	45.59	31	58.49		
Neoadjuvant Chemotherapy						
Yes	31	45.59	23	43.4	8	53.33
No	37	54.41	30	56.6	7	46.67

Table 1: Patient characteristics. n, number of patients.

3.2 Immune phenotype compared in colorectal liver and lung metastases

Both colorectal liver and lung metastases showed the strongest immune cell infiltrate at the invasive margin followed by the stroma. In contrast, the intratumoral fraction of immune cells was sparse. This finding was observed for $CD3^+$, $CD8^+$ and $PD\text{-}1^+$ immune cells. In all three topographic locations $CD3^+$ cells represented the highest number of leukocytes, followed by $CD8^+$ cells and $PD\text{-}1^+$ cells (Table 2, Figure 2).

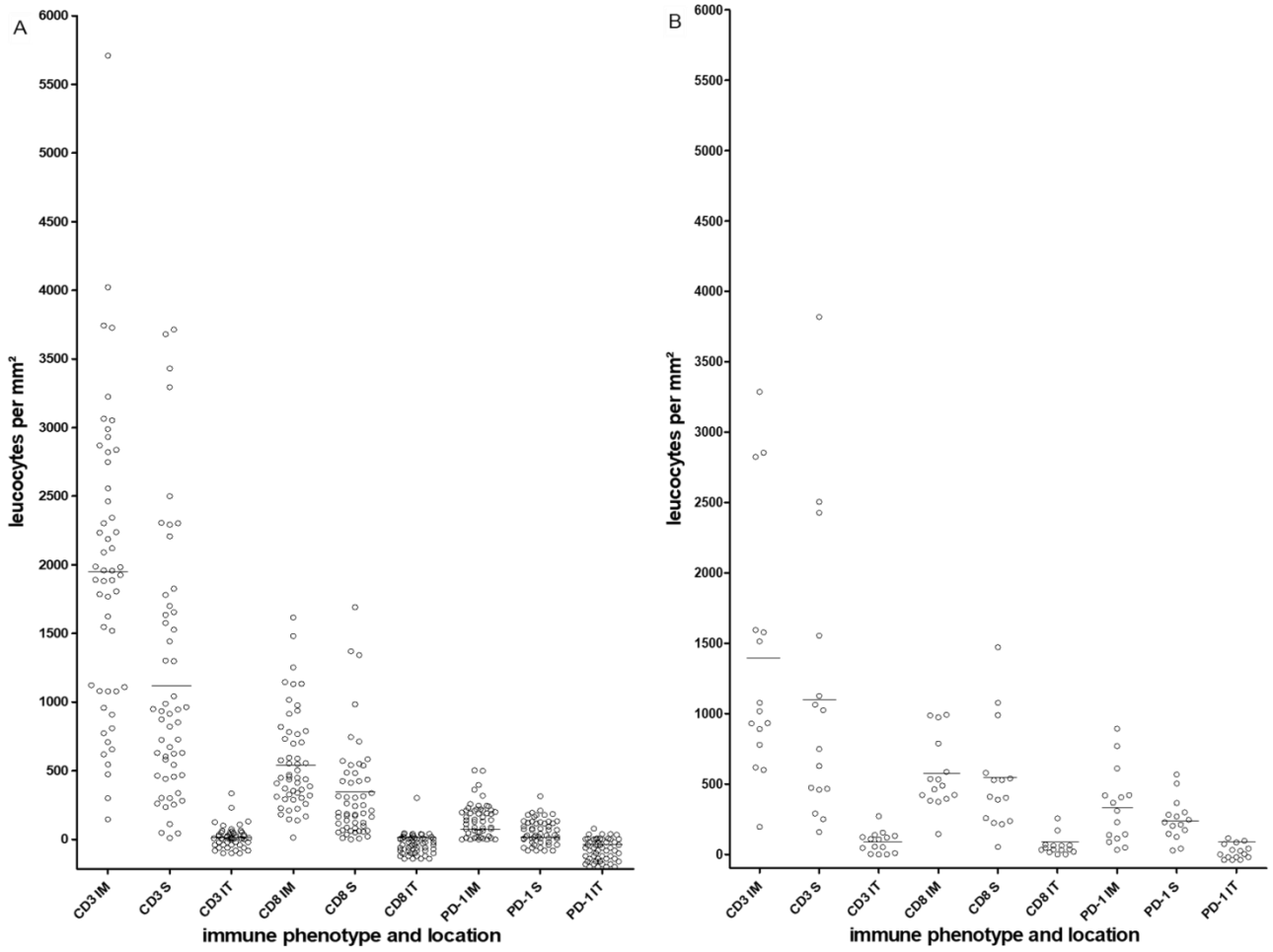

Figure 2: Heterogeneous distribution of $CD3^+$, $CD8^+$ and $PD\text{-}1^+$ cells at three topographical locations. A, liver metastases; B, lung metastases; bars represent the cut-offs (Means). Each open circle represents an individual metastatic lesion. IM, invasion margin; S, stromal; IT, intratumoral.

Comparison between liver and lung metastases revealed significant differences in the immune contexture. Lung metastases were characterised by a stronger infiltrate of $CD3^+$ IT (p=0.005), $CD8^+$ S (p=0.021), $CD8^+$ IT (p=0.001), $PD\text{-}1^+$ IM (p=0.007), $PD\text{-}1^+$ S (p=0.001) and $PD\text{-}1^+$ IT (p=0.01). In contrast liver metastases showed a stronger $CD3^+$ IM infiltrate (p=0.037) (Table 2).

		liver metastasis			lung metastasis			p-values*
Immuno Phenotype	**Location**	**n Positive/ n Analyzed**	**%**	**Mean Cell Counts (SD)**	**n Positive/ n Analyzed**	**%**	**Mean Cell Counts (SD)**	**Liver Vs. Lung**
CD3	**IM#**	52/52	100	1965 (918)	15/15	100	1379 (919)	**0.037**
	S	53/53	100	1142 (948)	15/15	100	1132 (1040)	0.923
	IT	36/53	67.9	35 (61)	13/15	86.7	87 (75)	**0.005**
CD8	**IM#**	52/52	100	558 (361)	15/15	100	565 (255)	0.539
	S	53/53	100	331 (357)	15/15	100	526 (381)	**0.021**
	IT	23/53	43.4	13 (43)	13/15	86.7	60 (68)	**0.001**
PD-1	**IM#**	47/52	90.4	144 (124)	15/15	100	332 (263)	**0.007**
	S	40/53	75.5	69 (74)	15/15	100	245 (149)	**0.001**
	IT	13/53	24.5	5 (14)	8/15	53.3	31 (41)	**0.01**

All counting performed in different topographical areas; IM, invasion margin; S, stromal; IT, intratumoral; *, Mann-Whitney-U-test was used; #, in one case the IM was not defined; n, number of samples; SD, standard deviation is given in parentheses.

Table 2: Comparison of the immune phenotypes in colorectal liver and lung metastases.

3.3 Immune infiltrate in metastatic lesions and clinicopathological parameters

The immune infiltrate in liver and lung metastases was correlated with patient related characteristics, treatment related parameters after surgery of the primary tumor and metastasis related clinical pathological parameters. All results are documented in detail in Supplementary Tables S1a-3c.

Chemotherapy, which was performed after surgery of the primary tumor, was found to have a significant modulating impact on the immune infiltrate in liver metastases. A high fraction of $CD3^+$ cells at the liver invasion front was detected after adjuvant chemotherapy ($CD3^+$ IM high: 19 out of 31, 61.29%). In contrast, in chemo-naive liver metastases the number of $CD3^+$ cells was low ($CD3^+$ IM low: 16 out of 21, 76.19%; p=0.011). Similar, $CD8^+$ cells were enriched at the invasion front after first-line chemotherapy ($CD8^+$ IM high: 16 out of 30, 53.33%), but were low if no chemotherapy was performed ($CD8^+$ IM low: 18 out of 22, 81.81%; p=0.02). The increase of the immune infiltrate was independent of the type of adjuvant drug treatment. No difference was found between oxaliplatin-based, irinotecan-based and antibody-based treatment in this small cohort. Interestingly, all but two (eight of ten cases, 80%) metachronous liver metastases, which received an adjuvant treatment, revealed a high $CD3^+$ infiltrate at the IM, despite a long progression free interval (mean 24.8 months, range >6 to 77 months).

The treatment decision factor KRAS was found to correlate with the extent of the $CD8^+$ IT infiltrate. Liver metastases originating from KRAS wildtype primary tumors often exposed a high $CD8^+$ infiltrate within the tumor nests

(CD8$^+$ IT high: 9 out of 27, 33.33%). Conversely, all KRAS mutated tumors (n=11) showed a low density of CD8$^+$ IT cells (p=0.038).

Correlation with metastasis related clinical pathological parameters revealed a significant association between the extent of the immune cell infiltrate and the tumor burden. Unilobular liver metastases showed a high CD8$^+$ IT infiltrate (CD8$^+$ IT high: 9 out of 23, 39.13%), while in liver metastases involving both lobes the CD8$^+$ IT infiltrate was low (CD8$^+$ IT low: 27 out of 30, 90%; p=0.019). Similarly, liver metastases with a maximum of two affected segments (CD8$^+$ IT high: 7 out of 17, 41.18%) were characterized by a strong CD8$^+$ IT infiltrate. Conversely, the number of CD8$^+$ IT cells in liver metastases with more than two segments involved was low (CD8$^+$ IT low: 31 out of 36, 86.11%; p=0.038).

In the small cohort of metachronous lung metastases a significant correlation was found between right-sided lung metastasis and a high CD8$^+$ S infiltrate (CD8$^+$ S high: 6 out of 8, 75%). Left-sided lung metastases on the other hand showed a low fraction of CD8$^+$ S cells (CD8$^+$ S low: 6 out of 7, 85.71%; p=0.041). Sidedness dependency was also observed for the PD-1 infiltrate. Right-sided lung metastases showed a high fraction of PD-1$^+$ cells at the invasive margin, which stood in contrast to left-sided lesions (p=0.041). In addition, PD-1$^+$ cell density at the invasive margin of lung metastases was high following neoadjuvant chemotherapy, which differed from the low PD-1$^+$ infiltrate in lung metastasis without previous treatment (p=0.041).

