Aus der

Klinik und Poliklinik für Urologie und Kinderurologie

des Universitätsklinikums Bonn

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# Identifizierung prädiktiver statischer und *on-treatment* Biomarker zur Vorhersage des Ansprechens auf Immuncheckpoint-Inhibition im Nierenzellkarzinom

Habilitationsschrift

zur Erlangung der *venia legendi*

der Hohen Medizinischen Fakultät

der Rheinischen Friedrich-Wilhelms-Universität Bonn

für das Lehrgebiet

„Experimentelle Urologie“

vorgelegt von

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2024

Datum des Habilitationskolloquiums: 13.07.2023

*Für meine Familie*

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## 1. Grundlage der kumulativen Habilitationsschrift

Die folgenden vier Originalarbeiten liegen der kumulativen Habilitationsschrift zugrunde, welche die wesentlichen Ergebnisse der Publikationen zusammenfasst und diskutiert:

I. **N. Klümper*** et al. LAG3 (LAG-3, CD223) DNA methylation correlates with LAG3 expression by tumor and immune cells, immune cell infiltration, and overall survival in clear cell renal cell carcinoma. *J. Immunother. Cancer*, e000552. doi: 10.1136/jitc-2020-000552. (2020).

II. **N. Klümper** et al. CTLA4 promoter hypomethylation is a negative prognostic biomarker at initial diagnosis but predicts response and favorable outcome to anti-PD-1 based immunotherapy in clear cell renal cell carcinoma. *J. Immunother. Cancer*, e002949. doi: 10.1136/jitc-2021-002949. (2021).

III. **N. Klümper*** et al. C-reactive protein flare-response predicts long-term efficacy to first-line anti-PD-1-based combination therapy in metastatic renal cell carcinoma. *Clin. Transl. Immunol*. 10(12):e1358. doi: 10.1002/cti2.1358. (2021).

IV. **N. Klümper*** et al. C-reactive protein flare predicts response to checkpoint inhibitor treatment in non-small cell lung cancer. *J Immunother. Cancer,* e004024. doi: 10.1136/jitc-2021-004024. (2022).

***** Geteilte Erstautorenschaft

# 2. Einleitung

## 2.1 Epidemiologie des Nierenzellkarzinoms

Nierenzellkarzinome (RCC) zählen zu den häufigsten Malignomen weltweit (Siegel et al., 2022). Nach dem Prostata- und Urothelkarzinom (UC) stellt das RCC in Deutschland die dritthäufigste urologische Tumorerkrankung dar (Erdmann et al., 2021).

Maligne Nierentumore liegen nach Daten des Robert Koch-Institutes aus den Jahren 2017/2018 mit einem Anteil von 2,4% bei Frauen und 3,5% bei Männern unter den zehn der am häufigsten diagnostizierten Krebsneuerkrankungen in Deutschland (Erdmann et al., 2021). Das mittlere Erkrankungsalter liegt für Frauen bei 71 Jahren und für Männer bei 68 Jahren.

## 2.2 Histologie des Nierenzellkarzinoms

Das klarzellige RCC (ccRCC) ist mit einem Anteil von etwa 70% der häufigste histologische Subtyp des RCC, das papilläre RCC (pRCC) ist mit 10-15% das zweithäufigste Karzinom des Nierentubulusepithels und das chromophobe RCC (chRCC) mit etwa 5% die dritthäufigste histopathologische Entität (Delahunt et al., 2013; Störkel et al., 1997).

## 2.3 Therapie des Nierenzellkarzinoms

Im lokalisierten Krankheitsstadium stellt die operative Therapie mit vollständiger Entfernung des Tumors die einzige kurative Therapiemodalität dar. Im metastasierten Krankheitsstadium handelt es sich um eine nicht heilbare Erkrankung. Die relative 5-Jahres-Gesamtüberlebensrate über alle Erkrankungsstadien, welche die Sterblichkeit in der Allgemeinbevölkerung berücksichtigt, beträgt für Männer und Frauen gleichermaßen etwa 75% (Erdmann et al., 2021).

Die medikamentöse Tumortherapie des metastasierten RCC (mRCC) stellt insbesondere aufgrund der hohen intrinsischen Chemo- und Strahlenresistenz eine Herausforderung dar (Bedke et al., 2021). Für das mRCC wurden in den letzten Jahren, insbesondere basierend auf wachsendem tumorbiologischem Verständnis der Erkrankung, zahlreiche neue, zielgerichtete Therapien zugelassen (Dutcher et al., 2020). Aktuell werden hauptsächlich zwei Medikamentenklassen für das mRCC eingesetzt – die Tyrosinkinase- (TKI) und Immuncheckpoint-Inhibitoren (ICI).

Im Jahr 2007 eröffnete die Zulassung des TKI Sunitinib die Dekade der oralen antiangiogenetischen Therapien für das ccRCC (Motzer et al., 2007). Die Basis für die hohe Vulnerabilität des Tumors gegenüber den antiangiogenetisch wirksamen Therapien liegt in der Karzinogenese des ccRCC (Gerlinger et al., 2012, 2014). Das früheste und mit etwa 80% häufigste genomische Event in der Tumorentstehung des ccRCC ist die funktionelle Inaktivierung des von-Hippel-Lindau Tumorsuppressorgens (VHL), welches zu einer konstitutiven Aktivierung des Hypoxie-Signalweges führt und das ccRCC zu einem hochvaskularisierten Tumor macht (The Cancer Genome Atlas Research Network, 2013). Neben Sunitinib wurden in den letzten Jahren multiple weitere TKI in der Behandlung des mRCC zugelassen (Bedke et al., 2021). Diese verschiedenen TKI entfalten ihre antiangiogenetische Wirkung über die Blockade des vaskulären endothelialen Wachstumsfaktor-Rezeptoren (VEGFR). Cabozantinib gehört seit kurzem zu den VEGFR-gesteuerten TKI und zeigt auch klinische Aktivität nach TKI-Vorbehandlung (Choueiri et al., 2015). Die Blockade weiterer Tyrosinkinasen neben VEGFR, wie c-Met und AXL, scheint die erhaltene Aktivität von Cabozantinib auch nach vorangegangener antiangiogenetischer Therapie zu erklären.

Die Erfolgsgeschichte der Immunonkologie ist maßgeblich durch die Entwicklung und klinische Einführung von ICI gegen die CTLA4 und PD-1/PD-L1 Achse geprägt, für die James Patrick Allison und Tasuku Honjo im Jahr 2018 mit dem Nobelpreis für Physiologie und Medizin ausgezeichnet wurden. Der Einsatz von ICI in der Behandlung des mRCC führte zu einer zunehmenden Verbesserung des Gesamtüberlebens (OS) (Bedke et al., 2021; Xu et al., 2020). Der anti-PD-1-Wirkstoff Nivolumab zeigte eine bemerkenswerte klinische Wirksamkeit mit einer objektiven Ansprechrate (ORR) von mehr als 20% bei TKI-refraktärer Erkrankung, sodass Nivolumab nach positiven Studiendaten der klinischen Phase-III-Studie CheckMate025 als erster ICI die Zulassung für das mRCC erhielt (Motzer et al., 2015).

Der gegen den Immuncheckpoint CTLA4 gerichtete humanisierte monoklonale Antikörper Ipilimumab wurde im Anschluss in Kombination mit Nivolumab für Patientinnen und Patienten (aus Gründen der besseren Lesbarkeit wird nachfolgend das generische Maskulinum verwendet) mit mRCC getestet. Diese Kombinationstherapie zeigte bereits große klinische Aktivität im malignen Melanom (Larkin et al., 2015, 2019). Im Rahmen der Phase-III-Studie CheckMate214 zeigte diese intensivierte ICI in der Erstlinie bei intermediärem und hohem Risiko nach IMDC Score im Vergleich zu Sunitinib eine

deutlich erhöhte objektive Ansprechrate (42% versus 27%) sowie einen Vorteil für das OS (Hazard Ratio: 0.65 (95% Konfidenzintervall: 0.54–0.78), p<0.001) (Albiges et al., 2020; Bedke et al., 2021; Motzer et al., 2018). Besonders bemerkenswert ist, dass bei einer Nachbeobachtungszeit von mindestens vier Jahren in der *intention-to-treat* Population von Patienten mit mittlerem/niedrigem Risiko gemäß IMDC-Score ein Plateau von 35% für das progressionsfreie Überleben (PFS) erreicht wurde, welches ein langfristiges Therapieansprechen darstellt. Dies ist eine Beobachtung, die es in der Ära der TKI-Monotherapie nicht gab (Albiges et al., 2020).

Nach Verdrängung der TKI in der Erstlinie durch Nivolumab und Ipilimumab folgten rasch die überzeugenden klinischen Daten zu der Kombinationstherapie von ICI+TKI (Bedke et al., 2021; Xu et al., 2020). Das Konzept der Kombination von VEGF-Signalblockade und ICI entstand nicht allein aus der Tatsache, dass diese beiden zielgerichteten Therapien bei mRCC wirksam sind, sondern vielmehr aus präklinischen Beobachtungen, die darauf hindeuteten, dass die VEGFR-Achse auch eine Rolle bei der Tumorimmunabwehr spielt (Hirsch et al., 2020). Der Einsatz von antiangiogenen TKI scheint daher zusätzlich zur PD-1-Blockade über immunmodulatorische Effekte des Tumormikromilieus eine synergistische therapeutische Wirkung zu entfalten (Rassy et al., 2020; Yasuda et al., 2013).

Basierend auf den positiven Daten der Studien Keynote-426, CLEAR und CheckMate-9ER werden in der Leitlinie der *European Association of Urology* (EAU) nun in chronologischer Reihenfolge die folgenden ICI+TKI-Kombinationstherapien in der Erstlinien des mRCC unabhängig vom IMDC-Risikoscore empfohlen: Keynote-426: Pembrolizumab plus Axitinib; CLEAR: Pembrolizumab plus Lenvatinib; CheckMate-9ER: Nivolumab plus Cabozantinib; (Bedke et al., 2021; Choueiri et al., 2021; Motzer et al., 2021; Rini et al., 2019). Die Therapielandschaft für Patienten mit mRCC entwickelte sich somit vor allem in der Erstlinien rasant weiter. Auf die FDA-Zulassung der Kombinationstherapie aus Nivolumab und Ipilimumab (ICI+ICI) für die Erstlinienbehandlung von Patienten mit fortgeschrittenem RCC mit mittlerem und niedrigem Risiko im April 2018 folgte die Zulassung der drei genannten ICI+TKI-Kombinationstherapien.

Derzeit werden in der Erstlinientherapie des mRCC mit mittlerem/ hohem Risiko daher vier in ihrer klinischen Wirksamkeit als gleichwertig eingestufte Kombinationstherapien eingesetzt. Im Wesentlichen können diese Therapiekonzepte als intensivierte ICI (anti-PD-1 + antiCTLA4) versus eine Kombination aus ICI und antiangiogener TKI (anti-PD-

1+VEGFR-TKI) klassifiziert werden. Da alle oben genannten Zulassungsstudien Sunitinib als Komparator verwendeten, welches in der Erstlinie nur noch für Patienten mit Kontraindikationen gegenüber Immuntherapie eingesetzt wird, und aktuell vergleichende, prospektive Studien zwischen diesen Erstlinientherapieoptionen ausstehen, ist es aktuell aufgrund fehlender prädiktiver Biomarker schwierig, die optimale Therapieoption für den Patienten zu wählen.

Eine Metaanalyse von Quhal et al. deutet darauf hin, dass Kombinationen aus ICI+TKI unabhängig von der IMDC-Risikogruppe eine bessere Ansprechrate sowie ein besseres PFS und OS im Vergleich zu ICI+ICI bieten (Quhal et al., 2021). Bei großer Tumorlast und Symptomatik werden im klinischen Kontext aufgrund der höheren Ansprechraten daher häufig ICI+TKI bevorzugt. Dennoch bietet ICI+ICI die beste Chance auf eine komplette Remission (Riaz et al., 2021). Die verlängerten Nachbeobachtungsdaten der Zulassungsstudien zeigten nur für die Nivolumab + Ipilimumab (ICI+ICI) Kombination ab etwa 30 Monaten ein Plateau bei circa einem Drittel der Studienpatienten, was auf eine außergewöhnliche Beständigkeit der Therapieantwort hindeutet (Albiges et al., 2020; Bedke et al., 2021). Die intensivierte Immuntherapie in der Erstlinie war zudem mit der niedrigsten Rate an behandlungsbedingten unerwünschten Ereignissen ≥3 assoziiert und zeigte hervorragende *Patient-Reported Outcomes* (Cella et al., 2019).

Dementsprechend gibt es aktuell für beide Therapiekonzepte gute klinische Argumente. Es muss jedoch dringend berücksichtigt werden, dass alle für die Erstlinientherapie zugelassenen Kombinationen, sowohl ICI+ICI als auch ICI+TKI, nicht für den Einsatz in der Zweitlinie zugelassen sind. Dadurch ergibt sich ein klinisches Dilemma, da die höhere Ansprechrate der ICI+TKI dem langfristigen Therapieerfolg der ICI+ICI bei vergleichsweise milderen Nebenwirkungen entgegensteht. In diesem Zusammenhang gibt es einen bisher nicht suffizient gedeckten Bedarf an robusten prädiktiven Biomarkern, welche eine für den individuellen Patienten maßgeschneiderte Therapiefestlegung anhand der Tumorbiologie ermöglicht.

### 2.4 Prädiktive statische Biomarker für Immuntherapie

Die Identifizierung robuster und einfach in den klinischen Alltag zu implementierender prädiktiver Biomarker für eine optimale Therapiestratifizierung ist von übergeordneter Bedeutung, um die derzeitig komplizierte Therapieentscheidung bei der Erstlinientherapie des mRCC zu rationalisieren. Ein prädiktiver Biomarker ist dadurch definiert,

dass das Vorhandensein oder die Veränderung des Biomarkers einen Patienten identifiziert, der mit größerer Wahrscheinlichkeit ein günstiges oder ungünstiges Ergebnis auf eine bestimmte Behandlung erfahren wird (Califf, 2018). Im Idealfall lässt sich dieser Biomarker vor Einleitung einer neuen Therapie bestimmen, sodass er eine Rationale für oder gegen eine bestimmte Therapieentscheidung schafft.