3.4 PD-L1 expression in liver and lung metastasis

High PD-L1 expression was found in 24 out of 53 (45.28%) liver metastases and in 13 out of 15 (86.67%) lung metastases. Strong PD-L1 expression in liver metastases was accompanied by a strong T cell infiltrate at the invasive margin (CD3$^+$ IM, p=0.05; CD8$^+$ IM, p=0.002; PD-1$^+$ IM, p=0.025), in the stroma (PD-1$^+$ S, p=0.014) and the intratumoral area (CD3$^+$ IT, p=0.002; CD8$^+$ IT, p=0.024; PD-1$^+$ IT, p=0.017; Supplementary Table S4). Liver metastases originating from KRAS wildtype primary CRC were identified PD-L1 positive (13 out of 27, 48.15%), while liver metastases with a KRAS mutated phenotype showed no PD-L1 expression (10 out of 11, 90.91%; p=0.03). In addition, high PD-L1 expression correlated with limited tumor load (1 liver metastases involved, p=0.02; unilobular liver metastasis, p=0.014; ≤2 liver segments affected, p=0.018). After neoadjuvant chemotherapy a reduced PD-L1 expression was observed in liver metastases (17 out of 23, 73.91%), whereas liver metastases without previous chemotherapy were characterized by a high PD-L1 expression (18 out of 30, 60%; p=0.025). No significant associations were found in the small cohort of lung metastases. The results of all correlations are summarized in Supplementary Table S5.

4. Discussion

The clinical-pathological relevance of the immune phenotype and the topographic distribution of the intrametastatic immune infiltrate was immunohistochemically analyzed in 53 liver metastases and 15 lung metastases of CRC. In both metastatic locations CD3$^+$, CD8$^+$ and PD-1$^+$ lymphocytes were detected in a high fraction at the invasive margin, and to a lesser extend in the stromal area. In contrast, intratumoral immune cells were rare. Correlation with routine clinical pathological factors identified a significant accumulation of CD3$^+$ and CD8$^+$ T-cells at the invasive margin of liver metastases after adjuvant chemotherapy. High density values of CD3$^+$ cells and CD8$^+$ cells were found independent of the

substance class used in the adjuvant treatment. Chemotherapy can provide broad-acting immune stimulating effects such as T-cell priming and recruitment [40]. Indeed, in the neoadjuvant setting oxaliplatin-based and anti-EGFR-based treatments strongly enhanced the immune infiltrate in liver metastatic lesions [30, 41]. These results indicate that standard of care therapy might induce neo-antigen expression elevating cancer immunogenicity [42]. Considering the large number of deviations in the clinical chemotherapeutic settings, the correlation between the extent of drug treatment and the extend of the $CD3^+$ and $CD8^+$ infiltrate needs to be further analyzed in preclinical models. In addition, chemotherapy might result at least in part in a less immune suppressive microenvironment allowing surrounding T-cells to infiltrate [26]. However, successful immunotherapy, for example treatment with bispecific antibodies [43], checkpoint-inhibitors [44] or CAR T-cells [45, 46] require immediate proximity between cancer cells and leukocytes. Therefore re-direction of the leukocytes from the invasive margin into the tumor nests is necessary and might be stimulated by additional therapeutics such as GM-CSF or CCL5 [47-49].

Correlation between the immunological factors and the extent of the liver metastatic disease revealed that high intratumoral infiltrates of $CD8^+$ cells and a high PD-L1 expression were more frequently observed in patients diagnosed with restricted liver metastases reflecting less aggressive metastatic behaviour. This finding suggests that $CD8^+$ cells might control tumor spread [50].

Furthermore, a subgroup of liver metastases originating from KRAS wildtype cancers was identified strongly PD-L1 positive. Simultaneous expression of two drugable targets suggests a biomarker driven patient stratification for dual inhibition combining a checkpoint-inhibitor with an anti-EGFR inhibitor. Indeed, there are currently several ongoing trials, namely the AVETUX study (NCT03174405) and CAVE study (EudraCT: 2017-004392-32) combining Cetuximab and Avelumab treatment. These significant correlations suggest adjuvant chemotherapy, KRAS wildtype and limited tumor burden as new stratification markers for colorectal liver metastasis characterized by a strong leukocyte infiltrate.

In addition to the liver metastases, immune phenotyping was performed in a small cohort of lung metastases. Significant differences in the immune contexture were identified between liver and lung metastases. Lung metastases revealed a significant stronger infiltrate of CD3, CD8 and PD-1 positive cells in both, the stromal and intratumoral compartment. This finding shows that lung metastases can be an attractive target for immune therapy.

In the present study, right-sided lung metastases showed a higher $CD8^+$ infiltrate in the stroma and $PD\text{-}1^+$ infiltrate at the invasion margin compared to left-sided lung metastases. Interestingly, it is known that patients with right-sided lung metastases have a better prognosis [51]. These results suggest immunological differences depending on the anatomical site of metastatic lesions similar as published for right-sided and left-sided primary colorectal cancer [52-55].

Correlation with therapeutic variables revealed a significant correlation between performance of neoadjuvant second-line chemotherapy and a high PD-1 infiltrate at the invasion margin. This finding supports chemotherapy as a precondition for immunotherapy not only in liver metastases but also in lung metastases of colorectal cancer. In contrast to LM, sidedness of metastasis and neoadjuvant treatment were identified as additional selection parameters for immunotherapy of LuM.

Moreover, lung metastases were observed more frequently PD-L1 positive compared to liver metastases. This finding supports treatment of colorectal lung metastasis with checkpoint inhibitors similar as reported for the standard therapy of non-small-cell lung cancer. The biological differences reported for the first time between liver and lung metastases might result in different treatment strategies of the CRC metastatic lesions. However, the results obtained in the present pilot study need to be confirmed in an enlarged cohort.

5. Conclusion

In conclusion, this pilot study identified differences of the immune contexture between liver and lung metastases of colorectal cancer. Most important, lung metastases revealed higher counts of intratumoral $CD3^+$, $CD8^+$ and $PD\text{-}1^+$ cells and a more frequent PD-L1 expression. This indicates that lung metastases are appropriate for immunotherapy. Routine clinical pathological parameters were identified as patient stratification markers for immunotherapy, i.e. KRAS status, tumor burden in liver metastases, sidedness of lung metastases and previous chemotherapy in both metastatic lesions. Thus, in addition to the known biological indicators, namely the MSI/MSS status, the PD-L1 status and the extent of lymphocyte infiltration [55], these clinical factors are suggested to be involved in precision immunotherapy. Based on these results, new treatment strategies can be proposed, specifically combination schemes of immunotherapy with EGFR inhibition and sequential treatment of chemotherapy with subsequent checkpoint inhibition.

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Conflict of Interest Disclosure Statement

The authors declare no potential conflicts of interest.

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Supplementary: All statistic data are documented in the supplementary tables S1-S5

Table S1a: CD3 infiltrate at the invasion margin in liver and lung metastases correlated with clinicopathological parameters

clinico pathological parameters		CD3 IM in liver metastases # N§	Low€	High€	p-value*	CD3 IM in lung metastases N§	Low€	High€	p-value*
patient related									
gender	male	52	19	14	0.568	15	8	4	0.525
	female		9	10			1	2	
Age	≤ Mean&	52	14	14	0.588	15	4	2	1.0
	> Mean		14	10			5	4	
post-surgery treatment related $									
adjuvant chemotherapy	yes	52	12	19	**0.011**	15	6	4	1.0
received	no		16	5			3	2	
KRAS status	wildtype	37	14	13	0.725	9	3	0	0.5
	mutated		6	4			4	2	
metastases related $									
Grading	G1/G2	47	22	16	0.27	15	5	6	0.103
	G3		3	6			4	0	
number of metastases	1	52	11	8	0.775	15	3	4	0.315
	>1		17	16			6	2	
diameter of largest metastases	≤ Mean&	52	17	15	1.0	15	7	5	1.0
	> Mean		11	9			2	1	
type of metastases	synchronous	52	19	15	0.774	all metachronous			
	metachronous		9	9					
R-status	R0	52	21	18	1.0	15	8	4	0.525
	R1/R2		7	6			1	2	
distinction of metastases	unilobular	52	12	11	1.0	15	3	2	1.0
	bi-/multilobular		16	13			6	4	
location in the metastatic organ	left	22	3	4	1.0	15	5	2	0.608
	right		8	7			4	4	
amount of segments recorded	≤ 2	52	8	9	0.562	no segments recorded			
	> 2		20	15					
neoadjuvant chemotherapy	yes	52	15	7	0.096	15	5	3	1.0
received	no		13	17			4	3	

CD3 IM, CD3 infiltrate at the invasion margin; *, Fisher-exact-test (two-tailed) was used; €, definitions for low and high are given in Table 2; #, in one case the IM was not definable; &, mean values are given in Table 1; §, number of cases analysed; $, clinicopathological parameters were not given for all patients

Table S1b: CD3 infiltrate in stromal of liver and lung metastases correlated with clinicopathological parameters

clinico pathological parameters		CD3 S in liver metastases N§	Low€	High€	p-value*	CD3 S in lung metastases N§	Low€	High€	p-value*
patient related									
gender	male	53	21	13	0.768	15	8	4	0.516
	female		13	6			3	0	
Age	≤ Mean&	53	19	9	0.58	15	4	2	1.0
	> Mean		15	10			7	2	
post-surgery treatment related $									
adjuvant chemotherapy	yes	53	20	12	1.0	15	8	2	0.56
received	no		14	7			3	2	
KRAS status	wildtype	38	16	11	0.488	all CD3 S low			
	mutated		8	3					
metastases related$									
Grading$	G1/G2	48	26	13	0.265	15	7	4	0.516
	G3		4	5			4	0	
number of metastases	1	53	13	6	0.768	15	5	2	1.0
	>1		21	13			6	2	
diameter of largest metastases	≤ Mean&	53	22	10	0.559	15	8	4	0.516
	> Mean		12	9			3	0	
type of metastases	synchronous	53	21	14	0.547	all metachronous			
	metachronous		13	5					
R-status	R0	53	24	15	0.746	15	10	2	0.154
	R1/R2		10	4			1	2	
distinction of metastases	unilobular	53	13	10	0.391	15	5	0	0.231
	bi-/multilobular		21	9			6	4	
location in the metastatic organ	left	22	4	3	1.0	15	6	1	0.569
	right		9	6			5	3	
amount of segments recorded	≤ 2	53	10	7	0.76	no segments recorded			
	> 2		24	12					
neoadjuvant chemotherapy	yes	53	14	9	0.775	15	5	3	0.569
received	no		20	10			6	1	

CD3 S, CD3 infiltrate in the stromal area; *, Fisher-exact-test (two-tailed) was used; €, definitions for low and high are given in Table 2; &, mean values are given in Table 1; §, number of cases analyzed; $, clinicopathological parameters were not given for all patients