Ein Biomarker wird prognostisch genannt, wenn er unabhängig von der Art der Behandlung lediglich mit der Krankheitsprognose verbunden ist. Ein prädiktiver Biomarker übt je nach Behandlung einen unterschiedlichen prognostischen Einfluss aus (Hu and Dignam, 2019).

Derzeit ist der im therapienaiven Tumorgewebe mittels Immunhistochemie (IHC) bestimmte PD-L1-Rezeptorstatus der einzige weitverbreitete prädiktive Biomarker für das mRCC. In einer Metaanalyse, die die Studiendaten der Erstlinientherapien verglich, zeigte Nivolumab in Kombination mit Ipilimumab in der Patientengruppe mit einer PD-L1-Tumorexpression von ≥1% im Vergleich zu ICI+TKI die besten Gesamtüberlebensdaten (Quhal et al., 2021). Der prädiktive Wert des PD-L1 Rezeptorstatus wird jedoch durch eine weitere Metaanalyse von immuntherapierten Patienten mit ccRCC infrage gestellt. Carretero-Gonzalez at al. konnten zwar eine Assoziation zwischen der Tumorexpression von PD-L1 mit einem verbesserten PFS zeigen, jedoch zeigte sich kein Vorteil für das OS (Carretero-González et al., 2020).

Die Robustheit des PD-L1 Status als prädiktiver Biomarker für die Immunonkologie wird zudem durch die Verwendung unterschiedlicher Antikörperklone und Expressionsscores - prozentualer Anteil der PD-L1 Expression auf den Tumorzellen (TPS), Immunzellen (IC) bzw. der kombinierte Positivitätsscore (CPS) - weiter geschmälert. Auch die hohe Variabilität zwischen den Laboren und Beobachtern beeinträchtigt die Robustheit des PD-L1 Status (Eckstein and Gupta, 2019; Eckstein et al., 2019; Gibney et al., 2016; Sommer et al., 2020). Das ccRCC ist außerdem durch eine ausgeprägte interläsionale und intratumorale Heterogenität gekennzeichnet, welches die robuste Bestimmung des PD-L1 Status im ccRCC durch den daraus entstehenden *Sampling Bias* zusätzlich erschwert (Callea et al., 2015; Gerlinger et al., 2012, 2014; Raimondi et al., 2020). Zudem ist es wichtig herauszustellen, dass der therapeutische Nutzen der ICI+TKI Kombinationstherapie unabhängig von dem PD-L1-Status zu beobachten war (Bedke et al., 2021). Zurzeit empfiehlt die EAU-Leitlinie mit einem Evidenzgrad von 2b daher die PD-L1-Expression nicht für die Therapieentscheidung zu verwenden.

Entsprechend gibt es aktuell in der translationalen Forschung – trotz intensiver Bemühungen – keinen robusten prädiktiven Biomarker für eine rationale Erstlinientherapieentscheidung im mRCC.

Neben PD-L1 wurden weitere prädiktive Biomarker zur Vorhersage des Ansprechens auf Immuntherapie vorgeschlagen, jedoch hat keiner dieser Marker den Einzug in den klinischen Alltag geschafft (Bai et al., 2020; Bedke et al., 2021; Franklin et al., 2022; Havel et al., 2019; Lesterhuis et al., 2017; Raimondi et al., 2020).

Eine hohe Tumormutationslast (*Tumor Mutational Burden*=TMB) hat sich unter anderem beim nicht-kleinzelligen Bronchialkarzinom (NSCLC) als vielversprechender Biomarker erwiesen (Strickler et al., 2021). Das RCC weist einen hohen Anteil an genomischen Insertionen und Deletionen auf, die aufgrund der möglichen Bildung von Neoantigenen eine hoch immunogene Mutationsklasse darstellen (Turajlic et al., 2017). Die Daten hinsichtlich des prädiktiven Potenzials der TMB zur Vorhersage des Ansprechens auf Immuntherapie sind für das mRCC jedoch widersprüchlich (Raimondi et al., 2020). Zudem ist die Bestimmung des TMB kostspielig, methodisch sehr aufwändig und nicht ubiquitär verfügbar, was die Implementation dieses potenziell prädiktiven Biomarkers in den klinischen Alltag weiter einschränkt.

Es wurden mehrere potenziell prädiktive Transkriptionssignaturen basierend auf der RNA-Sequenzierungstechnik (RNAseq) beschrieben. Eine verstärkte Angiogenese-Signatur war konsistent in mehreren Phase-III-Studien mit einem verbesserten Ansprechen auf den antiangiogenetisch wirksamen TKI Sunitinib (der Komparator in allen dieser Studien) assoziiert (McDermott et al., 2018; Motzer et al., 2020a, 2020b). Die Vorhersage des Immuntherapieansprechens anhand von transkriptionellen Signaturen war demgegenüber in den verschiedenen Biomarker-Studien basierend auf den Phase-III-Studienkollektiven weniger eindeutig. Die Vergleichbarkeit der unterschiedlichen Studiendaten ist darüber hinaus dadurch erschwert, dass unterschiedliche Expressionssignaturen verwendet wurden. In der JAVELIN Renal 101-Studie sagte eine immunmodulatorische Gensignatur (N=26 Gene) ein besseres PFS unter ICI+TKI (Avelumab plus Axitinib) voraus (Motzer et al., 2020a). Auf der Grundlage der IMmotion151-Studie wurde eine hohe T-Effektor/IFN-γ-Genexpressionssignatur mit einem besseren PFS für Atezolizumab plus Bevacizumab im Vergleich zu Sunitinib in Verbindung gebracht (McDermott et al., 2018). Der in der IMotion151-Studie verwendete Expressionsscore unterschied sich jedoch von der immunmodulatorischen Gensignatur

der JAVELIN Renal 101-Studie (Motzer et al., 2020a). In der klinischen Phase-III-Studie CheckMate214, die Nivolumab + Ipilimumab gegenüber Sunitinib verglich, war ein verlängertes PFS mit einer höheren Expression der charakteristischen Gen-Sets "*inflammatory response"* und *"epithelial mesenchymal transition"* verbunden (Motzer et al., 2020b). Die Identifizierung einer eindeutig prädiktiven Gensignatur ist daher aktuell ausstehend. Zur Schaffung einer validen Evidenz vor dem breiten Einsatz in der Klinik werden prospektive Studien die Robustheit dieser RNAseq-basierten Signaturen gegenüber der ausgeprägten intratumoralen Heterogenität des ccRCC untersuchen müssen (Callea et al., 2015; Gerlinger et al., 2012, 2014; Raimondi et al., 2020).

Die drei beschriebenen Studien verglichen eine ICI-basierte Kombinationstherapie (ICI+ICI oder ICI+TKI bzw. VEGF-Antikörper) mit dem Komparator Sunitinib in der Monotherapie. Ob diese Gensignaturen daher die aktuell unselektive Erstlinientherapieentscheidung ICI+ICI versus ICI+TKI unterstützen können ist fragwürdig. Prospektive vergleichende Studiendaten zwischen ICI+ICI versus ICI+TKI sind ausstehend, sodass aktuell keine optimale prospektiv-randomisierte Studienkohorte zur Erörterung dieser wichtigen Fragestellung verfügbar ist.

Insgesamt bleibt der PD-L1 Status weiterhin das einzig verfügbare prädiktive Instrument für den Kliniker, welches für die Erstlinientherapieentscheidung im mRCC herangezogen werden kann. Hier stellt das verbesserte OS für ICI+ICI in der Patientengruppe mit einer PD-L1-Tumorexpression von ≥1% im Vergleich zu ICI+TKI sicherlich eine starkes Argument dar (Quhal et al., 2021).

Biomarker auf der Grundlage von Methylierungssignaturen haben einige Vorteile gegenüber der IHC-basierten PD-L1 Proteinexpressionsmessung. Die DNA-Methylierung ist eine epigenetische Modifikation an den CpG-Dinukleotiden, die weniger dynamische Schwankungen als die mRNA- oder Proteinexpression aufweist (Luo et al., 2018). Darüber hinaus ist die Methylierung der DNA eine chemisch stabile kovalente Bindung und kann über Polymerase-Kettenreaktion (PCR)-basierte Analytik untersucherunabhängig quantifiziert werden. PCR-basierte Methoden haben zudem den Vorteil, dass die quantitative Messung aufgrund der Amplifikation auch bei kleinen Probenmengen (Biopsien, zirkulierende Tumorzellen, zellfreie DNA aus *Liquid Biopsies* (Blut, Urin)) möglich ist (Dietrich et al., 2009; Jung et al., 2017).

Eine Vielzahl von Studien konnte zeigen, dass die Expression von Immuncheckpoints wie PD-L1 in verschiedenen Tumorentitäten epigenetisch über DNA-Methylierung reguliert wird (Franzen et al., 2018; Gevensleben et al., 2016, 2016; Goltz et al., 2017a, 2017b; Micevic et al., 2019; Newell et al., 2021; Ralser et al., 2021). Die Arbeitsgruppe um PD Dr. Dietrich beschäftigt sich seit über einer Dekade mit der Untersuchung von DNA-Methylierungssignaturen, insbesondere mit der epigenetischen Regulation von Immuncheckpoints, für die es klinisch zugelassene (anti-PD-1, anti-PD-L1, anti-CTLA4) bzw. sich im Zulassungsverfahren befindende Antikörper (unter anderem anti-LAG3, (Maruhashi et al., 2020; Tawbi et al., 2022)) für die Immunonkologie gibt.

Der Immuncheckpoint LAG3 rückt aktuell in den Fokus der Immunonkologie. Zuletzt konnte erstmalig in einer randomisierten Studie ein Vorteil für das PFS für die Hinzunahme des anti-LAG3 Antikörpers Relatlimab im Melanom festgestellt werden (Tawbi et al., 2022). In der klinischen Phase II-III RELATIVITY-047-Studie zeigte die Kombinationstherapie von anti-LAG3 und anti-PD-1 bei Patienten mit einem unbehandelten, metastasierten oder inoperablen Melanom einen Vorteil hinsichtlich des PFS im Vergleich zur anti-PD-1 Monotherapie (Tawbi et al., 2022). In der Kombination traten keine neuen Toxizitätssignale auf. In dieser Studie wurde die immunhistochemisch gemessene Expression von LAG3 oder PD-L1 nicht als robuster prädiktiver Biomarker für die Vorhersage eines Vorteils von Relatlimab plus Nivolumab gegenüber einer Nivolumab-Monotherapie identifiziert, was erneut den untergeordneten Wert der Immunhistochemie für die Prädiktion des Immuntherapieansprechens unterstreicht. Beim Melanom zeigte jedoch der *LAG3*-Promotormethylierungsstatus (mLAG3), gemessen über einen HumanMethylation450 BeadChip und über einer eigenständig etablierten Methylierungs-spezifischen quantitativen PCR (mLAG3-qMSP), vielversprechende Daten (Fröhlich et al., 2020): Die LAG3 mRNA-Expression scheint über DNA-Methylierung des Promotors reguliert zu werden. Darüber hinaus korreliert mLAG3 mit der Zusammensetzung der Tumormikroumgebung und mit der bekanntermaßen für das Immuntherapieansprechen prädiktiven RNAseq-basierten inflammatorischen Interferon-γ-Signatur (Ayers et al., 2017). Derzeit werden auch klinische Studien zur Bewertung der Sicherheit und Wirksamkeit des anti-LAG3 Antikörpers Relatlimab in Kombination mit anti-PD-1 für Patienten mit mRCC durchgeführt (FRACTION-RCC: NCT02996110). Erste Daten der FRACTION-RCC Studie werden für Anfang 2023 erwartet. Darüber hinaus befindet sich der bispezifische Antikörper XmAb841 (You et al.,

2021), der gleichzeitig gegen die beiden Immuncheckpoints CTLA4 und LAG3 gerichtet ist, derzeit in klinischer Erprobung für das mRCC und weiteren anderen Tumorerkrankungen (DUET-4 Studie: NCT03849469, voraussichtliches Datum der Fertigstellung der Studie ist November 2025). Rationale dieser Kombinationstherapien ist die Förderung einer tumorselektiven T-Zell-Aktivierung.

Da ein verbessertes Verständnis der epigenetischen Regulation der LAG3-Expression aufgrund der laufenden Studien von großem Interesse für das RCC ist, **war ein Ziel dieser kumulativen Habilitationsschrift**, den Einfluss der *LAG3*-Promotormethylierung auf die Regulation der LAG3 Expression und Zusammensetzung des Tumormikromileus im ccRCC-Tumorgewebe zu untersuchen (Klümper et al., 2020).

Es konnte bereits nachgewiesen werden, dass der Metyhlierungsstatus von Immuncheckpoint-Genen auch das Ansprechen auf eine Immuntherapie vorhersagen kann. So konnte gezeigt werden, dass die Promotorhypomethylierung des Immuncheckpoints *CTLA4* (mCTLA4) das Ansprechen auf eine anti-PD-1 und anti-CTLA4-gerichtete Immuntherapie beim Melanom vorhersagen kann (Fietz et al., 2020; Goltz et al., 2018). Der in dieser Studie verwendete mCTLA4-qMSP basiert auf der Messung von DNA, die aus archiviertem, formalinfixiertem und paraffineingebettetem (FFPE) Tumorgewebe gewonnen wurde, was die Implementierung dieses Tests für den klinischen Einsatz vereinfachen würde, da die Verwendung von FFPE-Material für viele molekulare Analysen der klinische Standard ist.