Table S1c: CD3 infiltrate intratumoral of liver and lung metastases correlated with clinicopathological parameters

clinico pathological parameters		CD3 IT in liver metastases N§	Low€	High€	p-value*	CD3 IT in lung metastases N§	Low€	High€	p-value*
patient related									
gender	male	53	26	8	0.351	15	6	6	1.0
	female		12	7			1	2	
Age	≤ Mean&	53	20	8	1.0	15	4	2	0.315
	> Mean		18	7			3	6	
post-surgery treatment related $									
adjuvant chemotherapy	yes	53	21	11	0.351	15	4	6	0.608
received	no		17	4			3	2	
KRAS status	wildtype	38	18	9	0.452	9	2	1	1.0
	mutated		9	2			3	3	
metastases related$									
Grading	G1/G2	48	30	9	0.671	15	4	7	0.282
	G3		6	3			3	1	
number of metastases	1	53	13	6	0.756	15	3	4	1.0
	>1		25	9			4	4	
diameter of largest metastases	≤ Mean&	53	21	11	0.351	15	5	7	0.569
	> Mean		17	4			2	1	
type of metastases	synchronous	53	24	11	0.539	all metachronous			
	metachronous		14	4					
R-status	R0	53	28	11	1.0	15	6	6	1.0
	R1/R2		10	4			1	2	
distinction of metastases	unilobular	53	16	7	0.769	15	2	3	0.608
	bi-/multilobular		22	8			4	6	
location in the metastatic organ	left	22	5	2	1.0	15	4	3	1.0
	right		11	4			4	4	
amount of segments recorded	≤ 2	53	11	6	0.52	no segments recorded			
	> 2		27	9					
neoadjuvant chemotherapy	yes	53	19	4	0.14	15	4	4	1.0
received	no		19	11			3	4	

CD3 IT, CD3 infiltrate intratumoral; *, Fisher-exact-test (two-tailed) was used; €, definitions for low and high are given in Table 2; &, mean values are given in Table 1; §, number of cases analyzed; $, clinicopathological parameters were not given for all patients

Table S2a: CD8 infiltrate at the invasion margin in liver and lung metastases correlated with clinicopathological parameters

clinico pathological parameters		CD8 IM in liver metastases[#] N[§]	Low[€]	High[€]	p-value*	CD8 IM in lung metastases N[§]	Low[€]	High[€]	p-value*
patient related									
gender	male	52	21	12	0.771	15	7	5	0.505
	female		11	8			3	0	
Age	≤ Mean[&]	52	16	12	0.573	15	5	1	0.58
	> Mean		16	8			5	4	
post-surgery treatment related [$]									
adjuvant chemotherapy	yes	52	14	16	**0.020**	15	7	3	1.0
received	no		18	4			3	2	
KRAS status	wildtype	37	15	12	0.26	9	3	0	0.5
	mutated		8	2			4	2	
metastases related[$]									
Grading	G1/G2	47	23	15	1.0	15	7	4	1.0
	G3		6	3			3	1	
number of metastases	1	52	11	8	0.771	15	5	2	1.0
	>1		21	12			5	3	
diameter of largest metatstasis	≤ Mean[&]	52	17	15	0.149	15	8	4	1.0
	> Mean		15	5			2	1	
type of metastases	synchronous	52	20	14	0.766	all metachronous			
	metachronous		12	6					
R-status	R0	52	24	15	1.0	15	8	4	1.0
	R1/R2		8	5			2	1	
distinction of metastases	unilobular	52	11	12	0.09	15	4	1	0.6
	bi-/mulilobular		21	8			6	4	
location in metastatic organ	left	22	4	3	0.652	15	6	1	0.282
	right		6	9			4	4	
amount of segments recorded	≤ 2	52	9	8	0.544	no segments recorded			
	> 2		23	12					
neoadjuvant chemotherapy	yes	52	15	7	0.565	15	5	3	1.0
received	no		17	13			5	2	

CD8 IM, CD8 infiltrate at the invasion margin; *, Fisher-exact-test (two-tailed) was used; €, definitions for low and high are given in Table 2; #, in one case the IM was not definable; &, mean values are given in Table 1; §, number of cases analyzed; $, clinicopathological parameters were not given for all patients

Table S2b: CD8 infiltrate in stromal of liver and lung metastases correlated with clinicopathological parameters

clinico pathological parameters		CD8 S in liver metastases N§	Low€	High€	p-value*	CD8 S in lung metastases N§	Low€	High€	p-value*
patient related									
gender	male	53	21	13	0.547	15	6	6	1.0
	female		14	5			2	1	
Age	≤ Mean&	53	16	12	0.245	15	2	4	0.315
	> Mean		19	6			6	3	
post-surgery treatment related$									
adjuvant chemotherapy	yes	53	18	14	0.081	15	5	5	1.0
received	no		17	4			3	2	
KRAS status	wildtype	38	16	11	1.0	9	1	2	0.226
	mutated		7	4			5	1	
metastases related$									
Grading	G1/G2	48	27	12	0.247	15	5	6	0.569
	G3		4	5			3	1	
number of metastases	1	53	14	5	0.547	15	5	2	0.315
	>1		21	13			3	5	
diameter of largest metastases	≤ Mean&	53	21	11	1.0	15	5	7	0.2
	> Mean		14	7			3	0	
type of metastases	synchronous	53	20	15	0.072	all metachronous			
	metachronous		15	3					
R-status	R0	53	26	13	1.0	15	7	5	0.569
	R1/R2		9	5			1	2	
distinction of metastases	unilobular	53	14	9	0.565	15	3	2	1.0
	bi-/multilobular		21	9			5	5	
location in metastatic organ	left	22	4	3	1.0	15	6	1	**0.041**
	right		9	6			2	6	
amount of segments recorded	≤ 2	53	11	6	1.0	no segments recorded			
	> 2		24	12					
neoadjuvant chemotherapy	yes	53	13	10	0.249	15	5	3	0.315
received	no		22	8			5	2	

CD8 S, CD8 infiltrate in the stromal area; *, Fisher-exact-test (two-tailed) was used; €, definitions for low and high are given in Table 2; &, mean values are given in Table 1; §, number of cases analysed; $, clinicopathological parameters were not given for all patients

Table S2c: CD8 infiltrate intratumoral of liver and lung metastases correlated with clinicopathological parameters

clinico pathological parameters		CD8 IT in liver metastases N§	Low€	High€	p-value*	CD8 IT in lung metastases N§	Low€	High€	p-value*
patient related									
gender	male	53	29	5	0.09	15	8	4	0.525
	female		12	7			1	2	
Age	≤ Mean&	53	20	8	0.337	15	4	2	1.0
	> Mean		21	4			5	4	
post-surgery treatment related$									
adjuvant chemotherapy	yes	53	22	10	0.095	15	6	4	1.0
received	no		19	2			3	2	
KRAS status	wildtype	38	18	9	**0.038**	9	2	1	1.0
	mutated		11	0			5	1	
metastases related$									
Grading	G1/G2	48	30	9	1.0	15	6	5	0.604
	G3		7	2			3	1	
number of metastases	1	53	13	6	0.311	15	5	2	0.608
	>1		28	6			4	4	
diameter of largest metastases	≤ Mean&	53	23	9	0.323	15	7	5	1.0
	> Mean		18	3			2	1	
type of metastases	synchronous	53	26	9	0.73	all metachronous			
	metachronous		15	3					
R-status	R0	53	28	11	0.148	15	8	4	0.525
	R1/R2		13	1			1	2	
distinction of metastases	unilobular	53	14	9	**0.019**	15	3	2	1.0
	bi-/multilobular		27	3			6	4	
location in metastatic organ	left	22	3	4	0.343	15	5	2	0.608
	right		11	4			4	4	
amount of segments recorded	≤ 2	53	10	7	**0.038**	no segments recorded			
	> 2		31	5					
neoadjuvant chemotherapy	yes	53	19	4	0.519	15	4	4	0.608
received	no		22	8			5	2	

CD8 IT, CD8 infiltrate intratumoral; *, Fisher-exact-test (two-tailed) was used; €, definitions for low and high are given in Table 2; &, mean values are given in Table 1; §, number of cases analysed; $, clinicopathological parameters were not given for all patients

Table S3a: PD-1 infiltrate at the invasion margin in liver and lung metastases correlated with clinicopathological parameters

clinico pathological parameters		PD-1 IM in liver metastases[#] N[§]	Low[€]	High[€]	p-values*	PD-1 IM in lung metastases N[§]	Low[€]	High[€]	p-values*
patient related									
gender	male	52	18	15	0.774	15	6	6	1.0
	female		9	10			2	1	
Age	≤ Mean[&]	52	15	12	1.0	15	2	4	0.315
	> Mean		13	12			6	3	
post-surgery treatment related[$]									
adjuvant chemotherapay	yes	52	14	16	0.413	15	6	4	0.608
received	no		13	9			2	3	
KRAS status	wildtype	37	14	13	0.725	9	2	1	1.0
	mutated		6	4			4	2	
metastases related[$]									
Grading	G1/G2	47	22	16	0.270	15	4	7	0.077
	G3		3	6			4	0	
number of metastases	1	52	9	10	0.774	15	5	2	0.315
	>1		18	15			3	5	
diameter of largest metastases	≤ Mean[&]	52	15	17	0.404	15	5	7	0.2
	> Mean		12	8			3	0	
type of metastases	synchronous	52	18	16	1.0	all metachronous			
	metachronous		9	9					
R-status	R0	52	17	22	0.055	15	8	4	0.077
	R1/R2		10	3			0	3	
distinction of metastases	unilobular	52	11	12	0.78	15	4	1	0.282
	bi-/multilobular		16	13			6	4	
location in the metastatic organ	left	22	6	1	0.063	15	6	1	**0.041**
	right		5	10			2	6	
amount of segments recorded	≤ 2	52	7	10	0.378	no segments recorded			
	> 2		20	15					
neoadjuvant chemotherapy	yes	52	13	9	0.413	15	2	6	**0.041**
received	no		14	16			6	1	

PD-1 IM, PD-1 infiltrate at the invasion margin; *, Fisher-exact-test (two-tailed) was used; €, definitions for low and high are given in Table 2; #, in one case the IM was not definable; &, mean values are given in Table 1; §, number of cases analysed; $, clinicopathological parameters were not given for all patients

Table S3b: PD-1 infiltrate in stromal of liver and lung metastases correlated with clinicopathological parameters