In der CheckMate214-Studie, die letztlich zur Zulassung von ICI+ICI als Erstlinientherapie im mRCC führte (Motzer et al., 2018), wurde nur der prädiktive Wert der PD-L1-Expression bewertet. Entsprechend wurde die Hälfte des biologischen Mechanismus dieses Therapieansatzes, nämlich die Blockade des Immuncheckpoints CTLA4, nicht direkt für die Evaluation eines prädiktiven Biomarkers berücksichtigt. Bisher konnte für das mRCC kein robuster prädiktiver Biomarker identifiziert werden, der ein Therapieansprechen auf eine anti-CTLA4 gerichteten Therapie vorhersagen kann. In diesem Zusammenhang ist mCTLA4, welches prädiktives Potenzial im ICI-behandelten Melanom aufweist (Fietz et al., 2020; Goltz et al., 2018), ein vielversprechender Kandidat. Die *CTLA4*-Promotorhypomethylierung war mit einem verbesserten Ansprechen auf eine anti-CTLA-4-Monotherapie für Patienten mit metastasiertem Melanom assoziiert (Fietz et al., 2020). **Ein zweites Ziel dieser kumulativen Habilitationsschrift** ist es daher, die epigenetische Regulierung von *CTLA4* sowie den prädiktiven Wert von

mCTLA4 für das mRCC unter Immuntherapie zu untersuchen. Der Fokus liegt auf der Frage, ob mCTLA4 auch ein prädiktives Potenzial für das Ansprechen auf eine Immuntherapie bei mRCC hat (Klümper et al., 2021a), sodass seine Bewertung in Zukunft eine rationalere Entscheidung für oder gegen eine Hinzunahme des anti-CTLA4-Antikörpers Ipilimumab in der Erstlinienbehandlung bei Patienten mit mRCC ermöglichen könnte. Über eine verbesserte Patientenselektion könnte die optimale Therapieoption (ICI+ICI versus ICI+TKI) für den individuellen Patienten identifiziert werden und gleichzeitig Immuntherapie-vermittelte Toxizität eingespart werden (Bedke et al., 2021; Puzanov et al., 2017).

### 2.5 Prädiktive *on-treatment* Biomarker für Immuntherapie

Die meisten der bisher vorgeschlagenen prädiktiven Biomarker für das Ansprechen auf ICI werden statisch zu einem Zeitpunkt vor Beginn der Therapie bestimmt. In vielen Fällen werden diese Biomarker basierend auf (jahrelang) archiviertem Tumormaterial erhoben. Dies ist insbesondere im ccRCC aufgrund der ausgeprägten intratumoralen Heterogenität und klonalen Tumorevolution problematisch, da die unmittelbare Situation vor Therapiestart durch Verwendung von archiviertem Tumormaterial meist unzureichend abgebildet ist (Gerlinger et al., 2012, 2014). Es wurde beispielsweise häufig eine Diskrepanz zwischen der PD-L1-Expression von Tumorzellen im Primärtumor und in den korrespondierenden Metastasen beobachtet (Callea et al., 2015).

Zudem wird vermutet, dass aufgrund der Komplexität der Tumor-Immun-Interaktionen statische Biomarker möglicherweise nicht ausreichend sind, um das Ansprechen auf ICI genau vorherzusagen. In diesem Zusammenhang rücken dynamische, sogenannte *on-treatment* Biomarker in den Fokus der Biomarker-Forschung der Immunonkologie (Bratman et al., 2020; Lesterhuis et al., 2017; Powles et al., 2021).

*On-treatment* Biomarker messen dynamische longitudinale Veränderungen nach Start der Therapie und können so über unmittelbare Therapie-assoziierte Veränderungen einen prädiktiven Wert aufweisen. Dies scheint insbesondere für die Immunonkologie ein attraktiver Ansatz zu sein, da so die komplexen Wechselwirkungen zwischen Tumor und Immunsystem abgebildet und charakterisiert werden können. Ein weiterer Vorteil von *on-treatment* Biomarkern für die klinische Praxis ist, dass sie häufig blutbasierte Analysen darstellen und die Bestimmung zusätzlicher Parameter im Rahmen der üblichen erforderlichen Therapiekontrollen keine weitere Belastung für den Patien-

ten darstellen. Ein Nachteil ist jedoch, dass die Bestimmung von *on-treatment* Biomarkern den Beginn der Therapie voraussetzt und dem Kliniker somit keine prädiktiven Informationen vor der Einleitung der definitiven Therapie liefert. Durch die Verwendung eines *on-treatment* Biomarkers wäre somit die unselektive Erstlinientherapieentscheidung für Patienten mit mRCC nicht direkt gelöst. Andererseits ermöglicht eine frühzeitige Prädiktion von Therapieansprechen und -versagen ein verbessertes Therapiemonitoring mit der Option auf unmittelbare Therapieadjustierung.

Die Bestimmung von zirkulierender Tumor-DNA (ctDNA) im Blut rückt als longitudinaler Biomarker zunehmend in den Mittelpunkt (Cheng et al., 2021). Eine hohe ctDNA-Konzentration als statischer Biomarker vor Beginn der Therapie ist ein Surrogatmarker für die Tumorlast und wird mit einer schlechten Prognose in Verbindung gebracht (Bratman et al., 2020; Maia et al., 2017; Nabet et al., 2020; Powles et al., 2021). Die Messung der longitudinalen ctDNA-Dynamik in der onkologischen Therapie und in der Nachsorge ist ein vielversprechender Ansatz. Die Reduktion der ctDNA unter ICI sagt das Therapieansprechen und den klinischen Verlauf sehr genau voraus (Bratman et al., 2020; Nabet et al., 2020). In einer prospektiven klinischen Phase-II-Studie (NCT02644369), in der die ctDNA bei N=94 Patienten mit fortgeschrittenen soliden Tumoren, die mit Pembrolizumab behandelt wurden, longitudinal untersucht wurde, zeigten alle 12 Patienten, bei denen die ctDNA unter ICI vollständig eliminiert wurde, ein hervorragendes Ansprechen auf die Behandlung und Langzeitüberleben (Bratman et al., 2020). Daher scheint die Integration von ctDNA-Messungen in der klinischen Praxis ein vielversprechendes Instrument für eine sensible Therapieüberwachung zu sein. Die Methodik zur Bestimmung der ctDNA ist jedoch teuer, komplex und derzeit nicht allgemein verfügbar, was den unmittelbaren klinischen Transfer dieses vielversprechenden *on-treatment* Biomarkers aktuell noch erschwert (Cheng et al., 2021; Yi et al., 2017).

Differentialblutbildveränderungen, wie eine dynamische Veränderung der Neutrophilen-Lymphozyten-Ratio, sowie longitudinale Veränderungen der Laktatdehydrogenase (LDH) oder von Akute-Phase Proteinen wie Fibrinogen und dem C-reaktiven Protein (CRP) wurden als prognostische/prädiktive Biomarker für die Immuntherapie vorgeschlagen (Mezquita et al., 2021; Nenclares et al., 2021; Raza et al., 2021; Saito and Kihara, 2011). Diese Parameter stellen aufgrund ihrer breiten Verfügbarkeit als Routinediagnostika besonders attraktive und kostengünstige Biomarker dar, die schnell in

den klinischen Alltag implementiert werden könnten. Eine umfangreiche prospektive Validierung dieser potenziellen Biomarker ist vor dem breiten Einsatz in der klinischen Praxis jedoch noch ausstehend.

Eine besonders interessante, hochprädiktive und früh nach dem Immuntherapiestart auftretende longitudinale CRP-Kinetik wurde zuletzt für das mRCC erstbeschrieben (Fukuda et al., 2021). Die Gruppe um Fukuda et al. beschrieb das sogenannte CRP-Flare-Response-Phänomen, das durch einen frühen CRP-Anstieg nach Beginn der ICI-Behandlung mit anschließendem Abfall unter das Baseline-Niveau definiert ist. Diese frühe *on-treatment* CRP-Kinetik scheint die dynamische Phase der systemischen Entzündung nach Induktion der gewünschten antitumoralen Immunantwort durch ICI widerzuspiegeln. Die zugrundeliegende immunologische Erklärung ist ausstehend. Bemerkenswert ist, dass dieses neue Konzept eine genaue Vorhersage des Therapieerfolgs in einer kleinen retrospektiven Kohorte von N=42 mit anti-PD-1 (Nivolumab) behandelten Patienten mit mRCC ermöglichte (Fukuda et al., 2021). Da aktuell vorrangig ICI-Kombinationstherapien für das mRCC eingesetzt werden, ist **ein drittes Ziel dieser kumulativen Habilitationsarbeit** den prädiktiven Wert der frühen *on-treatment* CRP-Flare-Response in einer multizentrischen Kohorte von Patienten mit mRCC zu untersuchen, die ICI+ICI oder ICI+TKI (N=95) als zugelassene Erstlinientherapie erhielten (Klümper et al., 2021b). Die Patienten wurden gemäß der Definition von Fukuda et al. in die drei CRP-Kinetik Subgruppen eingeteilt (siehe Abbildung 1):

- CRP-Flare-Response ist definiert als ein mindestens zweifacher Anstieg des Baseline-CRP innerhalb von 30 Tagen nach Therapiestart mit anschließendem Abfall unterhalb des Ausgangswertes innerhalb von 3 Monaten.

- CRP-Response ist definiert als ein CRP-Abfall um 30% unter den Ausgangswert innerhalb von 3 Monaten bei mindestens einer Messung.

- Alle anderen Patienten werden als CRP Non-Responder klassifiziert.

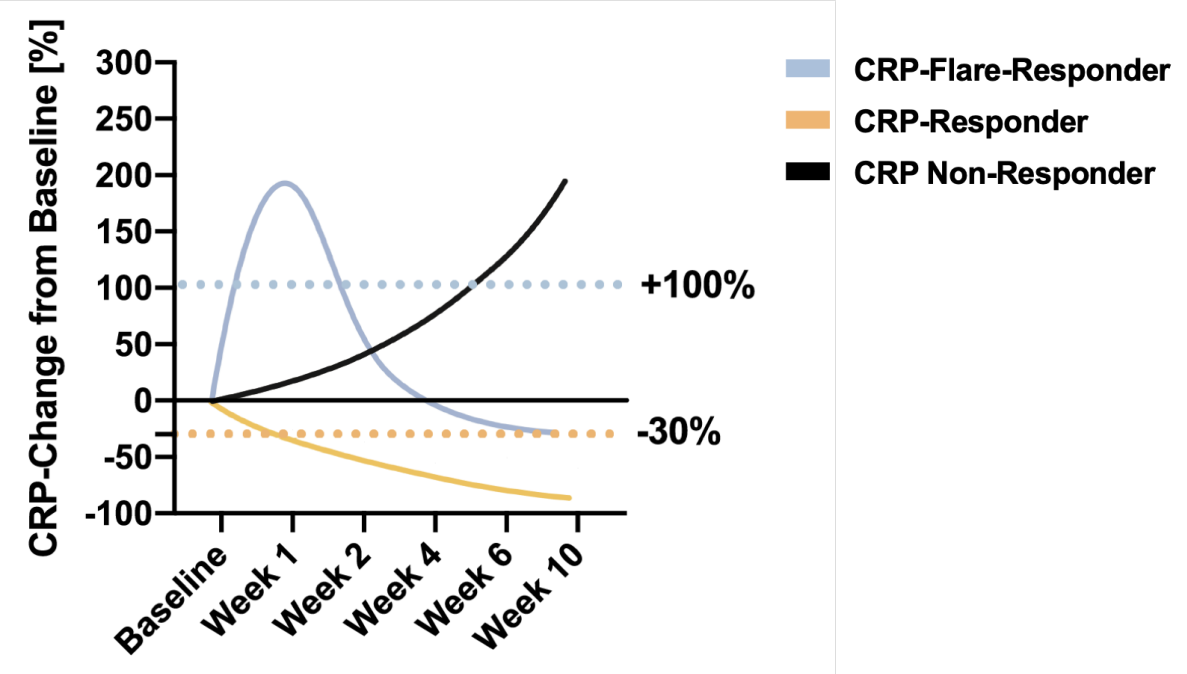


**Abbildung 1:** Schematische Darstellung der frühen *on-treatment* CRP-Kinetik Definition aus der Publikation IV (Klümper et al., 2022) dieser kumulativen Habilitationsarbeit, adaptiert von Fukuda et al. (Fukuda et al., 2021)

**Übergeordnete Zielsetzung dieser kumulativen Habilitationsarbeit** ist es, prädiktive (*on-treatment*) Biomarker, die das Ansprechen auf ICI-basierte Mono- bzw. Kombinationstherapien (ICI+ICI, ICI+TKI) im mRCC vorhersagen, zu identifizieren. Da viele der aktuell vorgeschlagenen prädiktiven Biomarker teure und umfangreiche Analysen beinhalten (u.a. TMB, RNAseq-basierte Transkriptionssignaturen, ctDNA-Dynamik), ist es ein weiteres Ziel, kosteneffiziente Biomarker herauszuarbeiten, die einen breiten Einsatz für den *standard of care* (SOC) zulassen würden. Aufgrund seiner breiten Verfügbarkeit und der relativ geringen Kosten scheint die *on-treatment* CRP-Kinetik somit ein hervorragender, leicht in den klinischen Alltag zu implementierender Biomarker für die Vorhersage des Ansprechens auf eine Immuntherapie zu sein.

Da die frühe *on-treatment* CRP-Kinetik möglicherweise ein biologisches, Tumortyp-unabhängiges Phänomen widerspiegelt, sollte dieses bisher relativ unbekannte Phänomen auch in anderen Tumorentitäten untersucht werden, bei denen ICI-basierte Therapien als SOC eingesetzt werden. **Im Rahmen dieser kumulativen Habilitationsarbeit** wird der prädiktive Wert der CRP-Flare Kinetik für Patienten mit NSCLC und UC untersucht, die ICI im Rahmen der Standardtherapie erhielten. Darum wurde die

Hypothese aufgestellt, dass die frühe CRP-Kinetik unabhängig von der Tumorentität ein vielversprechendes Instrument für eine verbesserte Therapieüberwachung in der Ära der Immunonkologie darstellen könnte.

Das CRP-Flare-Response-Phänomen wurde bei ICI-behandelten Patienten mit NSCLC in einer retrospektiven Entdeckungs- (N=105) und prospektiven Validierungskohorte (N=108) (Berner et al., 2019) untersucht. Die Patienten der prospektiven *immune monitoring of immune therapy* Studie (IMIT NSCLC), die von Juli 2016 bis Januar 2021 in vier Schweizer onkologischen Zentren durchgeführt wurde, wurden insbesondere früh nach Beginn der Immuntherapie regelmäßig untersucht. Im Rahmen dieser Studie konnte daher die longitudinale CRP-Kinetik aufgrund der frühen und regelmäßigen Studienbesuche sehr detailliert untersucht werden. Während die ursprüngliche Definition der frühen CRP-Kinetik einen prädiktiven Wert bis zur ersten radiologischen Bildgebung nach 8-12 Wochen ermöglichte (Fukuda et al., 2021; Klümper et al., 2021b), konnte für das NSCLC gezeigt werden, dass unsere Modifikation der frühen *on-treatment* CRP-Kinetik eine robuste Vorhersage des Therapieansprechens bereits vier Wochen nach Therapiestart zulässt. Diese Modifikation erhöht deren klinischen Wert signifikant, da sich ein großes therapeutisches Fenster für frühe Therapieanpassungen öffnet, beispielsweise im Rahmen von Biomarker-stratifizierten Interventionsstudien (Hu and Dignam, 2019).

# 3. Ergebnisse

## 3.1 Klümper, N. et al. (2020). LAG3 (LAG-3, CD223) DNA methylation correlates with LAG3 expression by tumor and immune cells, immune cell infiltration, and overall survival in clear cell renal cell carcinoma. J Immunother Cancer 8, e000552

LAG3 ist ein vielversprechendes immuntherapeutisches Angriffsziel für Patienten mit mRCC. Ziel dieser Studie war es, die epigenetische Regulierung von *LAG3* im ccRCC zu untersuchen. Es wurde die quantitative *LAG3*-Methylierung (mLAG3) mit der transkriptionellen Aktivität, der Immunzellinfiltration und dem klinischen Verlauf von ccRCC-Patienten in zwei verschiedenen ccRCC-Kohorten (Entdeckungs- und Validierungskohorte) assoziiert. Der Methylierungsstatus des Tumors wurde mit dem vom normalen tumornahen Gewebe (NAT) verglichen. Die Entdeckungskohorte umfasste N=533 ccRCC-Patienten und N=160 NAT und wurde über das *The Cancer Genome Atlas (TCGA) Research Network* bezogen. In einer unabhängigen Validierungskohorte (UKB Kohorte, N=118) wurde mLAG3 mittels quantitativer methylierungsspezifischer PCR (mLAG3-qMSP) untersucht und mit der immunhistochemisch gemessenen LAG3-Proteinexpression und Infiltration von Immunzellsubtypen (CD4+, CD8+, CD45+ IHC) assoziiert. Darüber hinaus korrelierte mLAG3 mit der LAG3 mRNA Expression mononukleärer Zellen des peripheren Blutes (PBMCs) und konventioneller ccRCC-Tumorzelllinien. Die Untersuchungen zeigten unterschiedliche Methylierungsprofile in PBMCs, NAT, ccRCC-Zelllinien und ccRCC-Tumorgewebe. Die Methylierung des Promotors korrelierte invers mit der LAG3-mRNA- (TCGA-Kohorte) und Proteinexpression (UKB-Kohorte). Darüber hinaus korrelierte die LAG3-Methylierung stark mit der inflammatorischen Interferon-γ-Signatur (TCGA-Kohorte) und intratumoralen Immunzellinfiltration (UKB-Kohorte). Die LAG3-mRNA-Expression (TCGA-Kohorte), der Methylierungsstatus beider Kohorten und die tumorintrinsische Proteinexpression (UKB-Kohorte) waren signifikant mit dem OS assoziiert. Die Daten der Studie deuten auf eine epigenetische Regulierung der LAG3-Expression in Tumor- und Immunzellen durch die DNA-Methylierung hin. Über die Erhebung von mLAG3 wurde eine Patientengruppe mit starker Inflammation im Tumor mit eingeschränkter Prognose identifiziert. Diese Ergebnisse stellen die Grundlage für weitere Untersuchungen zu mLAG3 als prädiktiven Biomarker für das Ansprechen auf anti-LAG3-basierte ICI dar, welcher sich aktuell in der klinischen Testung befindet.

Journal for ImmunoTherapy of Cancer

# *LAG3* (*LAG-3, CD223*) DNA methylation correlates with LAG3 expression by tumor and immune cells, immune cell infiltration, and overall survival in clear cell renal cell carcinoma

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**To cite:** Klümper N, Ralser DJ, Bawden EG, *et al*. *LAG3* (*LAG-3, CD223*) DNA methylation correlates with LAG3 expression by tumor and immune cells, immune cell infiltration, and overall survival in clear cell renal cell carcinoma. *Journal for ImmunoTherapy of Cancer* 2020;**8**:e000552. doi:10.1136/jitc-2020-000552

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Accepted 11 February 2020

Check for updates

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## ABSTRACT

**Background** Lymphocyte activating 3 (LAG3, LAG-3, CD223) is a promising target for immune checkpoint inhibition in clear cell renal cell carcinoma (KIRC). The aim of this study was to investigate the epigenetic regulation of *LAG3* in KIRC by methylation.

**Methods** We correlated quantitative *LAG3* methylation levels with transcriptional activity, immune cell infiltration, and overall survival in a cohort of n=533 patients with KIRC and n=160 normal adjacent tissue (NAT) samples obtained from The Cancer Genome Atlas (TCGA). Furthermore, we analyzed *LAG3* methylation in peripheral blood mononuclear cells (PBMCs) and KIRC cell lines. We validated correlations between LAG3 expression, immune cell infiltrates, survival, and methylation in an independent KIRC cohort (University Hospital Bonn (UHB) cohort, n=118) by means of immunohistochemistry and quantitative methylation-specific PCR.

**Results** We found differential methylation profiles among PBMCs, NAT, KIRC cell lines, and KIRC tumor tissue. Methylation strongly correlated with LAG3 mRNA expression in KIRCs (TCGA cohort) and KIRC cell lines. In the UHB cohort, methylation correlated with LAG3-positive immune cells and tumor-intrinsic LAG3 protein expression. Furthermore, *LAG3* methylation strongly correlated with signatures of distinct immune cell infiltrates, an interferon-γ signature (TCGA cohort), and immunohistochemically quantified $CD45^+$, $CD8^+$, and $CD4^+$ immune cell infiltrates (UHB cohort). LAG3 mRNA expression (TCGA cohort), methylation (both cohorts), and tumor cell-intrinsic protein expression (UHB cohort) was significantly associated with overall survival.

**Conclusion** Our data suggest an epigenetic regulation of LAG3 expression in tumor and immune cells via DNA methylation. LAG3 expression and methylation is associated with a subset of KIRCs showing a distinct clinical course and immunogenicity. Our study provides rationale for further testing *LAG3* DNA methylation as a predictive biomarker for response to LAG3 immune checkpoint inhibitors.

## BACKGROUND

Clear cell renal cell carcinoma (KIRC) represents 80% of kidney malignancies and is among the most common malignancies worldwide.[1] Although overall 5-year survival rates are approximately 75%, treatment of advanced and metastatic KIRC remains a challenge due to limited therapy options. For years, resistance to chemotherapy and radiation has been a major issue rendering surgical cytoreductive procedure the only effective therapy for advanced and metastatic KIRC. Development and clinical implementation of novel systemic therapies, such as multitarget tyrosine kinase inhibitors, has improved the long-term survival of advanced stage KIRC and recently brought into question the therapy regime with cytoreductive surgical therapy.[2] The development of immune checkpoint inhibitors targeting the cytotoxic T-lymphocyte associated protein 4 (CTLA-4) or the programmed cell death 1 (PD-1) receptor/PD-1 ligand 1 (PD-L1)-axis have led to improved clinical outcomes in advanced and metastatic disease stages across various malignancies such as melanoma or non-small cell lung cancer.[3 4] Recent studies have also demonstrated efficacy in targeting PD-1/PD-L1 and CTLA-4 in patients with metastatic KIRC.[5 6] Despite the great success of immunotherapies targeting the PD-1/PD-L1 axis, many patients do not respond or ultimately develop resistance under treatment. As a result, other inhibitory receptors are being investigated as alternative immune therapy targets.[7] One of these potential targets is the immune checkpoint lymphocyte activating 3 (LAG3, LAG-3, CD223).[8 9]

The regulation of *LAG3* in KIRC on an epigenetic level, however, is largely unknown and might involve DNA methylation. DNA methylation is an important epigenetic mechanism involved in many fundamental biological processes, that is, differentiation (including T cell differentiation), X chromosome inactivation, and imprinting. Methylation of the gene promoter region is frequently associated with transcriptional silencing while gene body methylation is often a hallmark of transcriptionally active genes.[10 11] Elucidation of the epigenetics of immune-checkpoint receptor genes might help in understanding mechanisms of response and resistance to the respective inhibitors, ultimately paving the way for the development of predictive biomarkers.

Methylation has been shown to regulate expression of PD-1 and PD-L1 in various malignancies.[12–16] We therefore hypothesized that LAG3 expression might also be regulated epigenetically via DNA methylation. Understanding the epigenetic regulation of LAG3 expression is of major interest, as it could be useful for the stratification of patients who may benefit from immunotherapeutic LAG3 inhibition.

## MATERIAL AND METHODS

The results shown here are partly based on data generated by *The Cancer Genome Atlas Research Network* (TCGA, http://cancergenome.nih.gov/). The KIRC TCGA cohort comprises in total n=741 samples consisting of n=533 tumor samples and n=208 normal adjacent tissues (NAT). For the majority of the KIRC cohort, clinical follow-up and overall survival (OS) data were available (n=534, mean follow-up period 3.68 years, range 0–12.4 years). The mean age at initial diagnosis was 61.1 (range 26–90). For validation, a second cohort comprising n=118 formalin-fixed and paraffin-embedded KIRC tumors from patients treated at the University Hospital Bonn was included (UHB cohort). The mean follow-up period was 4.6 years (range 0–14.1 years), and the mean age at initial diagnosis was 62.7 (range 28–84).

### Methylation analysis

Methylation data of the TCGA KIRC cohort were generated using the Infinium HumanMethylation450 BeadChip (Illumina, San Diego, California, USA) and were downloaded from the UCSC Xena browser (http://xena.ucsc.edu, KIRC n=318, NAT n=160). Beta values, approximately considered per cent methylation, for 16 CpG sites targeted by Infinium HumanMethylation450 BeadChip beads within *LAG3* were analyzed (figure 1).

Infinium HumanMethylation450 BeadChip DNA methylation data of peripheral blood mononuclear cells (PBMCs, n=25) and human KIRC cell lines (n=12) were downloaded from NCBI (National Center for Biotechnology Information) Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/, GSE82221, GSE89649).[17–20]

Methylation analysis of the UHB cohort was performed using bisulfite-specific quantitative real-time PCR employing methylation-unspecific primers and probe pairs specifically and competitively binding methylated and unmethylated template DNA, respectively. This quantitative methylation-specific PCR (qMSP) is described in detail by Lehmann and Kreipe.[21] Tumor tissue was annotated and macrodissected from tissue sections mounted on glass slides and subsequently lysed and bisulfite

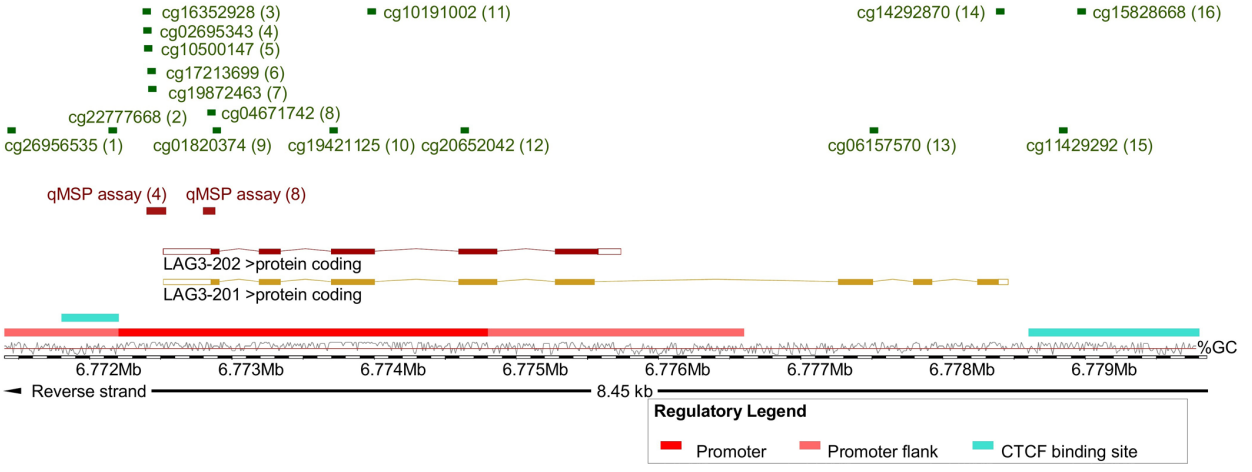


**Figure 1** Overview of analyzed methylation sites and genomic organization of *LAG3*. Shown is chromosome 12, position 6 771 404–6 779 853, including the *LAG3* gene, its transcripts and regulatory elements (promoter, promoter flank, and CCCTC-binding factor (CTCF) binding site), and the investigated loci. The *LAG3* methylation target sites of the HumanMethylation450 BeadChip beads (1-16) and qMSP assays (4 and 8) are based on Genome Reference Consortium Human Build 38 patch release 13 (GRCh38.p13). The illustration (modified) was exported from www.ensemble.org (release 98).[50] Beads are numbered as follows: cg26956535 (1), cg22777668 (2), cg16352928 (3), cg02695343 (4), cg10500147 (5), cg17213699 (6), cg19872463 (7), cg04671742 (8), cg01820374 (9), cg19421125 (10), cg10191002 (11), cg20652042 (12), cg06157570 (13), cg14292870 (14), cg11429292 (15), and cg15828668 (16). LAG3, lymphocyte activating 3; qMSP, quantitative methylation-specific PCR.

converted using the innuCONVERT Bisulfite All-In-One Kit (Analytik Jena, Jena, Germany) according to the manufacturer's protocol. We developed two qMSP assays (qMSP assays 4 and 8) that target CpG sites as probed by Illumina HumanMethylation450 BeadChip beads 4 and 8, respectively (figure 1). Uncalibrated methylation levels, approximately considered per cent methylation, were computed using cycle threshold (CT) values obtained from the probes specifically binding to methylated ($CT_{methylated}$) and unmethylated ($CT_{unmethylated}$) DNA, respectively (methylation [%]=$100\%/(1+2^{CT_{methylated}-CT_{unmethylated}})$). We performed 20 µL triplicate PCR reactions using buffer composition as previously described[22] containing 20 ng bisulfite converted DNA (quantified via UV-VIS spectrophotometry) and 0.2 µM each probe and 0.2 µM each primer (qMSP assay 4 forward primer: aacccccctcaaactttccacta, reverse primer: gttttgttggtttttgggtttttatttt, $probe_{methylated}$: 6-FAM-tagggtttacggtttcgtttcgt-BHQ-1, $probe_{unmethylated}$: HEX-gtattttagggtttatggttttgttttgta-BHQ-1; qMSP assay 8 forward primer: ctttccttttctaacctcctttta, reverse primer: gtaagtttaggaattgagttttttatatt, $probe_{methylated}$: 6-FAM-tggtt tgggtagcgttgagttt-BHQ-1, $probe_{unmethylated}$: HEX-atggtttg ggtagtgttgagttttt-BHQ-1). qMSP was carried out using a 7900HT Fast Real-Time PCR system (Applied Biosystems, Waltham, Massachusetts, USA) with the following temperature profile: 10 min at 95°C and 40 cycles with 15 s at 95°C, 2 s at 62°C, and 60 s at 58°C (qMSP assay 4) or 53°C (qMSP assay 8).