		PD-1 S in liver metastases				PD-1 S in lung metastases			
clinico pathological parameters		N§	Low€	High€	p-values*	N§	Low€	High€	p-values*
patient related									
gender	male	53	16	18	0.390	15	5	7	0.2
	female		12	7			3	0	
Age	≤ Mean&	53	15	13	1.0	15	3	3	1.0
	> Mean		13	12			5	4	
post-surgery treatment related$									
adjuvant chemotherapy	yes	53	14	18	0.160	15	5	5	1.0
received	no		14	7			3	2	
KRAS status	wildtype	38	16	11	0.491	9	2	1	1.0
	mutated		5	6			4	2	
metastases related$									
Grading	G1/G2	48	22	17	0.279	15	4	7	0.077
	G3		3	6			4	0	
number of metastases	1	53	9	10	0.580	15	4	3	1.0
	>1		19	15			4	4	
diameter of largest metastases	≤ Mean&	53	16	16	0.779	15	5	7	0.2
	> Mean		12	9			3	0	
type of metastases	synchronous	53	20	15	0.402	all metachronous			
	metachronous		8	10					
R-status	R0	53	20	19	0.763	15	7	5	0.569
	R1/R2		8	6			1	2	
distinction of metastases	unilobulae	53	11	12	0.586	15	3	2	1.0
	bi-/multilobular		17	13			5	5	
location in metastatic organ	left	22	4	3	1.0	15	5	2	0.315
	right		7	8			3	5	
amount of segments recorded	≤ 2	53	7	10	0.377	no segments recorded			
	> 2		21	15					
neoadjuvant chemotherapy	yes	53	13	10	0.782	15	3	5	0.315
received	no		15	15			5	2	

PD-1 S, PD-1 infiltrate in the stromal area; *, Fisher-exact-test (two-tailed) was used; €, definitions for low and high are given in Table 2; &, mean values are given in Table 1; §, number of cases analysed; $, clinicopathological parameters were not given for all patients

Table S3c: PD-1 infiltrate intratumoral of liver and lung metastases correlated with clinicopathological parameters

clinico pathological parameters		PD-1 IT in liver metastases N§	Low€	High€	p-values*	PD-1 IT in lung metastases N§	Low€	High€	p-values*
patient related									
gender	male	53	30	4	0.436	15	6	6	0.229
	female		15	4			3	0	
Age	≤ Mean&	53	23	5	0.708	15	3	3	0.622
	> Mean		22	3			6	3	
post-surgery treatment related$									
adjuvant chemotherapy	yes	53	26	6	0.455	15	5	5	0.58
received	no		19	2			4	1	
KRAS status	wildtype	38	21	6	0.648	9	2	1	1.0
	mutated		10	1			4	2	
metastases related$									
Grading	G1/G2	48	35	4	0.312	15	6	5	0.604
	G3		7	2			3	1	
number of metastases	1	53	16	3	1.0	15	4	3	1.0
	>1		29	5			5	3	
diameter of largest metastases	≤ Mean&	53	26	6	0.455	15	6	6	0.229
	> Mean		19	2			3	0	
type of metastases	synchronous	53	28	7	0.24	all metachronous			
	metachronous		17	1					
R-status	R0	53	33	6	1.0	15	7	5	1.0
	R1/R2		12	2			2	1	
distinction of metastases	unilobular	53	20	3	1.0	15	2	3	0.329
	bi-/multilobular		25	5			3	7	
location in metastatic organ	left	22	6	1	1.0	15	6	1	0.119
	right		14	1			3	5	
amount of segments recorded	≤ 2	53	14	3	0.701	no segments recorded			
	> 2		31	5					
neoadjuvant chemotherapy	yes	53	21	2	0.441	15	5	3	1.0
received	no		24	6			4	3	

PD-1 IT, PD-1 infiltrate intratumoral; *, Fisher-exact-test (two-tailed) was used; €, definitions for low and high are given in Table 2; &, mean values are given in Table 1; §, number of cases analysed; $, clinicopathological parameters were not given for all patients

Table S4: PD-L1 of liver and lung metastases correlated with the immune phentotypes

		PD-L1 in liver metastases				PD-L1 in lung metastases			
parameters		n#	Low€	High€	p-value*	n#	Low€	High€	p-value*
CD3 IM	low	52	19	9	**0.050**	15	2	7	0.486
	high		9	15			0	6	
CD3 S	low	53	21	13	0.250	15	2	9	1.000
	high		8	11			0	4	
CD3 IT	low	53	26	12	**0.002**	15	1	6	1.000
	high		3	12			1	7	
CD8 IM	low	52	23	9	**0.002**	15	2	8	0.524
	high		5	15			0	5	
CD8 S	low	53	21	14	0.384	15	1	7	1.000
	high		8	10			1	6	
CD8 IT	low	53	26	15	**0.024**	15	1	8	1.000
	high		3	9			1	5	
PD-1 IM	low	52	19	8	**0.025**	15	2	6	0.467
	high		9	16			0	7	
PD-1 S	low	53	20	8	**0.014**	15	2	6	0.467
	high		9	16			0	7	
PD-1 IT	low	53	28	17	**0.017**	15	2	7	0.486
	high		1	7			0	6	

IM, invasion margin; S, stromal; IT, intratumoral; *, Fisher-exact-test (two-tailed) was used; #, number of samples;
€, definition of PD-L1 expression: low, ≤1%; high >1%

Table S5: PD-L1 of liver and lung metastases correlated with clinicopathological parameters

clinico pathological parameters		PD-L1 in liver metastases N[§]	Low[€]	High[€]	p-values*	PD-L1 in lung metastases N[§]	Low[€]	High[€]	p-values*
patient related									
gender	male	53	20	14	0.566	15	1	11	0.371
	female		9	10			1	2	
Age	≤ Mean[&]	53	15	13	1.0	15	1	5	1.0
	> Mean		14	11			1	8	
post-surgery treatment related[$]									
adjuvant chemotherapy	yes	53	16	16	0.416	15	2	8	0.524
received	no		13	8			0	5	
KRAS status	wildtype	38	14	13	**0.03**	9	1	2	1.0
	mutated		10	1			1	5	
metastases related[$]									
Grading	G1/G2	48	23	16	0.477	15	1	10	0.476
	G3		4	5			1	3	
number of metastases	1	53	6	13	**0.02**	15	0	7	0.467
	>1		23	11			2	6	
diameter of largest metastases	≤ Mean[&]	53	17	15	1.0	15	1	11	0.371
	> Mean		12	9			1	2	
type of metastases	synchronous	53	20	15	0.772	all metachronous			
	metachronous		9	9					
R-status	R0	53	22	17	0.76	15	2	10	1.0
	R1/R2		7	7			0	3	
distinction of metastases	unilobular	53	8	15	**0.014**	15	0	5	0.524
	Bi-/multilobular		21	9			2	8	
location in metastatic organ	left	22	4	3	0.343	15	1	6	1.0
	right		4	11			1	7	
amount of segments recorded	≤ 2	53	5	12	**0.018**	no segments recorded			
	> 2		24	12					
neoadjuvant chemotherapy	yes	53	17	6	**0.025**	15	2	6	0.467
received	no		12	18			0	7	

*, Fisher-exact-test (two-tailed) was used; &, mean values are given in Table 1; §, number of cases analysed; $, clinicopathological parameters were not given for all patients; €, definition of PD-L1 expression: low, ≤1%; high >1%

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Article

High Dual Expression of the Biomarkers CD44v6/α2β1 and CD44v6/PD-L1 Indicate Early Recurrence after Colorectal Hepatic Metastasectomy

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Simple Summary: Distant metastasis in colorectal cancer still correlates with poor prognosis, emphasizing the high need for new diagnostic and therapeutic strategies. In the present study, liver and lung metastases revealed profound differences in the expression pattern of metastasis-driving protein biomarkers. This suggests the adaption of the therapy to the biology of the metastatic organ site. High expression of the cell adhesion molecule CD44v6 and high dual expression of CD44v6, combined with the cell adhesion molecules integrin α2β1, as well as the checkpoint inhibitor molecule PD-L1, correlated significantly with early recurrence after hepatectomy, in a substantial number of liver metastatic patients. These findings suggest the need for the implementation of biological risk factors into clinical risk scores, aiming to make the prognosis of the individual patient more precise. Further, dual expression of protein biomarkers that are druggable, such as CD44v6/α2β1 and CD44v6/PD-L1, can identify high-risk patients for targeted therapy that might provide a survival benefit.

Abstract: Considering the biology of CRC, distant metastases might support the identification of high-risk patients for early recurrence and targeted therapy. Expression of a panel of druggable, metastasis-related biomarkers was immunohistochemically analyzed in 53 liver (LM) and 15 lung metastases (LuM) and correlated with survival. Differential expression between LM and LuM was observed for the growth factor receptors IGF1R (LuM 92.3% vs. LM 75.8%, $p = 0.013$), EGFR (LuM 68% vs. LM 41.5%, $p = 0.004$), the cell adhesion molecules CD44v6 (LuM 55.7% vs. LM 34.9%, $p = 0.019$) and α2β1 (LuM 88.3% vs. LM 58.5%, $p = 0.001$) and the check point molecule PD-L1 (LuM 6.1% vs. LM 3.3%, $p = 0.005$). Contrary, expression of HGFR, Hsp90, Muc1, Her2/neu, ERα and PR was comparable in LuM and LM. In the LM cohort ($n = 52$), a high CD44v6 expression was identified as an independent factor of poor prognosis (PFS: HR 2.37, 95% CI 1.18–4.78, $p = 0.016$). High co-expression of CD44v6/α2β1 (HR 4.14, 95% CI 1.65–10.38, $p = 0.002$) and CD44v6/PD-L1 (HR 2.88, 95% CI 1.21–6.85, $p = 0.017$) indicated early recurrence after hepatectomy, in a substantial number of patients (CD44v6/α2β1: 11 (21.15%) patients; CD44v6/PD-L1: 12 (23.1%) patients). Dual expression of druggable protein biomarkers may refine prognostic prediction and stratify high-risk patients for new therapeutic concepts, depending on the metastatic location.

Keywords: colorectal cancer; liver metastases; lung metastases; protein biomarker; dual expression; early recurrence; poor prognosis

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1. Introduction

According to international guidelines [1–3], metastasectomy currently offers the best chance for long-term survival for selected colorectal cancer patients. Additional standard chemotherapy for patients with resectable liver metastases resulted in the prolongation of

disease-free survival (DFS) and progression-free survival (PFS) but revealed no significant improvement in overall survival (OS) [4,5]. In patients with resectable pulmonary metastases, the outcome of peri-operative chemotherapy is inconclusive [6,7]. However, despite curative-intent metastasectomy, more than half of the patients suffer recurrence [8,9]. This highlights the urgent need for the implementation of new strategies to identify high-risk patients suitable for personalized therapy, aiming to improve treatment outcome and survival [10].

Colorectal cancer preferentially metastasizes to the liver, followed by the lung and the peritoneum and, more rarely, in bone, ovary and the brain [11–13]. The metastatic pattern depends on the sidedness of the primary colorectal tumor. Elucidating the underlying mechanisms of the metastatic organotropism, profound molecular differences were observed between right-sided and left-sided CRC cancers. Similarly, the tumor microenvironment seems to have a deep impact on the metastatic site [14]. Indeed, for primary metastatic colorectal cancer, a growing body of molecular data is available, resulting in the continuous development of targeted therapies and improvement in survival [15,16].

Comparative analysis of primary CRC and corresponding metastatic sites revealed maintenance of the main driver mutations in both liver and lung metastases, some of which are approved for CRC therapy, such as RAS, BRAF and MSI [17–19]. In contrast, genomic [20–22], transcriptomic [23] and proteomic [24] profiling identified molecular differences between primary tumor, liver and lung metastases that might have potential therapeutic implications for specific metastatic sites. Moreover, distant metastases in different organs revealed discordant responses to standard chemotherapy [25], all together, supporting the concept of inter- and intratumor heterogeneity, which is one of the key factors in tumor progression, therapeutic resistance, and poor patient outcome.

In the present study, a panel of protein biomarkers was selected, which drive the complex metastatic process of primary colorectal cancer and lead to poor prognosis. In contrast, little information is available on the expression pattern of these prognostic factors in liver and lung metastases. The protein biomarker panel encompassed the growth factor receptors epidermal growth factor receptor (EGF-R) and hepatocyte growth factor receptor (HGF-R) [26], human epidermal growth factor receptor (Her2/neu) [27], insulin-like growth factor 1 receptor (IGF-1R) [28], estrogen receptor alpha (Erα) [29] and progesterone receptor (PR) [30], the cell adhesion molecules CD44v6 [31], Muc1 [32] and integrin α2β1 [33], the chaperone heat shock protein 90 (Hsp90) [34], and the immune checkpoint molecule programmed death ligand 1 (PD-L1) [35]. Interestingly, the protein biomarkers selected are drug targets, for which drugs are already approved or for which clinical trials are ongoing, in primary colorectal cancer or other cancer types. This could open up new options for second and further line treatments in colorectal cancer.

The present study aimed (1) to identify the phenotypic heterogeneity in tumor biology between colorectal liver and lung metastases and (2) to stratify patients with a high risk for early recurrence after hepatic metastasectomy.

2. Materials and Methods

2.1. Patient Cohort

The patient cohort consists of 68 patients with metastatic colorectal cancer, receiving metastasectomy with curative intent at the Department of General, Visceral, and Transplant Surgery, Ludwig-Maximilians-University, Munich, Germany. A liver metastasis (LM, $n = 53$) or a lung metastasis (LuM, $n = 15$) was analyzed from each patient. Double-coded tissues and the corresponding data used in this study were provided by the Biobank of the Department of General, Visceral, and Transplant Surgery, Ludwig-Maximilians-University Munich, Munich, Germany. This Biobank operates under the administration of the Human Tissue and Cell Research (HTCR) Foundation. The framework of HTCR Foundation, which includes obtaining written informed consent from all donors, has been approved by the ethics commission of the Faculty of Medicine at the LMU (approval number 025-12) as well as the Bavarian State Medical Association (approval number 11142) in

Germany. All liver metastases were diagnosed as the first relapse of the individual patient. Lung metastases represented first (n = 3), second (n = 8) and later stage relapse (n = 4). Survival analysis was performed for 52 patients diagnosed with liver metastases. One patient was lost to follow up. Follow-up period of the patient cohort was from December 2010 until February 2018.

2.2. Immunohistochemistry and Evaluation of Biomarker Expression

Fresh tumor samples including adjacent benign reference tissue were collected according to international biobanking standards. After surgery the tumor samples were immediately snap frozen in liquid nitrogen. Serial cryosections (5 µm) were performed and air dried over night at room temperature. Sections were either fixed in acetone, or for the ERα und PR staining in formalin solution (10%). Immunohistochemistry was performed using the standard avidin-biotin-peroxidase complex method [36–38]. Briefly, unspecific Fc receptors were blocked with 10% AB-serum in D-PBS, pH 7.4 for 20 min. Endogenous biotin was blocked using the Avidin-/Biotin-blocking Kit for 15 min. The primary antibodies (Table 1) were incubated for one hour. Some antibodies were detected with the secondary biotinylated antibody (111-065-114; wc 7.0 µg/mL; JacksonImmunoResearch, West Grove, PA, USA for anti-rabbit and 315-065-048; wc 0.75 µg/mL; JacksonImmunoResearch for anti-mouse) for 30 min, followed by the peroxidase-conjugated streptavidin (016-030-084; wc 1.0 µg/mL; affymetrix eBiosciences, Santa Clara, CA, USA) for another 30 min. Other primary antibodies were detected with the amplification Kit ZytoChem Plus (HRP060; Zytomed Systems, Bargteheide, Germany) according to the instructions of the manufacturer (marked in Table 1 with Kit: +). For visualization of the antigen–antibody reaction all slides were developed in a 3-Amino-9-ethylcarbazole solution containing 35% hydrogen peroxide (AEC staining) for eight minutes in darkness. Counterstaining was performed with Mayer's hemalum solution. All incubation steps were performed in a humid chamber at room temperature. Specificity of the staining was controlled by the corresponding isotype controls (Table 1). Cancer cells were visualized by EpCAM and pan-cytokeratin expression.

For the evaluation of biomarker expression, the size of the measurement field was standardized using a normalized grid at 100× magnification (Olympus microscope BX50, Olympus, Hamburg, Germany). The biomarker-positive tumor area was determined in relation to the total tumor area. The percentage of biomarker-positive tumor cells was expressed by semiquantitative estimation in 10% increments. Staining results were evaluated by two independent observers (FW, BM). External monitoring was performed by local pathologists (Institute of Pathology, LMU Munich, Munich, Germany, T. Kirchner) and for Her2/neu expression by J. Rüschoff (Institute of Pathology Nordhessen, Kassel, Germany, Rüschoff) [39].

For some biomarkers standardized cut-off values are given, namely ERα and PR [40], Her2/neu [39,41], Muc1 [42,43], and PD-L1 [36,44]. In the absence of standardized cut-offs for other biomarkers, cut-offs were assessed using the biphasic distribution, which was statistically defined using the mean antigen expression in liver or lung metastases. Biomarker expression below the calculated cut-off was defined as low expression, and biomarker expression above the calculated cut-off was defined as high expression. The same cut-off values were used for single biomarker analysis and the evaluation of dual biomarker expression. In addition to the tumor tissue, antigen expression was evaluated on the adjacent benign liver and lung tissues.

Table 1. Antibody Panel for Immunophenotyping of Colorectal Liver and Lung Metastases.

Biomarker	Antibody/Clone	Species	Isotype	Working Concentration (µg/mL)	Kit	Source
HGF-R	Sp44	rabbit	IgG1	2.12	-	Spring Bioscience/Biomol, Pleasanton, CA, USA
IGF1-R	24–31	mouse	IgG1	4.0	+	Invitrogen, Carlsbad, CA, USA
EGR-R	H11	mouse	IgG1	2.94	-	Dako, Santa Clara, CA, USA
Her2/neu	4B5	rabbit	IgG1	1.5	-	Ventana, Roche, Basel, Switzerland
Erα	ID5	mouse	IgG1	2.5	+	Dako
PR	PgR 636	mouse	IgG1	2.5	+	Dako
Muc1	Ma55.2	mouse	IgG1	0.5	-	Monosan, Uden, The Netherlands
CD44v6	VFF-18	mouse	IgG1	1.0	-	eBioscience Affymetrix
α2β1	BHA2.1	mouse	IgG1	2.5	-	Millipore, Burlington, MA, USA
Hsp90	AC88	mouse	IgG1	10.0	+	Abcam, Cambridge, UK
PD-L1	MIH1	mouse	IgG1	10.0	+	Affymetrix
Positive controls						
Epcam	Ber-EP4	mouse	IgG1	5.0	-	Dako
Pan Cytokeratin	KL-1	mouse	IgG1	0.32	-	Zytomed Systems
isotype controls						
MOPC-21	MOPC-21	mouse	IgG1	5.0	-	Sigma-Aldrich, St. Louis, MO, USA
MOPC-21	MOPC-21	mouse	IgG1	4.0	+	Sigma-Aldrich
MOPC-21	MOPC-21	mouse	IgG1	10.0	+	Sigma-Aldrich
Rabbit mAb	DA1E	rabbit	IgG1	2.12	-	Cell Signaling, Danvers, MA, USA

2.3. Statistical Analysis

All statistical analyses were performed with IBM SPSS v. 23. Mean biomarker expression between liver and lung metastases was compared using the Mann–Whitney U-test. The prognostic impact of single and dual biomarker expression was evaluated using Kaplan–Meier analysis (log rank test, 'pairwise over strata') and multivariate Cox regression analysis (biomarker expression used as 'categorical covariate', 'First' as reference category). OS was defined as the time from metastasectomy until the last follow-up or death of the patient. PFS was defined as the time from metastasectomy until the next progression. A p-value of ≤ 0.05 was considered as significant.

3. Results

3.1. Patient Characteristics

In the present study, 53 liver metastases and 15 lung metastases surgically resected from colorectal cancer patients were analyzed. Men were more frequently affected than women (LM: ratio 1.79:1; LuM: ratio 4:1). Most (66.04%) liver metastases were detected at primary diagnosis (synchronous), whereas all lung metastases were documented at a later time (metachronous). Liver and lung metastases were diagnosed as single organ metastases. However, at the organ site, tumor disease was frequently extensive (number of nodules within the metastatic organ >1; LM: 64.15%, LuM: 53.33%; multilobular involvement; LM: 56.6%, LuM: 66.67%). Still, most patients were resected with curative intent (R0; LM: 73.58%, LuM: 80%). Further, 32 of 53 (60.38%) patients diagnosed with liver metastases received first-line chemotherapy (5-FU as single agent: 34.38%, oxaliplatin-based: 43.75%, irinotecan-based: 15.63%, others: 6.25%) and 23 of 53 (43.40%) received neoadjuvant chemotherapy before liver metastasectomy. Of these, 10 of 15 (66.67%) patients were treated with front line chemotherapy (5-FU as single agent: 10%, oxaliplatin-based: 80%, others: 10%) and 8 of 15 (53.33%) patients received neoadjuvant chemotherapy, right before surgery of the

lung metastasis studied. Complete treatment records were not available for all patients with lung metastases.

Patient characteristics are summarized in detail in Table 2.