### mRNA expression data

TCGA transcriptome sequencing data (RNA-Seq v2) were downloaded from the UCSC Xena browser (http://xena.ucsc.edu) (KIRC n=533, NAT n=72). Log2 transformed RNA sequencing data generated by IlluminaHiSeq (Illumina) were used for bioinformatical analysis. Transcriptome data of GSE82221 was generated by HumanHT-12 v4.0 Gene Expression BeadChip (Illumina), GSE89649 by Human Transcriptome Array 2.0 (transcript (gene) version) (Affymetrix, Santa Clara, California, USA).

### Mutation status

The mutation status of *VHL*, *PBRM1*, *SETD2*, *BAP1*, and *KDM5C* in the KIRC TCGA cohort as determined via whole-exome sequencing was downloaded from the cBioportal website (http://www.cbioportal.org).[23 24]

### Immune cell infiltration

Quantitative RNA-Seq signatures of infiltrating B cells, dendritic cells, neutrophils, macrophages, $CD4^+$, and $CD8^+$ T cells in the KIRC TCGA cohort were obtained from Li *et al*.[25] We further used CD8A, CD8B, and CD4 mRNA expression data from the TCGA cohort as surrogate for the infiltration by the respective T cell subsets. In the UHB cohort, infiltrates of $CD45^+$ leukocytes, $CD4^+$, and $CD8^+$ T cells were quantified via immunohistochemistry (IHC) as described below.

### Immunohistochemistry

Immunohistochemical staining of CD45, CD8, and CD4 was performed as previously described using the following monoclonal antibodies and dilutions:[26] anti-CD45 clones 2B11+PD7/26 (#M070101, Dako/Agilent Technologies, Santa Clara, California, USA), 1:100; anti-CD8 clone C8/144B (#M710301, Dako/Agilent Technologies), 1:50; anti-CD4 clone SP35, #503–3354 (Zytomed Systems GmbH, Berlin, Germany), 1:20. $CD45^+$, $CD8^+$, and $CD4^+$ immune cell infiltrates were evaluated as percentage of positive cells from all cells in the tumor.

For LAG3 IHC antigen retrieval was performed in pH 6 Target Retrieval Solution (#S169984-2, Dako/Agilent Technologies) for 10 min at 100°C. After blocking, the 5 µm sections were incubated with the primary monoclonal LAG3 antibody (clone IHC103, #IHC103-1, GenomeMe Lab, Richmond, Canada) at 4°C overnight and washed with 550 mM TBS. Visualization was performed employing the Dako REAL Detection System Alkaline Phosphatase/RED (Dako/Agilent Technologies, #K5005). Staining was contrasted with Mayer's Hemalum solution (Merck Millipore, Billerica, MA, USA, #HX73030749). Absence or presence of $LAG^+$ immune cells and quantitative (H-score) tumor cell-intrinsic LAG3 expression was evaluated by an experienced pathologist (MT).

### Statistics

Statistical analyses were performed using Microsoft Excel, GraphPad PRISM and SPSS V.25.0. Spearman's $\rho$ correlation coefficients were calculated. Depending on the distribution group, comparisons were done using parametric two-sided student's *t*-test or nonparametric Mann-Whitney *U* test. Survival analyses of dichotomized variables were performed by Kaplan-Meier and continuous log2-transformed variables were used for Cox proportional hazards analyses. P values refer to logrank and Wald tests, respectively. Two-sided $p<0.05$ were considered as statistically significant.

## RESULTS

The genomic organization of *LAG3* is depicted in figure 1. The *LAG3* gene encodes for two protein coding transcripts sharing the same transcription start site. Transcript LAG3-202 represents a truncated version of the full-length transcript LAG3-201 lacking the last three exons. An extended promoter and its flanks are predicted in the region of the transcription start site. Binding sites of the transcriptional repressor CTCF (CCCTC-binding factor) are predicted within the promoter and downstream from *LAG3*. In total, we investigated 16 CpG sites probed by beads from the Infinium HumanMethylation450 BeadChip. CpG sites 1–12 are located in the promoter and promoter flank region, CpG site 2 is found in the promoter-embedded CTCF binding site, beads 13 and 14 target CpG sites within the gene body downstream from LAG3-202 and CpGs 15 and 16 are situated in the

downstream CTCF binding site. We further developed qMSP assays that target promoter CpG sites 4 and 8. In total, we analyzed a cohort comprised of n=318 patients with KIRC and n=24 NAT obtained from TCGA, a cohort of n=118 KIRCs from the University Hospital Bonn (UHB cohort), isolated PBMCs (n=25), and human KIRC cell lines (n=12) with regard to *LAG3* gene methylation and expression.

### *LAG3* is broadly hypomethylated in KIRC versus NAT

First, we investigated *LAG3* methylation status in KIRC tissue compared with NAT. Fourteen out of 16 CpG sites were differentially methylated between KIRC and NAT (table 1, figure 2A). Interestingly, CpG sites located within the promoter and gene body region showed significant hypomethylation in KIRC versus NAT. Contrarily, CpG site 15 and 16, located downstream of *LAG3* within the CTCF binding site, were hypermethylated in KIRC. Of note, promoter CpG sites (CpG sites 3–8 and 10–12) showed high correlation coefficients towards each other indicating a high degree of co-methylation (figure 3E). On the other hand, CpG sites 14 and 15 showed an inverse methylation pattern compared with sites 3–8 and 10–12. We validated the level of comethylation of CpG sites 4 and 8 via qMSP in the independent UHB cohort (TCGA cohort: Spearman's $\rho$=0.62, p<0.001; UHB cohort: $\rho$=0.66, p<0.001).

### LAG3 expression correlates with methylation

Second, we analyzed the extent to which *LAG3* mRNA expression correlated to CpG methylation (table 1). In KIRC tissue, 12 out of 16 analyzed CpG sites significantly correlated with *LAG3* mRNA expression. Interestingly, *LAG3* promoter and body hypomethylation were associated with increased *LAG3* mRNA expression, indicated by strong negative correlation coefficients (figure 3A–C). Conversely, methylation downstream of the *LAG3* gene (figure 3D) correlated with *LAG3* mRNA upregulation. This effect was most pronounced at CpG site 15 located within the binding site of the transcriptional repressor CTCF. We found a significant inverse correlation between methylation and mRNA expression in NAT at one CpG site (CpG site 10, table 1). A negative correlation at CpG site 11 was present in KIRC cell lines. Of note, we detected a significant positive correlation at CpG site 13 in KIRC cell lines whereas methylation of this CpG site did not correlate with mRNA expression in KIRC tumors. Since the microenvironment of KIRC tumors comprises many cell types, the lack of correlation at this site in KIRC tumors might be due to oppositely directed correlations in immune cells compared with KIRC cells. Accordingly, we found a strong negative correlation between methylation and expression at site 13 in PBMCs. Other CpG sites (8, 9, 12, and 15) revealed similar correlations in PBMCs compared with KIRC tumors. An additional significant correlation in PBMCs was found for CpG site 1 which was not observed in KIRC tumors, cell lines or NAT. Despite the low number of analyzed cell lines, PBMC, and NATs necessitating a thorough consideration of correlation coefficients and p values, our data clearly show differences in correlates of mRNA expression with *LAG3* methylation among distinct cell lineages, suggesting differences in epigenetic regulation between NAT, PBMCs, and KIRC tumor cell lines.

Next, we analyzed the correlation between methylation (CpG site 4 and 8) and expression of LAG3 on the protein level in our independent UHB KIRC cohort (n=118) with IHC and qMSP. CpG site 4 was selected as hypomethylation of this CpG site showed the strongest negative correlation with LAG3 mRNA expression in the TCGA KIRC cohort. Methylation of CpG site 8, on the other hand, was strongly correlated with reduced mRNA expression in PBMCs. Interestingly, a subset of tumors (32/118; 27.1%, figure 4A) exhibited tumor cell-intrinsic LAG3 protein expression, however, on a low expression level. LAG3-positive immune cells were rare and mainly present in the tumors with concomitant tumor cell-intrinsic LAG3 expression indicated by high correlation coefficient (22/118; 18.6%; Spearman's $\rho$=0.62, p<0.001). Of note, LAG3 protein expression was also observed in the proximal tubules in the normal adjacent kidney (figure 4A) and in the urothelium of the renal pelvis. Methylation status of CpG sites 4 and 8 negatively correlated with LAG3 protein expression by tumor cells (CpG 4: Spearman's $\rho$=–0.42, p<0.001; CpG 8: Spearman's $\rho$=–0.27, p=0.004). Thus, we were able to confirm the proposed epigenetic regulation of LAG3 on the translational level in an independent cohort consistent with the results obtained from the KIRC TCGA.

### *LAG3* promoter hypomethylation and expression is associated with immune cell infiltration

The tumor microenvironment can be infiltrated by various distinct immune cell subsets. We correlated RNAseq signatures of B cells, $CD4^+$ and $CD8^+$ T cells, neutrophils, macrophages, and dendritic cells with *LAG3* methylation levels. We found an association of *LAG3* promoter hypomethylation with higher infiltration of immune cells. In contrast, hypermethylation of CpG site 15, which is located in the downstream CTCF binding site, was correlated with increased immune cell infiltration (figure 2). This observation was supported by strong correlation between *LAG3* promoter hypomethylation and elevated mRNA levels of the cluster of differentiation markers CD4, CD8A, and CD8B (figure 2A). These analyses are based on whole tumor tissue comprising tumor cells as well as stroma and infiltrating immune cells. *LAG3* methylation in isolated PBMCs, comprising lymphocytes and monocytes, showed strong *LAG3* promoter hypomethylation. Renal cell carcinoma cell lines, devoid of immune cells or stroma, on the other hand, were hypermethylated at the *LAG3* promoter (figure 2B). Consistent with this finding, the arithmetic mean *LAG3* methylation of the whole tumor, representing a composition of both tumor cells and immune infiltrates, was greater than that of PBMCs and less than that of the renal cell carcinoma cell lines (figure 2).

Further, we evaluated the relationship between *LAG3* methylation and an IFN-γ signature, indicated by IFN-γ,

**Table 1** *LAG3* methylation levels and correlation with mRNA expression and overall survival

| | | Mean methylation level | | | Spearman's correlation between methylation and mRNA expression | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Tumor, n=318 | | NAT, n=24 | | KIRC cell lines, n=12 | | PBMCs, n=25 | | Overall survival, n=318 | |
| Bead (CpG site) | Number | NAT (%), n=160 | Tumor (%), n=318 | P value | Spearman's ρ | P value | Spearman's ρ | P value | Spearman's ρ | P value | Spearman's ρ | *P*-value | HR (95% CI) | P value |
| cg26956535 | 1 | **90.5** | **88.3** | **<0.001** | −0.07 | 0.20 | −0.21 | 0.33 | −0.02 | 0.95 | **−0.50** | **0.012** | 0.28 (0.03 to 2.52) | 0.26 |
| cg22777668 | 2 | **33.0** | **23.0** | **<0.001** | −0.08 | 0.14 | 0.16 | 0.46 | −0.20 | 0.54 | −0.18 | 0.40 | 0.45 (0.04 to 4.66) | 0.50 |
| cg16352928 | 3 | **74.2** | **65.7** | **<0.001** | **−0.55** | **<0.001** | 0.01 | 0.96 | −0.46 | 0.13 | −0.18 | 0.39 | **0.05 (0.01 to 0.22)** | **<0.001** |
| cg02695343 | 4 | **78.7** | **65.2** | **<0.001** | **−0.60** | **<0.001** | −0.02 | 0.91 | −0.12 | 0.71 | −0.06 | 0.77 | **0.04 (0.01 to 0.18)** | **<0.001** |
| cg10500147 | 5 | **78.5** | **59.3** | **<0.001** | **−0.54** | **<0.001** | −0.12 | 0.59 | −0.26 | 0.42 | −0.05 | 0.80 | **0.13 (0.04 to 0.38)** | **<0.001** |
| cg17213699 | 6 | **93.2** | **78.1** | **<0.001** | **−0.59** | **<0.001** | −0.11 | 0.63 | −0.40 | 0.20 | 0.23 | 0.26 | **0.13 (0.04 to 0.39)** | **<0.001** |
| cg19872463 | 7 | **90.9** | **73.8** | **<0.001** | **−0.56** | **<0.001** | −0.09 | 0.69 | −0.19 | 0.56 | 0.24 | 0.24 | **0.23 (0.08 to 0.69)** | **0.01** |
| cg04671742 | 8 | **84.5** | **78.4** | **<0.001** | **−0.49** | **<0.001** | −0.01 | 0.96 | 0.02 | 0.95 | **−0.61** | **0.001** | 0.21 (0.03 to 1.55) | 0.13 |
| cg01820374 | 9 | **58.4** | **39.6** | **<0.001** | **−0.26** | **<0.001** | 0.37 | 0.08 | −0.04 | 0.90 | **−0.61** | **0.001** | 0.52 (0.08 to 3.38) | 0.50 |
| cg19421125 | 10 | **72.2** | **76.5** | **<0.001** | **−0.55** | **<0.001** | **−0.47** | **0.04** | −0.18 | 0.58 | 0.03 | 0.88 | **0.03 (0.01 to 0.18)** | **<0.001** |
| cg10191002 | 11 | 61.5 | 61.6 | 0.95 | **−0.53** | **<0.001** | −0.40 | 0.05 | **−0.63** | **0.03** | 0.21 | 0.32 | **0.02 (0.01 to 0.23)** | **<0.001** |
| cg20652042 | 12 | **83.5** | **76.4** | **<0.001** | **−0.62** | **<0.001** | 0.17 | 0.44 | −0.43 | 0.17 | **−0.52** | **0.007** | 0.20 (0.01 to 3.88) | 0.29 |
| cg06157570 | 13 | **78.9** | **75.4** | **<0.001** | −0.07 | 0.20 | 0.23 | 0.28 | **0.65** | **0.02** | **−0.44** | **0.026** | **6.69 (1.53 to 29.3)** | **0.01** |
| cg14292870 | 14 | 38.3 | 37.0 | 0.23 | **0.44** | **<0.001** | 0.35 | 0.09 | 0.34 | 0.29 | 0.01 | 0.96 | **7.81 (1.92 to 31.8)** | **<0.001** |
| cg11429292 | 15 | **8.1** | **17.3** | **<0.001** | **0.79** | **<0.001** | −0.24 | 0.26 | 0.39 | 0.22 | **0.60** | **0.002** | **9.67 (1.29 to 72.4)** | **0.03** |
| cg15828668 | 16 | **5.0** | **5.5** | **<0.001** | −0.02 | 0.72 | −0.01 | 0.98 | 0.22 | 0.48 | −0.02 | 0.95 | 0.91 (0.57 to 1.49) | 0.54 |

*LAG3* methylation levels in NATs and KIRC tumors, correlations of methylation and mRNA expression levels (in tumors, NATs, PBMCs, and KIRC cell lines), and association of methylation and overall survival. Shown are the results from 16 CpG sites within the *LAG3* gene locus. Significant data are shown in boldface.
KIRC, clear cell renal cell carcinoma; LAG3, lymphocyte activating 3; NAT, normal adjacent tissue; PBMC, peripheral blood mononuclear cell.

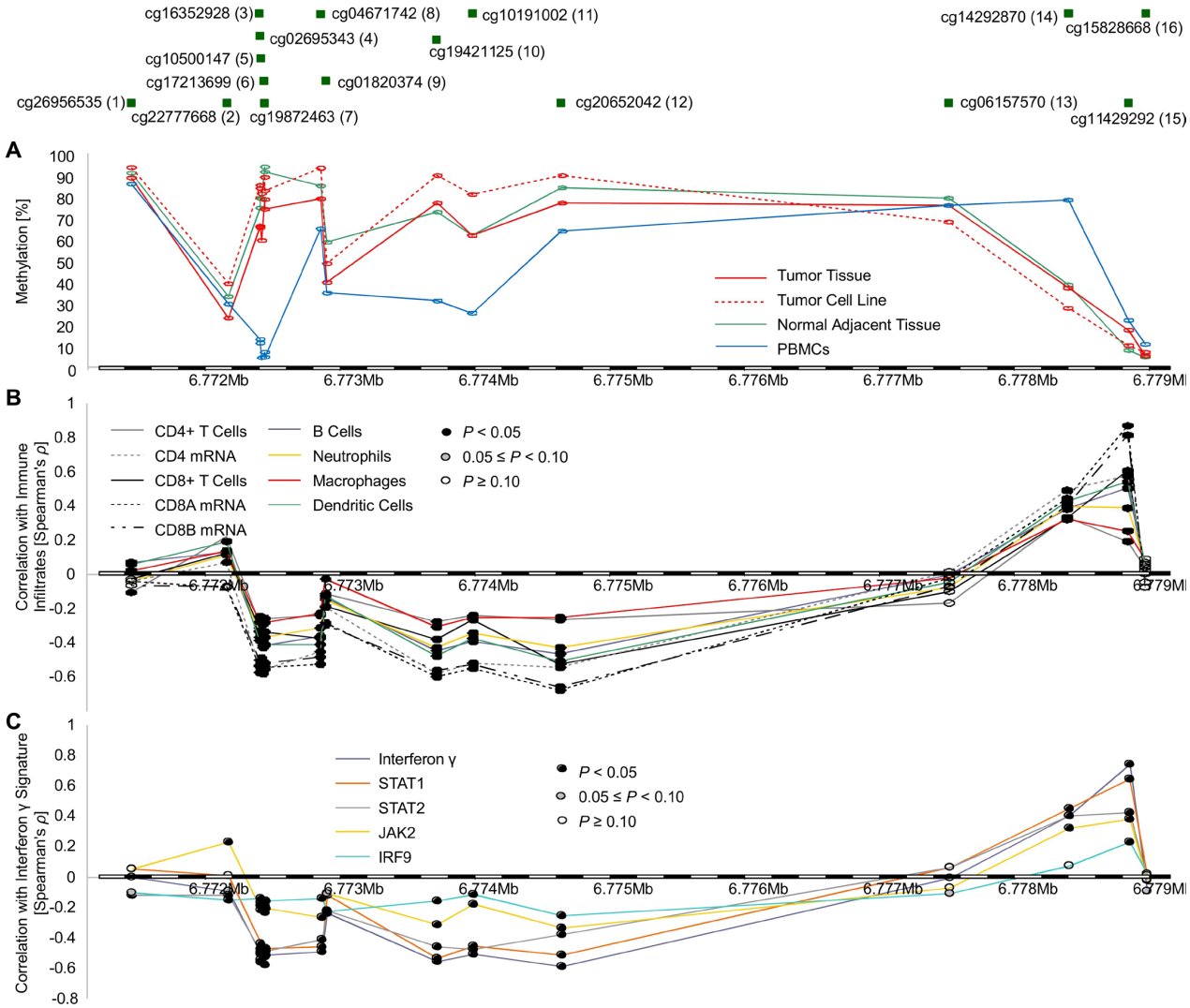


**Figure 2** *LAG3* methylation in KIRC, isolated tumor cell lines, and PBMCs and its correlation with immune cell infiltrates and IFN-γ signature. Correlation of *LAG3* methylation status in KIRC, NAT, PBMCs (GSE82221), and KIRC cell lines (GSE89649) (A) with leukocyte subtypes (CD4, CD8A, and CD8B mRNA; and absolute immune cell infiltrates adopted from Li *et al*[21]) (B), and IFN-γ signature (C). (A)–(C) are illustrated with regard to CpG sites targeted by HumanMethylation450 BeadChip. KIRC, clear cell renal cell carcinoma; LAG3, lymphocyte activating 3; NAT, normal adjacent tissue; PBMC, peripheral blood mononuclear cell.

STAT1, STAT2, JAK2, and IRF9 mRNA expression. Widespread promoter hypomethylation and body hypermethylation were strongly associated with increased IFN-γ signature. Consistent with results presented above, methylation of CpG sites 14 and 15 showed positive correlations with an IFN-γ signature (figure 2C).

We next evaluated the correlation of *LAG3* methylation status of CpG sites 4 and 8 with immune cell infiltration in the UHB cohort. In addition to the methodology described above, which is entirely based on RNA signatures of immune cell infiltrates, the proportion of $CD45^+$, $CD4^+$, and $CD8^+$ immune cells was evaluated via IHC. We were able to confirm that *LAG3* methylation status correlated with pan-leukocyte infiltration measured by CD45 IHC, as well as with infiltration of $CD4^+$ and $CD8^+$ T cells (figure 4). Further, strong infiltration of $CD45^+$, $CD8^+$, and $CD4^+$ immune cells was seen in the LAG3 expressing KIRC tumors.

### Common driver mutations are associated with *LAG3* methylation

In the KIRC TCGA cohort, the most frequent driver mutations are located in genes regulating oxygen metabolism and chromatin modification, namely *VHL*, *PBRM1*, *SETD2*, *BAP1*, and *KDM5C* (TCGA mutation frequency approximately: 49.9%, 30.6%, 11.3%, 8.9%, 6.0%, respectively). *VHL*-mutation status showed no correlation to *LAG3* methylation. In contrast, methylation at several CpG loci were associated with mutation status of the most frequently mutated chromatin modifier genes, especially *PBRM1* and *BAP1* (table 2).

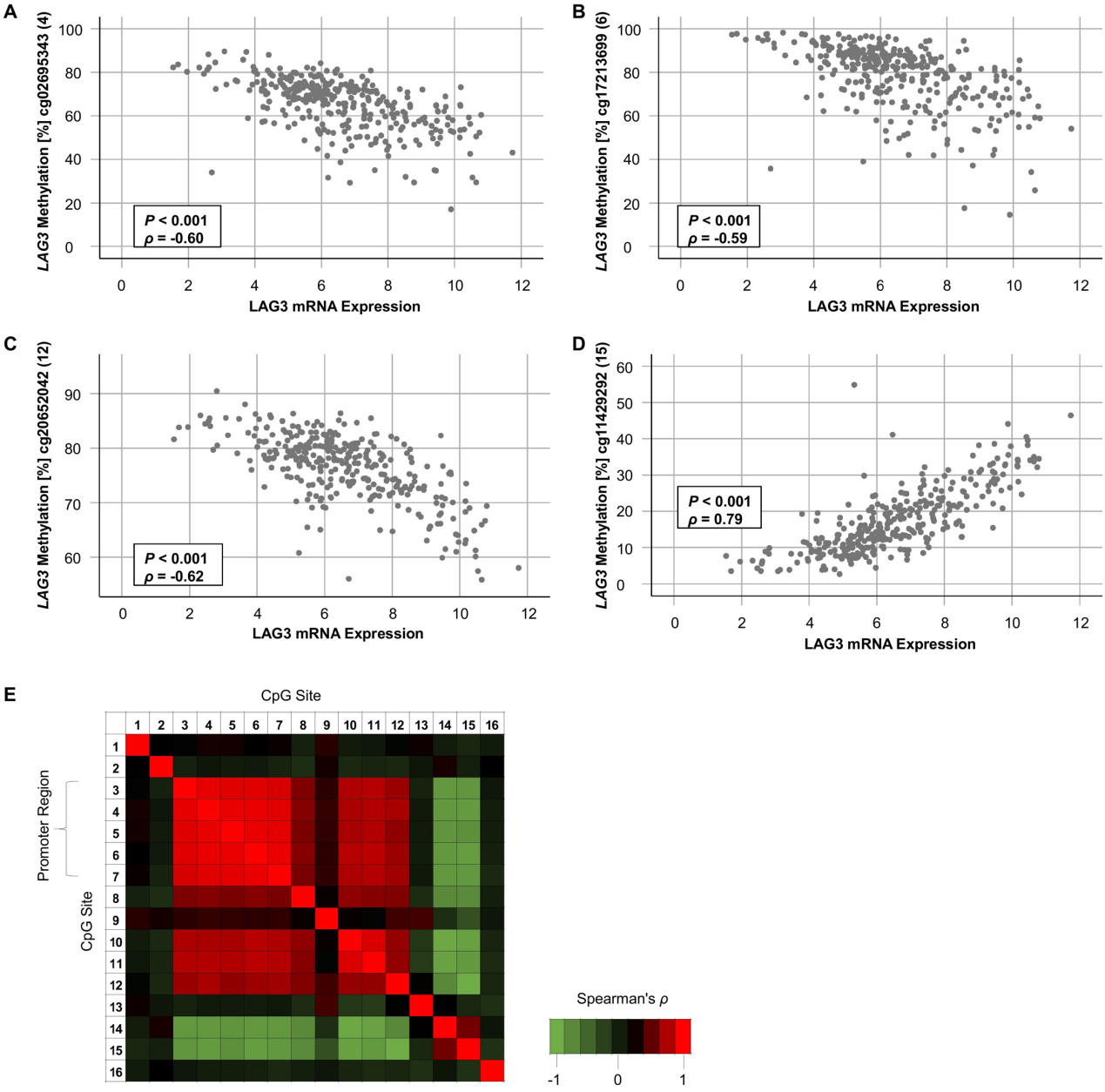


**Figure 3** Correlation of *LAG3* methylation and mRNA expression. (A–D) Scatter plots showing *LAG3* methylation with respect to LAG3 mRNA expression of the four CpG sites with highest correlation coefficients targeted by HumanMethylation450 BeadChip beads cg02695343 (4, (A)), cg17213699 (6, (B)), cg20652042 (12, (C)), and cg11429292 (15, (D)) in n=318 KIRC samples. (E) Correlation heatmap of methylation levels of 16 *LAG3* CpG sites. LAG3, lymphocyte activating 3; KIRC, clear cell renal cell carcinoma.

### *LAG3* promoter hypomethylation and expression predict overall survival in KIRC

Next, we evaluated whether *LAG3* methylation levels in KIRC are correlated with OS. In univariate Cox regression analyses, differential methylation of 10 out of 16 CpG sites significantly correlated with OS (table 1). *LAG3* promoter hypomethylation was associated with unfavorable OS (CpG loci 3–7, 10, and 11) while higher methylation of CpG sites 13, 14, and 15, located in the gene body and the downstream CTCF binding site, significantly correlated to poor OS (table 1, figure 5). These results were validated via qMSP methylation analyses of CpG loci 4 and 8 in the UHB cohort. Hypomethylation of CpG sites 4 and 8 was significantly associated with worse OS measured by Cox regression (CpG site 4: HR (95% CI) 0.39 (0.02 to 0.66), p=0.001; CpG site 8: HR (95% CI) 0.11 (0.02 to 0.88), p=0.037).

As previously shown by Giraldo *et al*.[27] *LAG3* mRNA overexpression was associated with unfavorable OS in KIRC.