Table 2. Patient Characteristics.

Parameters	Liver Metastases		Lung Metastases	
	n	**%**	***n***	**%**
patient related				
sex				
male	34	64.15	12	80.00
female	19	35.85	3	20.00
age (years)				
median	64		62	
mean	64		59	
range	30–89		37–74	
metastasis related				
grading				
G1/G2	39	81.25	11	73.33
G3	9	18.75	4	26.67
missing	5		0	
number of metastases *				
1	19	35.85	7	46.67
>1	34	64.15	8	53.33
diameter of the largest metastases (cm)				
median	3.5		1.8	
mean	4.29		2.25	
range	1.3–21.7		0.9–3.3	
type of metastasis				
synchronous	35	66.04	0	0.00
metachronous	18	33.6	15	100.00
R-status				
R0	39	73.58	12	80.00
R1	14	26.42	3	20.00
distinction of metastasis				
unilobular	23	43.4	5	33.33
multilobular	30	56.6	10	66.67
anatomical site				
left sided	7	13.21	7	46.67
right sided	15	28.30	8	53.33
both sided	31	58.49		
neoadjuvant chemotherapy #				
yes	23	43.40	8	53.33
no	30	56.60	7	46.67
therapy options				
oxaliplatin-based	11	47.83	1	12.5
irinotecan-based	7	30.43	5	62.5
others	5	21.74	2	25.0

n, number of patients; **R-status**, residual status after surgery; *, nodules within the metastatic organ; #, administered directly before metastasectomy.

Survival analysis was performed in the patient cohort with liver metastases but was omitted in patients with lung metastases because of small sample size. Patients diagnosed with multiple (>1) LM had a significantly shorter PFS compared to patients diagnosed with a single liver metastasis (multiple metastases, PFS: 6.5 months; single metastasis, PFS: 10 months; log-rank, $p = 0.014$). Patients with synchronous LM relapsed much faster compared to patients with metachronous LM (synchronous, PFS: 7 months; metachronous, PFS: 16 months; log rank, $p = 0.001$). None of the patient characteristics revealed an impact on OS.

3.2. Differential Biomarker Expression in Colorectal Liver and Lung Metastases

Liver and lung metastases were comparatively analyzed with a panel of metastasis-related protein biomarkers. A differential expression pattern between liver and lung metastases was observed for the growth factor receptors IGF-1R (LuM 92.3% vs. LM 75.8%, $p = 0.013$) and EGF-R (LuM 68% vs. LM 41.5%, $p = 0.004$), showing a significantly higher fraction of positive cancer cells in the lung metastases, respectively. Similar results were obtained for the cell adhesion molecules CD44v6 (LuM 55.7% vs. LM 34.9%, $p = 0.019$) and integrin α2β1 (LuM 88.3% vs. LM 58.5%, $p = 0.001$), as well as for the check point molecule PD-L1 (LuM 6.1% vs. LM 3.3%, $p = 0.005$). In contrast, no significant difference was observed for the growth factor receptor HGF-R and the chaperon molecule Hsp90, both showing a high fraction of positive cancer cells in almost all distant metastases. Conversely, all but one metastatic lesion were found negative for the hormone receptors ERα and PR. One individual liver metastasis demonstrated 30% ERα positive cancer cells. Moreover, in colorectal liver and lung metastases, a minor fraction of the cancer cells were found positive for the cell adhesion molecule Muc1 and growth factor receptor Her2/neu. In fact, only one liver metastasis (60% Her2/neu positive cancer cells) qualified for anti-Her2/neu therapy. The number of biomarker-positive lesions and the means of biomarker expression are given in Table 3. The distribution of biomarker expression is shown for liver and lung metastases (Figure 1).

Table 3. Positivity and Distribution of Biomarkers in Liver and Lung Metastases.

Biomarker	Number of Positive Lesions				Number of Positive Cancer Cells (%)					Number of Positive Lesions above Cut-Offs				
	Liver		Lung		Median			Mean		Liver		Lung		
	n = 53	%	*n* = 15	%	Liver	Lung	*p*-Value	Liver	Lung	Cut Off *	*n* = 53	%	*n* = 15	%
HGF-R	52	98.1	15	100	95	95	0.166	87.7	95.3				n.t.	
IGF-1R	50	94.3	15	100	90	100	**0.013**	75.8	92.3	>80	29	54.7	12	80
EGF-R	45	84.9	15	100	40	70	**0.004**	41.5	68.0	>50	25	47.2	12	80
Her2/neu	19	35.8	8	53.3	0	1	0.575	5.7	1.7	>50	1	1.9	0	0
ERα	1	1.9	0	0	0	0	n.t.	0.6	0	≥1			n.t.	
PR	0	0	0	0	0	0	n.t.	0	0	≥1			n.t.	
Muc1	26	49.1	9	60	0	1	0.614	6.8	5.9	+/−	26	49.1	9	60
CD44v6	45	84.9	15	100	30	60	**0.019**	34.9	55.7	>30	23	43.4	10	66.7
α2β1	46	86.8	15	100	70	90	**0.001**	58.5	88.3	>80	20	37.7	11	73.3
Hsp90	51	96.2	15	100	75	80	0.475	68.7	73.9	>70	26	49.1	9	60
PD-L1	24	45.3	13	86.7	0	1	**0.005**	6.1	3.25	>1	24	45.3	11	73.3

n, number of patients; **n.t.**, not tested; *, calculation of the cut-offs is given in the Materials and Methods Section.

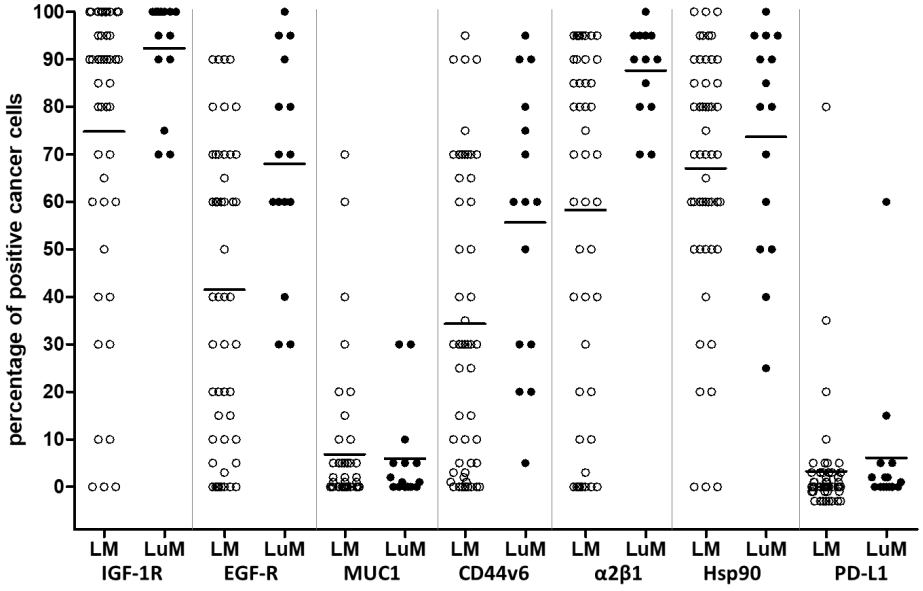

Figure 1. Biomarker Expression Pattern of Liver and Lung Metastases: horizontal bars, Means. Each dot represents a metastatic lesion; empty dots represent liver metastases (**LM**); filled dots represent lung metastases (**LuM**).

Figure 1. Biomarker Expression Pattern of Liver and Lung Metastases: horizontal bars, Means. Each dot represents a metastatic lesion; empty dots represent liver metastases (**LM**); filled dots represent lung metastases (**LuM**).

Biomarker analysis showed most of the benign liver tissues positive for HGF-R, EGF-R, and Hsp90. IGF-1R and PD-L1 were detected in a fraction of benign liver samples (IGF-1R: 11 out of 52, 21.2%; PD-L1: 10 out of 52, 19.2%). Interestingly, benign liver tissue was negative for Muc1, CD44v6 and the integrin α2β1. In contrast, all biomarkers tested were detected on benign lung tissue, although the integrin α2β1 (10 out of 15, 66.6%) and Muc1 (8 out of 15, 53%) were observed on a reduced number of adjacent lung tissues. Data obtained in benign tissue samples are summarized in Table S1. Figure 2 demonstrates the significantly different staining patterns by each biomarker of liver and lung metastases.

3.3. Prognostic Impact of Biomarker Expression in Colorectal Liver Metastases

The prognostic impact of the biomarkers was analyzed in patients with liver metastases. CD44v6, but none of the other biomarkers tested, was identified as an indicator for early recurrence. Liver metastases with a high fraction (>30%, n = 22) of CD44v6+ tumor cells significantly correlated with a shorter (median 7.0 months) PFS compared to LM with a low CD44v6 expression (≤30% CD44v6+ cells, n = 30; median 15.5 months; log rank p = 0.01). Recurrent liver metastases with a high proportion of CD44v6+ cancer cells showed more frequent multi-organ metastases (6 out of 19, 31.58%), compared to liver metastases with a low proportion of CD44v6+ cancer cells (3 out of 22, 13.65%). Almost all multi-organ metastases involved liver and lung, regardless of the extent of CD44v6 expression. Cox regression analysis confirmed the independent prognostic impact of CD44v6 on PFS (Table 4). No significant correlation was found between CD44v6 expression in LM and OS.

Table 4. Multivariate Survival Analysis of CD44v6 Expression in Colorectal liver Metastases.

Variable	Groups	Cox Regression		
		HR	*p*-Value	95% CI
age (median in years)	>64/≤64	1.424	0.357	0.671–3.021
number of metastases *	>1/≤1	1.221	0.572	0.610–2.454
type of metastases	synchronous/metachronous	4.206	**0.004**	1.572–11.254
CD44v6 expression	>30%/≤30%	2.369	**0.016**	1.175–4.777

HR, Hazard ratio; *p*-value was calculated for progression free survival; **CI**, confidence interval; *, nodules within the metastatic organ.