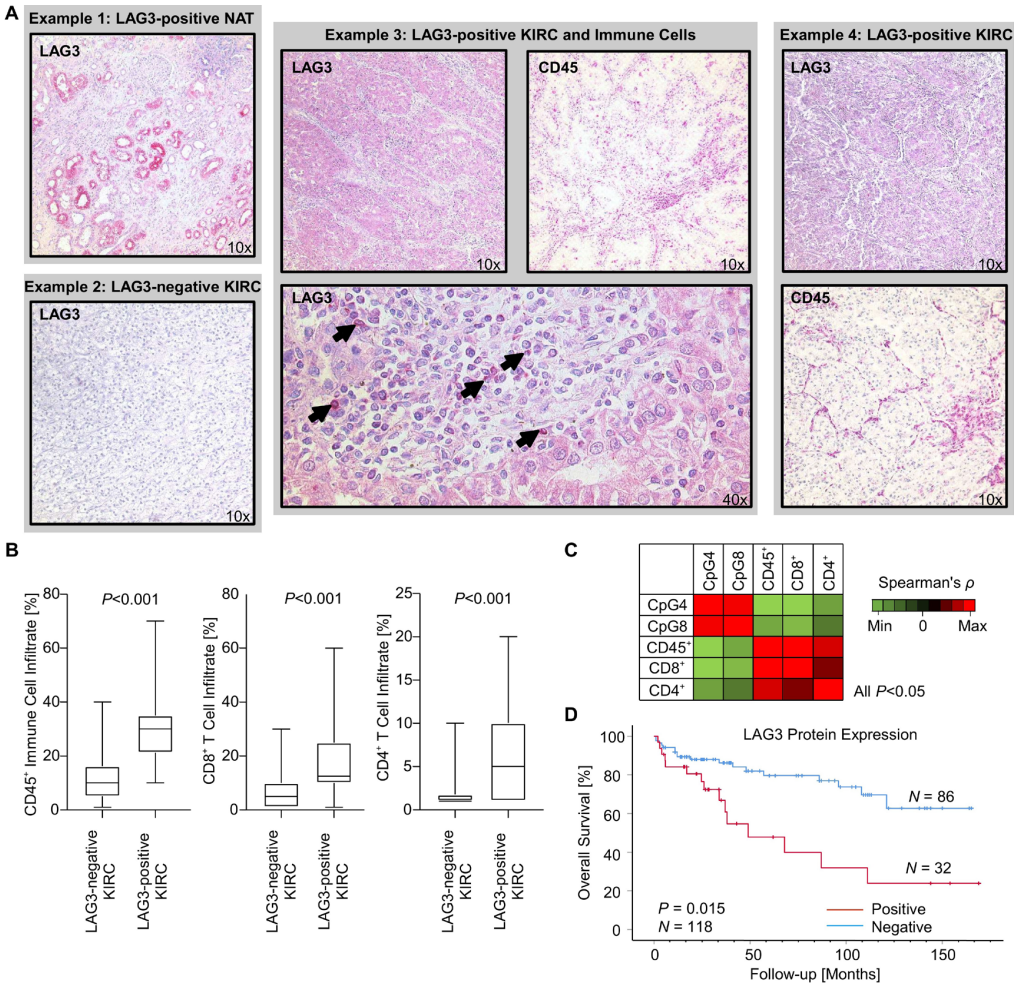


**Figure 4** LAG3 protein expression, immune cell infiltration, and methylation levels of CpG sites 4 and 8 in the UHB cohort (n=118 KIRC). (A) Representative images of immunohistochemical stainings against LAG3 and pan-leukocyte marker CD45 in four distinct cases of the UHB cohort. Example 1: The proximal tubules of NAT exhibit LAG3 expression. Example 3: Tumor-intrinsic LAG3 expression of a highly immune infiltrated KIRC tumor. The arrowheads point to LAG3$^+$ immune cells within the tumor microenvironment of case 3. (B) Boxplots displaying immune cell infiltration of LAG3-negative vs LAG3-positive KIRC. (C) Correlation heatmap of methylation levels of CpG sites 4 and 8 within *LAG3* and immune cell infiltration markers. (D) Kaplan-Meier survival analysis of patients with KIRC dichotomized according to tumor-intrinsic LAG3 protein expression. KIRC, clear cell renal cell carcinoma; LAG3, lymphocyte activating 3; UHB, University Hospital Bonn.

In the UHB cohort, the LAG3-expressing and strongly immune-infiltrated KIRC subset was also associated with strongly reduced OS (figure 4D). *LAG3* methylation status and overexpression on the transcriptional and translational level were therefore linked to unfavorable OS in two different KIRC cohorts.

## DISCUSSION

Despite the resounding success of multiple antibody-based therapies that inhibit the PD-1/PD-L1 axis, there are numerous nonresponding patients. Since this axis represents only one of a large number of inhibitory immune checkpoints, investigation of other potential targets that can be blocked to enhance the anticancer immune response is warranted. One of these targets is the immune checkpoint receptor, LAG3.

In the present study, we investigated DNA methylation at single CpG site resolution within the *LAG3* gene. We correlated *LAG3* methylation with transcriptional activity, protein expression, most frequent somatic mutations, overall patient survival, and tumor immune microenvironment in KIRC tissue. Our results suggest a strong epigenetic regulation of *LAG3* via promoter methylation. Molecular, clinicopathological, and immunogenicity correlates with *LAG3* promoter DNA methylation.

Distinct binding sites for transcriptional repressor CTCF are predicted within the promoter and downstream from *LAG3*. CTCF is known to be heavily involved in transcriptional regulation of regulation of chromatin

**Table 2** Associations of LAG3 methylation and mRNA expression (mean levels) with *VHL*, *PBRM1*, *SETD2*, *KDM5C*, and *BAP1* mutation status in KIRC

| Analyte (CpG site/bead, mRNA) | Number | *VHL* | | | *PBRM1* | | | *SETD2* | | | *KDM5C* | | | BAP1 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Wild type (n=179) | Mutant (n=140) | P value | Wild type (n=228) | Mutant (n=91) | P value | Wild type (n=287) | Mutant (n=32) | P value | Wild type (n=301) | Mutant (n=18) | P value | Wild type (n=296) | Mutant (n=23) | P value |
| LAG3 mRNA | NA | 6.481 | 6.68 | 0.36 | **6.717** | **6.19** | **0.028** | 6.58 | 6.42 | 0.65 | 6.56 | 6.65 | 0.86 | **6.51** | **7.34** | **0.045** |
| cg26956535 | 1 | 88.4 | 88.1 | 0.76 | 88.3 | 88.1 | 0.80 | **89.2** | **79.7** | **<0.001** | 88.3 | 86.8 | 0.41 | 88.3 | 87.7 | 0.72 |
| cg22777668 | 2 | 23.8 | 22.1 | 0.07 | 23.6 | 21.7 | 0.073 | **23.4** | **20.2** | **0.035** | **23.3** | **18.5** | **0.014** | 23.0 | 23.1 | 0.98 |
| cg16352928 | 3 | 65.5 | 66.0 | 0.67 | **64.4** | **69.0** | **0.002** | 66.0 | 63.5 | 0.28 | 65.8 | 64.3 | 0.61 | **66.3** | **58.1** | **0.002** |
| cg02695343 | 4 | 65.3 | 65.1 | 0.91 | **64.1** | **68.1** | **0.007** | 65.6 | 61.6 | 0.079 | 65.3 | 63.4 | 0.52 | **65.9** | **57.0** | **0.001** |
| cg10500147 | 5 | 59.3 | 59.2 | 0.97 | **57.8** | **63.0** | **0.010** | **60.0** | **53.2** | **0.026** | 59.4 | 56.7 | 0.48 | **60.0** | **50.3** | **0.006** |
| cg17213699 | 6 | 78.3 | 77.9 | 0.83 | **76.9** | **81.1** | **0.021** | 78.6 | 74.1 | 0.097 | 78.3 | 75.7 | 0.46 | **78.8** | **69.0** | **0.002** |
| cg19872463 | 7 | 73.8 | 73.8 | 0.97 | **72.6** | **76.9** | **0.023** | 74.3 | 69.1 | 0.068 | 73.9 | 72.1 | 0.62 | **74.4** | **66.4** | **0.016** |
| cg04671742 | 8 | 78.2 | 78.6 | 0.67 | **77.5** | **80.6** | **0.006** | 78.2 | 80.1 | 0.26 | 78.4 | 77.7 | 0.73 | 78.7 | 75.0 | 0.06 |
| cg01820374 | 9 | 39.2 | 40.2 | 0.39 | **38.6** | **42.3** | **0.002** | 39.8 | 38.1 | 0.36 | 39.9 | 35.7 | 0.08 | 39.6 | 39.4 | 0.91 |
| cg19421125 | 10 | 76.1 | 76.9 | 0.51 | **75.5** | **78.9** | **0.008** | 76.4 | 77.3 | 0.66 | 76.6 | 74.8 | 0.48 | **77.1** | **69.3** | **0.001** |
| cg10191002 | 11 | 61.1 | 62.2 | 0.24 | **60.8** | **63.3** | **0.015** | 61.6 | 61.3 | 0.88 | 61.6 | 60.9 | 0.72 | **62.0** | **56.4** | **0.002** |
| cg20652042 | 12 | 76.4 | 76.4 | 0.95 | **75.6** | **78.4** | **<0.001** | 76.3 | 77.2 | 0.46 | 76.4 | 76.8 | 0.79 | 76.6 | 74.8 | 0.19 |
| cg06157570 | 13 | 74.7 | 76.2 | 0.10 | **74.4** | **77.8** | **0.001** | 75.2 | 76.5 | 0.40 | **75.6** | **70.8** | **0.015** | **75.0** | **79.5** | **0.012** |
| cg14292870 | 14 | 37.6 | 36.3 | 0.35 | **38.2** | **34.1** | **0.005** | 37.3 | 34.6 | 0.23 | 37.3 | 33.3 | 0.17 | **36.5** | **44.0** | **0.003** |
| cg11429292 | 15 | 16.9 | 17.9 | 0.32 | **18.1** | **15.5** | **0.018** | 17.5 | 15.7 | 0.27 | 17.3 | 17.5 | 0.94 | **17.0** | **22.4** | **0.005** |
| cg15828668 | 16 | 5.6 | 5.4 | 0.31 | 5.7 | 5.2 | 0.13 | 5.6 | 5.2 | 0.46 | 5.5 | 5.2 | 0.62 | 5.5 | 5.2 | 0.53 |

*LAG3* methylation was determined at 16 different CpG sites targeted by HumanMethylation450 BeadChip beads (figure 1). Significant data are shown in boldface.
KIRC, clear cell renal cell carcinoma; LAG3, lymphocyte activating 3; NA, not applicable.

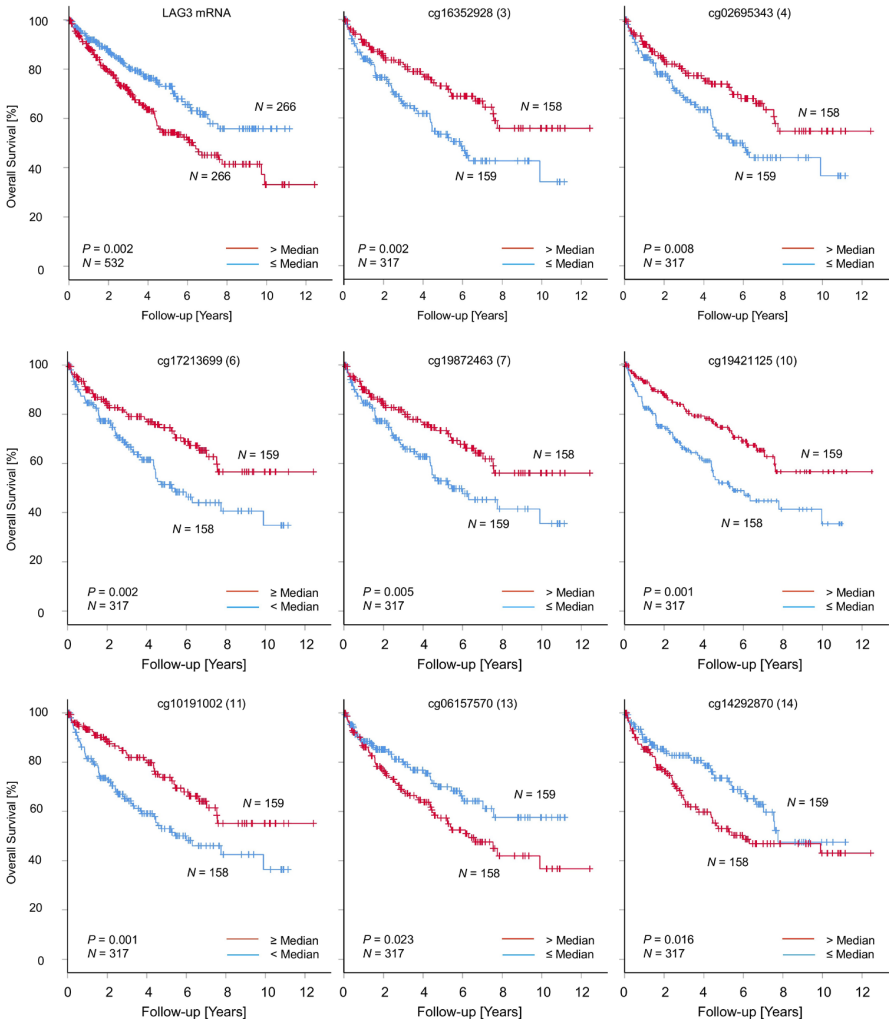


**Figure 5** Kaplan-Meier analysis regarding overall survival in patients with KIRC stratified according to *LAG3* methylation and mRNA expression. Methylation and mRNA expression levels were dichotomized by median *LAG3* methylation and mRNA expression, respectively. The prognostic significant CpG sites and LAG3 mRNA expression are depicted. KIRC, clear cell renal cell carcinoma; LAG3, lymphocyte activating 3.

architecture.[28] Differential methylation of the CTCF binding sites within *LAG3* correlates with its expression; however, further functional investigations are needed to clarify the mechanism of this differential regulation.

LAG3 is expressed on activated T cells, natural killer cells, B cells, and plasmacytoid dendritic cells and binds with high affinity to MHC class II receptors. Research has shown that LAG3 negatively regulates cellular proliferation, activation, and homeostasis of T cells, similar to the function of CTLA-4 and PD-1. Furthermore, it has been demonstrated that LAG3 plays a role in regulatory T cell (Treg) suppressive function and maintenance of $CD8^+$ T cell exhaustion during chronic viral infection.[29 30] In addition, LAG3 is involved in the process of maturation and activation of dendritic cells.

In an oncological context, LAG3 inhibition activates effector T cells, similar to PD-1 inhibitors, and additionally inhibits suppressive Tregs. This combined effect has the potential to enhance the antitumor response of the tumor-infiltrating immune cells.[8 9] Hence, LAG3 is considered as a potential target in immunotherapy. Currently, clinical trials evaluating the safety and efficacy of multiple therapeutic monoclonal LAG3 antibodies such as relatlimab (Bristol-Myers Squibb) or MK-4280 (Merck/MSD Sharp & Dohme) alone (ClinicalTrials.gov Identifier: NCT01968109) or in combination with nivolumab and/or ipilimumab (NCT01968109, NCT03459222, NCT02996110) in KIRC are ongoing.