3.4. CD44v6-Related Dual Biomarker Expression in Colorectal Liver Metastases

Co-expression analysis was performed on CD44v6 and the metastasis-related biomarkers. Univariate analysis identified three pairs of highly expressed biomarkers associated with short PFS. Patients with liver metastases with strong expression of CD44v6 and integrin α2β1 showed a shorter mean PFS (3 months) compared to the group with only high expression of CD44v6 (7 months) (Table 5, Figure 3). Multivariate Cox regression analysis identified the combination of a high CD44v6 and a high integrin α2β1 expression (HR: 4.135, 95% CI: 1.648–10.375, p = 0.002) and the combination of a high CD44v6 and a high PD-L1 expression (HR: 2.882, 95% CI: 1.213–6.848, p = 0.017), as independent prognostic factors for short progression-free survival (Table 6). High co-expression was detected in a substantial number of patients; i.e., CD44v6 high (>30% positive tumors cells) combined with integrin α2β1 high (>80% positive tumor cells) in 11 out of 52 (21.15%) patients, CD44v6 high combined with Hsp90 high (>70% positive tumor cells) in 14 out of 52 (26.92%) patients and CD44v6 high combined with PD-L1 high (>1% positive cells) in 12 out of 52 (23.1%) patients.

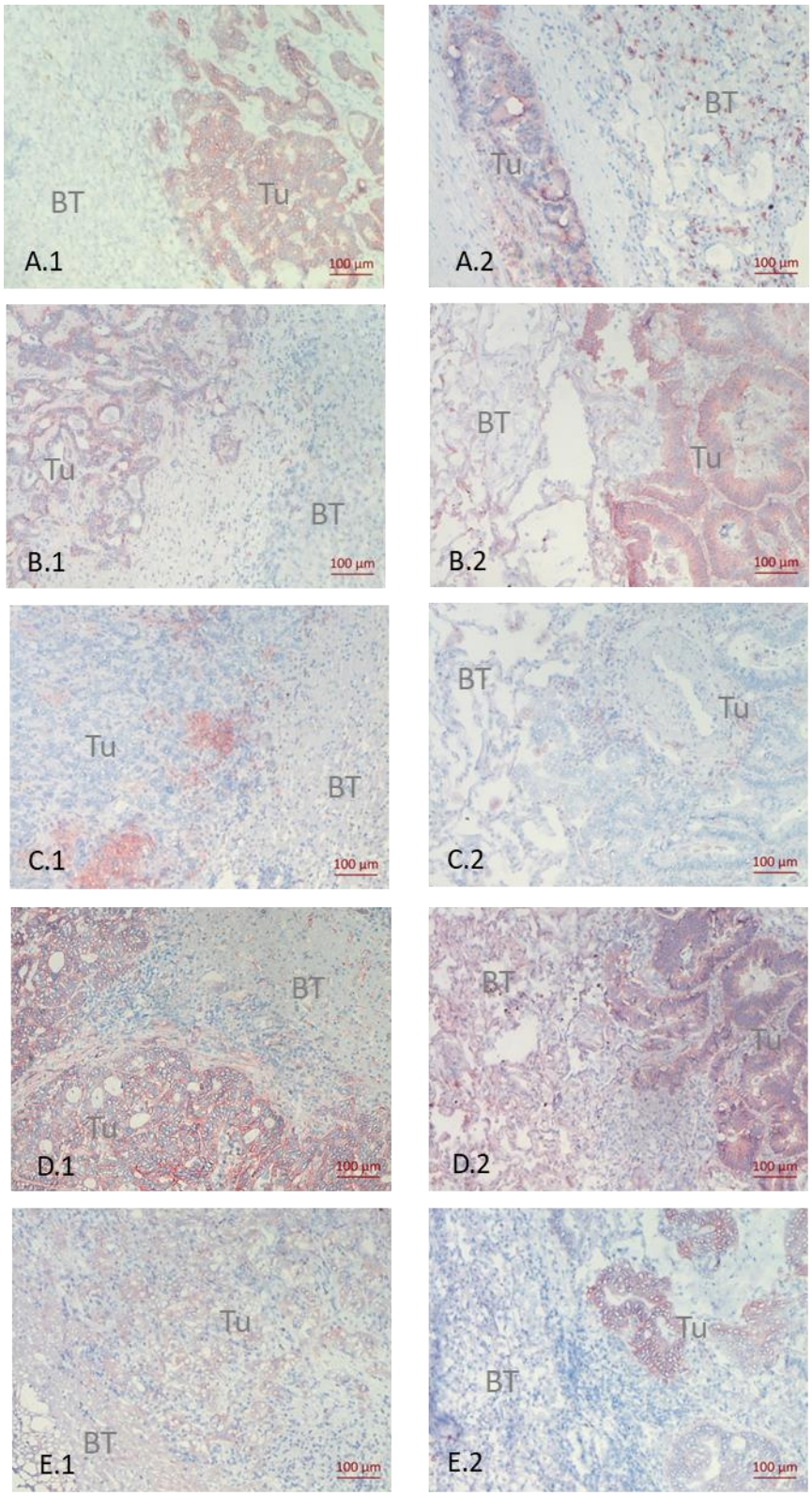

Figure 2. Immunohistochemical Staining of Different Biomarkers. Differential biomarker expression between liver (**1**) and lung (**2**) metastases demonstrated by immunohistochemistry. (**A**), CD44v6; (**B**), α2β1; (**C**), PD-L1; (**D**), IGF-1R; (**E**), EGFR; **Tu**, tumor tissue; **BT**, Benign tissue.

Table 5. Univariate Survival Analysis of CD44v6-Related Dual Biomarker Expression in Colorectal Liver Metastases.

Combination	Number of Patients (n)	Log Rank *p*-Value	Median PFS (month)
CD44v6 high *	22	**0.01**	7
CD44v6 low	30		15.5
CD44v6 high/IGF1-R high	15	0.142	7
CD44v6 high/IGF1-R low or CD44v6 low/IGF1-R high	20		9
CD44v6 low/IGF1-R low	17		17
CD44v6 high/EGF-R high	11	0.217	6
CD44v6 high/EGF-R low or CD44v6 low/EGF-R high	24		11.5
CD44v6 low/EGF-R low	17		9
CD44v6 high/Muc1 high	11	0.574	8
CD44v6 high/Muc1 low or CD44v6 low/Muc1 high	23		11
CD44v6 high/Muc1 low	18		7.5
CD44v6 high/α2β1 high	11	**0.002**	3
CD44v6 high/α2β1 low or CD44v6 low/α2β1 high	18		9
CD44v6 low/α2β1 low	23		24
CD44v6 high/Hsp90 high	14	**0.022**	7
CD44v6 high/Hsp90 low or CD44v6 low/Hsp90 high	21		9
CD44v6 low/Hsp90 low	17		17
CD44v6 high/PD-L1 high	12	**0.023**	7
CD44v6 high/PD-L1 low or CD44v6 low/PD-L1 high	21		14
CD44v6 low/PD-L1 low	19		11

PFS, progression-free survival; cut-off values defining high and low for the individual biomarker are given in Table 3; *, calculation of the cut-offs is given in the Materials and Methods Section.

Table 6. Multivariate Survival Analysis of CD44v6-Related Dual Biomarker Expression in Colorectal Liver Metastases.

Variable	Groups	Cox Regression (PFS)		
		HR	*p*-Value	95% CI
age (median in years)	>64/≤64	1.561	0.256	0.724–3.366
number of metastases *	>1/≤1	1.398	0.358	0.684–2.855
type of metastases	synchronous/metachronous	3.813	**0.008**	1.407–10.332
CD44v6/α2β1 expression	high/high vs. low/low	4.135	**0.002**	1.648–10.375
	high/low and low/high vs. low/low	1.784	0.145	0.819–3.886
age (median in years)	>64/≤64	1.129	0.773	0.496–2.568
number of metastases	>1/≤1	1.321	0.460	0.632–2.762
type of metastases	synchronous/metachronous	3.345	**0.013**	1.289–8.680
CD44v6/Hsp90 expression	high/high vs. low/low	2.039	0.085	0.906–4.586
	high/low and low/high vs. low/low	1.412	0.443	0.585–3.404
age (median in years)	>64/≤64	1.290	0.493	0.623–2.675
number of metastases	>1/≤1	1.341	0.418	0.659–2.728
type of metastases	synchronous/metachronous	4.154	**0.004**	1.584–10.893
CD44v6/PD-L1 expression	high/high vs. low/low	2.882	**0.017**	1.213–6.848
	high/low and low/high vs. low/low	0.872	0.723	0.409–1.860

HR, Hazard ratio; **PFS**, progression free survival; **CI**, confidence interval; *, nodules within the metastatic organ; cut-off values defining high- and low-level expression for the individual biomarker are given in Table 3.

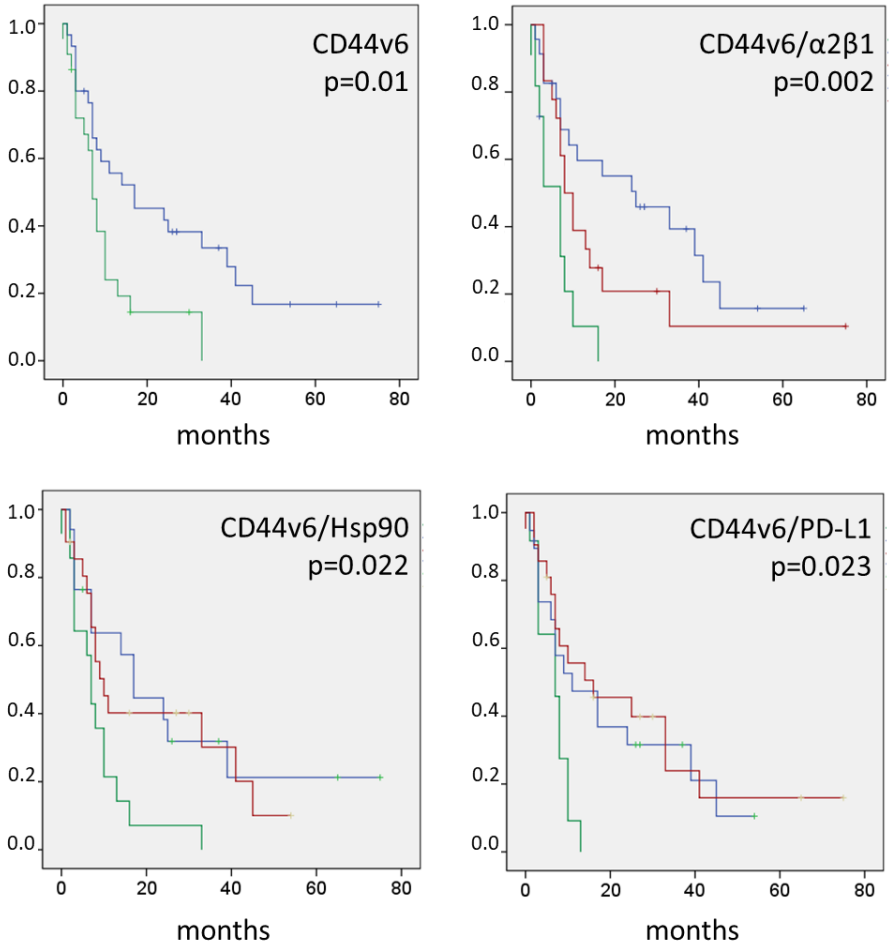

Figure 3. Kaplan–Meier Curves of CD44v6-Related Biomarker Expression in Colorectal Liver Metastases. **Blue lines**, low/low expression; **green lines**, high/high expression; **red lines**, high/low and low/high expression; log-rank *p*-values are given; cut-off values defining high- and low-level expression for the individual biomarker are given in Table 3.

Figure 3. Kaplan–Meier Curves of CD44v6-Related Biomarker Expression in Colorectal Liver Metastases. **Blue lines**, low/low expression; **green lines**, high/high expression; **red lines**, high/low and low/high expression; log-rank *p*-values are given; cut-off values defining high- and low-level expression for the individual biomarker are given in Table 3.

4. Discussion

It is well published that primary colorectal cancer differs in its biology, depending on sidedness [45]. This also includes treatment-relevant characteristics, such as the RAS [46,47], MSI [48] and BRAF status [49]. In the present study, biomarker heterogeneity was identified between colorectal liver and lung metastases, namely for the cell adhesion molecules α2β1, CD44v6, the growth factor receptors IGF-1R, EGF-R and the immune checkpoint biomarker PD-L1. These site-specific differences in biomarker expression might reflect the complex multifactorial interactions between disseminated cancer cells and the target organ microenvironment [50]. Cancer cells with a unique tumor biology are homing to metastatic niches with a microenvironment promoting colonization, survival, and proliferation [51,52]. Liver and lung metastases reveal biological differences, for example, in the cellular composition of the microenvironment [36,52–54], the ECM signature [52,55,56] and the secretome profile [52,57]. Quantitative differences in protein biomarker expression were found between liver and lung metastases, showing a significantly higher proportion of IGF-1R-, EGF-R-, CD44v6-, α2β1-, and PD-L1-positive cancer cells in the lung. This observation confirms published data, showing a higher frequency of genetic drivers, such as KRAS alterations and MET amplification in lung metastases [20,58]. At the same time, lung metastases exhibit an increased immunosuppressive microenvironment and prometastatic inflammation [36,59]. These findings suggest distinct colorectal cancer mechanisms, involving both specific cancer cells with a higher propensity to metastasize to the lung and a lung-specific environment that facilitates metastasis of specific cancer cells.

Table 6. Multivariate Survival Analysis of CD44v6-Related Dual Biomarker Expression in Colorectal Liver Metastases.

Variable	Groups	Cox Regression (PFS)		
		HR	*p*-Value	95% CI
age (median in years)	>64/≤64	1.561	0.236	0.724–3.366
number of metastases	>1/≤1	1.898	0.358	0.651–2.855
type of metastases	synchronous/metachronous	3.813	**0.008**	1.407–10.332
CD44v6/α2β1 expression	high/high vs. low/low	4.133	**0.002**	1.648–10.375
	high/low and low/high vs. low/low	1.784	0.145	0.819–3.886
age (median in years)	>64/≤64	1.129	0.773	0.496–2.568
number of metastases	>1/≤1	1.321	0.460	0.632–2.762
type of metastases	synchronous/metachronous	3.345	**0.013**	1.289–8.680
CD44v6/Hsp90 expression	high/high vs. low/low	2.039	0.085	0.906–4.586
	high/low and low/high vs. low/low	1.412	0.443	0.585–3.404
age (median in years)	>64/≤64	1.290	0.493	0.623–2.675
number of metastases	>1/≤1	0.541	0.418	0.659–2.728
type of metastases	synchronous/metachronous	4.154	**0.004**	1.584–10.895
CD44v6/PD-L1 expression	high/high vs. low/low	2.882	**0.017**	1.213–6.848
	high/low and low/high vs. low/low	0.872	0.723	0.409–1.860

Targeting metastasis-relevant biomarker expression will open up new therapeutic opportunities, adjusted to specific metastatic localizations. This is in deep contrast to the current guideline, which recommends the concept of treating distant metastasis with the same therapy, independent from the metastatic organ site.

The protein biomarker expression pattern in liver metastases was tested for prognostic relevance. A high (>30%) fraction of CD44v6+ liver metastatic cells was identified as an independent prognostic factor mediating short progression-free survival. This finding supports CD44v6 as a metastatic driver. Multiple underlying molecular mechanisms have been described for CD44v6-mediated progression in colorectal cancer. Examples are interactions with the extracellular matrix components osteopontin and hyaluronic acid and the binding of different cytokines, such as HGF, EGF and VEGF [31,60]. Co-expression analysis identified two new independent risk factors associated with poor prognosis of CRC patients with liver metastases. Most interesting, high dual expression of CD44v6 and integrin α2β1 represents an indicator of early recurrence, defined as tumor relapse within six months after liver resection for colorectal metastases [61,62]. Direct and extracellular matrix-mediated molecular crosstalk between CD44v6 and various integrins, including α2β1, was found to promote cancer cell proliferation and invasion, tumor angiogenesis and chemoresistance, all involved in a considerable shortening of progression-free survival compared to the single CD44v6 expression [63–65]. In addition, dual expression of CD44v6 and PD-L1, indicating the crosstalk between tumor cells and the tumor microenvironment, significantly correlated with short survival. The subset of CD44v6+ colorectal cancers simultaneously expressing PD-L1 might represent stem-like properties and contributes to immune evasion mediating poor prognosis [66,67]. Similarly, co-mutations in RAS, TP53 and SMAD4, as well as in APC and PIK3CA, resulted in a worse outcome after hepatectomy compared to single mutations [19]. Therefore, our findings support the strategy of combining prognostic protein biomarkers to render the prediction of outcome more precise [68,69]. Further, these new factors might be included in clinical risk scores, similar as reported for the KRAS status in the GAME score [70] and the KRAS/NRAS/BRAF status in the CERR score [71], which resulted in the refinement to predict recurrence after resection of CRC liver metastases. In contrast to some of the most investigated therapeutic biomarkers, namely BRAF, MSI-high, and Her2/neu, all detected in a very small patient cohort [19,20], dual expression of the druggable targets CD44v6/α2β1 and CD44v6/PD-L1 was identified in about 20% of the liver metastatic patients.

In addition, these novel findings might have an impact on the development of new therapeutic strategies for liver metastatic CRC patients. Currently, new anti-CD44v6 treatment strategies, such as half antibodies conjugated nanoparticles [72], peptides (NCT03009214) and CD44v6-specific CAR gene-engineered T cells (NCT04427449, [73]) are under investigation and might also become a treatment option for CRC patients with CD44v6-positive liver metastases. Combination of two biomarkers might help to stratify patients more precisely for targeted therapy compared to single biomarker expression. For example, Shek et al., 2021, reported that only a subgroup of PD-L1-positive mCRCs responded to checkpoint inhibitor therapy [74]. In addition, dual expression of druggable biomarkers will further promote the promising concept of multiple target inhibition, aiming to improve treatment outcome and reduce the risk of drug resistance. Recently, the combination of the BRAF inhibitor Encorafenib with the EGF-R inhibitor Cetuximab has been reported as the new standard for the treatment of metastatic BRAF-mutated colorectal cancer [75]. Currently, a number of clinical trials are ongoing in advanced colorectal cancer, simultaneously inhibiting different targets. This includes combination therapy of the EGF-R inhibitor Panitumumab with the multi-kinase inhibitor Cabozantinib [76]. Further, anti-PD-L1 checkpoint inhibitors have been combined with targeted therapies, aiming to improve the response to immunotherapy [77]. In the present study, dual expression of PD-L1 and CD44v6 was found to correlate with poor prognosis and might represent a new therapeutic option for combination therapy. The second interesting pair of therapeutic targets identified in the present study was the co-expression of CD44v6 and the integrin α2β1. Both cell ad-

hesion molecules were found to mediate chemoresistance [65,78]. Simultaneous inhibition of both targets might result in the circumvention of chemoresistance and represent a new anti-metastatic strategy of targeted therapy. Consideration of metastasis-driving protein biomarkers that predict early recurrence after hepatectomy might play a critical role in the clinical management of patients diagnosed with liver metastases [79]. The findings in the present study need to be confirmed in a larger, prospective trial.

5. Conclusions

A differential expression pattern of the druggable protein biomarkers α2β1, CD44v6, IGF-1R, EGF-R and PD-L1 was identified between colorectal liver and lung metastases. High expression of CD44v6, CD44v6/α2β1, and CD44v6/PD-L1 correlated significantly with early recurrence after hepatic metastasectomy. Dual biomarker expression may render the prognostic prediction more precise and stratify high-risk patients for new therapeutic concepts, depending on the metastatic organ site.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers14081939/s1, Table S1: Positivity and Distribution of Biomarkers in Benign Tissue of Liver and Lung Metastases.

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Data Availability Statement: Data corresponding to the analyzed tissues were delivered in anonymized form by the HTCR Foundation.

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Table S1: Positivity and Distribution of Biomarkes in Benign Tissue of Liver and Lung Metastases

	number of positive lesions				percentage of positive benign tissue (%)				number of positive lesions above cut-offs			log-rank (p value)	
	liver		lung		Median		Mean		cut off	liver		liver	
Biomarker	n=52*	%	n=15	%	liver	lung	liver	lung	(%)	n=52*	%	PFS	OS
HGF-R	38	73.1	11	73.3	100	40	72.4	41.0	70	36	69.2	0.167	0.665
IGF-1R	11	21.2	13	86.7	0	100	17.2	78.0	20	10	19.2	0.494	0.342
EGF-R	46	88.5	15	100	100	80	86.3	75.3	80	44	84.6	0.505	0.613
Her2/neu	20	38.5	5	33.3	0	0	23.3	5.3	20	14	26.9	0.393	0.748
ERα		patchy staining			n.t.		n.t.			n.t.		n.t.	n.t.
PR		patchy staining			n.t.		n.t.			n.t.		n.t.	n.t.
Muc1	0	0	8	53.3	0	20	0	19.3		n.t.		n.t.	n.t.
CD44v6	0	0	12	80	0	30	0	28.7		n.t.		n.t.	n.t.
α2β1	0	0	10	66.7	0	20	0	37.3		n.t.		n.t.	n.t.
Hsp90	46	88.5	12	80	100	60	83.9	54.7	80	36	69.2	0.922	0.068
PD-L1	10	19.2	13	86.7	0	30	7.0	50.7	10	7	13.5	0.416	0.275

Legend:n.t., not tested; *, one benign liver tissue was noch available; PFS, progression free survival; OS, overall survival

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