Clinical studies evaluating the efficacy of immune checkpoint inhibitors targeting the PD-1/PD-L1 axis have proposed different potential markers such as tumor-intrinsic immune checkpoint expression, the intensity of intratumoral $CD8^+$ T cell infiltrates, or an enhanced interferon-γ signature to be predictive for therapy response.[31–35] Currently, immunohistochemically determined receptor status is the only widely used biomarker

prior immune checkpoint inhibition. However, the use of PD-L1 IHC as a predictive biomarker is confounded by multiple difficulties, such as the usage of different antibodies and expression scores, as well as interlaboratory and interobserver variability.[36] The use of methylation as a biomarker has several advantages: DNA methylation is a stable epigenetic modification which is not as dynamic as mRNA or protein expression. In addition, it is chemically stable and can also be measured investigator-independently. Moreover, a quantitative measurement is also possible in small sample amounts (microdissected cells, biopsies), a situation often faced in the clinical setting.[37 38] DNA methylation status is a particular promising biomarker for immunotherapies as demonstrated in previous reports.[12–16]

Results of the present study suggest that *LAG3* gene expression in KIRC is strongly regulated epigenetically, that is, *LAG3* methylation correlates with *LAG3* mRNA expression. In particular, hypomethylation of the promoter region negatively correlates with enhanced mRNA expression in the KIRC TCGA cohort whereas two methylation sites, one in the body of the *LAG3* gene, and one downstream within a putative binding site of the transcriptional repressor CTCF, positively correlate with mRNA expression. These findings on epigenetic regulation are consistent with our findings in KIRC tumor samples (UHB cohort). Overall, *LAG3* promoter hypomethylation was concordantly associated with LAG3 protein expression. Results from IHC demonstrated that LAG3 is expressed intrinsically by a subset of KIRC tumor cells and sparsely by immune infiltrates. This LAG3-expressing KIRC subset was characterized by adverse clinical outcome, high immune cell infiltration, and $LAG3^+$ immune cell infiltration indicating those tumors as immunologically "hot tumors." Consistent with our observations, distinct populations of phenotypically exhausted $CD8^+$ T cells have previously been shown to exist in highly immune-infiltrated KIRC tumors.[39] Furthermore, soluble LAG3, which is known to function as a dendritic cell activator, was also recently shown to be associated with advanced KIRC stage.[40] Tumor-specific LAG3 upregulation might be an immune evasion phenotype similarly to PD-L1 upregulation or upregulation of other immune checkpoints. This hypothesis needs further functional evaluation. However, the finding of KIRC cell-intrinsic LAG3 expression is contradictory to a recent study on immune checkpoints in the microenvironment of primary and metastatic KIRC that revealed LAG3 expression by tumor infiltrating immune cells in a small subset of KIRC but not by the tumor itself.[27] Previously, the immune checkpoint PD-1, which was thought to be solely expressed on immune cells, was also found to be expressed cancer cell-intrinsically in different tumor entities including renal cancers.[41] Furthermore, it has already been shown that LAG3 is solely expressed on immune cells and in the central nervous system.[42] Additionally, we found LAG3 expression in proximal tubules of NAT, the site in which malignant transformation occurs for KIRC,[43 44] and in the urothelium of the renal pelvis. While the finding of cancer cell-intrinsic LAG3 expression in KIRC is interesting, further validation and functional analyses are required.

Based on data on *LAG3* DNA methylation shown here, *LAG3* methylation status could be used as an observer-independent predictive biomarker for stratification of patients who could benefit from LAG3 or other immune checkpoint inhibition therapy. However, this is speculative and further prospective studies are needed to evaluate the extent to which therapy response of LAG3 inhibition is predicted by promoter hypomethylation. Therefore, we recommend integration of *LAG3* promoter methylation in biomarker programs of clinical trials to evaluate the efficacy of immune checkpoint inhibition in patients with KIRC.

Correlations of LAG3 mRNA expression and methylation in the KIRC TCGA cohort, comprising whole tumor tissue, NAT, PBMC, and KIRC cell lines showed significant differences between sample type. This suggests a cell type-specific epigenetic regulation of *LAG3*. For example, the methylation status of CpG site 13 showed a differential correlation with LAG3 mRNA in PBMCs compared with KIRC cell lines. This could be a hint for an alternative *LAG3* promoter that indicates a leukocyte-specific expression of a particular splice variant. LAG3 variants that display different effects on immunological microenvironments have already been described.[45] Widespread tissue-specific and cell type-specific methylation patterns in diverse biological processes are already described, highlighting the complexity of epigenetics.[46]

In KIRC, the most frequently mutated genes comprise chromatin modifier genes as *PBRM1* and *SETD2*.[47 48] Interestingly, recent studies have shown associations between therapy response to ICI and *PBRM1* mutation status in KIRC and mesothelioma.[47 49] Genomic alterations in *SETD2* have already been associated with an altered activation of the interferon signaling pathway. Our data also showed a differential *LAG3* methylation status in the context of mutations within chromatin modifier genes.

Further, our data demonstrate that promoter hypomethylation predicts unfavorable OS in KIRC. This finding highlights clinical significance. However, the main focus of our study is on the epigenetic regulation of LAG3 via DNA methylation in KIRC. *LAG3* promoter hypomethylation is associated with increased immune cell infiltration and an interferon-γ signature. In the context of PD-1/PD-L1 directed immune checkpoint inhibition, interferon-γ signature is suggested as a critical driver for antitumoral immune efficacy and thereby a potential biomarker for ICI therapy response.[35] Using GEO datasets, *LAG3* methylation in KIRC cell lines and PBMCs was investigated independently. Our results showed that compared with tumor cell lines, PBMCs are hypomethylated in the *LAG3* promoter region. The KIRC TCGA data are based on whole tumor tissue comprising tumor cells, stroma, and immune infiltrates. *LAG3* promoter hypomethylation could thereby serve as a surrogate biomarker for

high immune cell infiltration. However, a limitation of this data is that only isolated immune cells from peripheral blood of healthy individuals were examined. In this context, further studies are needed to clarify whether *LAG3* methylation in the tumor is a surrogate biomarker for immunogenicity, that is, infiltration by immune cells and an interferon-γ signature, or whether LAG3 expression of the tumor cells themselves represents a more aggressive phenotype. One aspect of this is highlighted by our results from IHC staining, which showed that LAG3-expressing tumors are associated with poorer survival.

## CONCLUSION

Our results suggest an epigenetic regulation of *LAG3* in KIRC by DNA methylation. Understanding the epigenetic regulation of *LAG3* in KIRC might help to develop biomarkers for the stratification of patients eligible for anti-LAG3 immunotherapies. Our study provides rationale to include *LAG3* methylation testing as a potential predictive biomarker into the biomarker program of clinical trials with anti-LAG3 immune checkpoint inhibitors.

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**Acknowledgements** We thank the BioBank Bonn of the Bonn University Medical Faculty and the University Hospital Bonn for the support of this study.

**Contributors** NK, DJR, JE and DD were involved in the study design and concept. NK, DJR, and DD drafted the manuscript. NK, DJR, RZ and DD performed the experiments. Immunohistochemical stainings were analyzed by NK, MT and JL. NK and DD performed statistical analyses. JL, GK, MR, EGB, MH and JE revised the manuscript for critical intellectual content. All authors read and approved the final version of the manuscript.

**Funding** This study was supported by a Ferdinand Eisenberger grant of the Deutsche Gesellschaft für Urologie (German Society of Urology), grant ID KIN1/FE-19 (NK). The main funding source was the University Hospital Bonn.

**Competing interests** DD owns patents and patent applications on biomarker technologies and methylation of immune checkpoint genes as predictive and prognostic biomarkers (DE 10 2016 005 947.8, DE 10 2015 009 187.5, DE 10 2017 125 780.2, PCT/EP2016/001237). The patents are licensed to Qiagen GmbH (Hilden, Germany). DD is a consultant of Qiagen. The University Hospital Bonn (PI Dimo Dietrich) receives research funding from Qiagen. The other authors have declared that no conflict of interest exists.

**Patient consent for publication** Not required.

**Ethics approval** All patients registered in the TCGA Research Network and UHB cohort had signed informed consent in accordance with the declaration of Helsinki. The study protocol for the UHB cohort was approved by the Institutional Review Board (vote no. 187/16).

**Provenance and peer review** Not commissioned; externally peer reviewed.

**Data availability statement** Data are available in a public, open access repository. Data are available on reasonable request. The results shown here are based on data generated by The Cancer Genome Atlas project (TCGA, http://cancergenome.nih.gov/) and the Gene Expression Omnibus project (GEO, https://www.ncbi.nlm.nih.gov/geo/). Data based on the UHB cohort is available on reasonable request.

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## 3.2 Klümper, N. et al. (2021a). CTLA4 promoter hypomethylation is a negative prognostic biomarker at initial diagnosis but predicts response and favorable outcome to anti-PD-1 based immunotherapy in clear cell renal cell carcinoma. J Immunother Cancer 9, e002949.

Robuste prädiktive Biomarker für eine rationale Erstlinientherapieentscheidung (ICI+ICI versus ICI+TKI) bei Patienten mit mRCC fehlen bisher, obwohl sie dringend benötigt werden. In dieser Studie wurde untersucht, inwieweit sich der *CTLA4*-Methylierungsstatus (mCTLA4) als prädiktiver Biomarker für anti-PD-1-basierte ICI im ccRCC eignet. Im therapienaiven ccRCC-Tumorgewebe wurde mCTLA4, die CTLA4 mRNA Expression sowie die Immunzellinfiltration erhoben und nachfolgend mit dem klinischen Verlauf zweier Nephrektomie-Kohorten aus dem *The Cancer Genome Atlas (TCGA) Research Network* (TCGA-Kohorte, N=533) und dem Universitätsklinikum Bonn (UKB-Kohorte, N=116) verglichen. Darüber hinaus wurde in einer multizentrischen ICI-behandelten RCC-Kohorte (RCC-ICI-Kohorte, N=71) in therapienaivem Tumorgewebe mCTLA4 mittels qMSP sowie die CD8+ T-Zell-Infiltration und PD-L1-Expression mittels IHC untersucht. Die Patienten der RCC-ICI-Kohorte wurden entweder mit einer anti-PD-1-Kombinationstherapie in der Erstlinie (N=25) oder mit einer ICI-Monotherapie nach einer vorherigen antiangiogenetischen TKI-Therapie behandelt. Die *CTLA4*-Promotorhypomethylierung korrelierte in den in kurativer Intention behandelten ccRCC-Kohorten (TCGA-Kohorte und UKB-Kohorte) signifikant mit der CTLA4-mRNA-Expression, der Lymphozyteninfiltration und einem verkürzten OS. In der RCC-ICI-Kohorte sagte die *CTLA4*-Promotorhypomethylierung das Ansprechen auf die Immuntherapie voraus. In der mit ICI behandelten Kohorte war die Hypomethylierung von *CTLA4* mit einem verlängerten PFS und OS assoziiert und blieb im multivariablen Cox-Regressionsmodell ein unabhängiger prognostischer Faktor. Der initial negative prognostische Wert der *CTLA4*-Hypomethylierung beim primären ccRCC wurde also im metastasierten Setting aufgrund des besseren Immuntherapieansprechens überkompensiert. mCTLA4 war hinsichtlich des prädiktiven Potenzials der PD-L1 Expression in der RCC-ICI-Kohorte überlegen. Die Studie zeigt somit, dass mCTLA4 ein vielversprechender prädiktiver Biomarker für die Vorhersage des Ansprechens auf ICI für Patienten mit mRCC zu sein scheint. Diese Ergebnisse bedürfen einer prospektiven Validierung, um das definitive Potential von mCTLA4 für eine rationale Erstlinientherapieentscheidung (ICI+TKI versus ICI+ICI) für Patienten mit mRCC zu bestimmen.

Journal for ImmunoTherapy of Cancer

# *CTLA4* promoter hypomethylation is a negative prognostic biomarker at initial diagnosis but predicts response and favorable outcome to anti-PD-1 based immunotherapy in clear cell renal cell carcinoma

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**To cite:** Klümper N, Ralser DJ, Zarbl R, *et al*. *CTLA4* promoter hypomethylation is a negative prognostic biomarker at initial diagnosis but predicts response and favorable outcome to anti-PD-1 based immunotherapy in clear cell renal cell carcinoma. *Journal for ImmunoTherapy of Cancer* 2021;**9**:e002949. doi:10.1136/jitc-2021-002949

Accepted 29 July 2021

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## ABSTRACT

**Background** In metastatic clear cell renal cell carcinoma (ccRCC), different combination therapies, each including anti-PD-1 immune checkpoint blockade (ICB), are applied as first-line treatment. Robust predictive biomarkers for rational upfront therapy decisions are lacking, although they are urgently needed. Recently, we showed that *CTLA4* promoter methylation predicts response to ICB in melanoma. Here, we aimed to investigate *CTLA4* methylation in ccRCC and its utility to serve as a predictive biomarker for anti-PD-1 based ICB in metastatic ccRCC.

**Methods** *CTLA4* methylation was analyzed with regard to transcriptional gene activity (mRNA expression), intratumoral immune cell composition, and clinical course in two ccRCC cohorts obtained from The Cancer Genome Atlas (TCGA cohort, n=533) and the University Hospital Bonn (UHB Non-ICB Cohort, n=116). In addition, *CTLA4* methylation as well as $CD8^+$ T cell infiltrates and PD-L1 expression were evaluated in pre-treatment samples from a multicenter cohort (RCC-ICB Cohort, n=71). Patients included in the RCC-ICB Cohort were treated with either first line anti-PD-1 based combination therapy (n=25) or monotherapy post–tyrosine kinase inhibition in second line or later. Analyses were performed with regard to treatment response according to RECIST, progression-free survival (PFS), event-free survival (EFS), and overall survival (OS) following treatment initiation.

**Results** *CTLA4* promoter hypomethylation was significantly correlated with *CTLA4* mRNA expression, lymphocyte infiltration, and poor OS in both primary ccRCC cohorts (TCGA: HR 0.30 (95% CI 0.18 to 0.49), p<0.001; UHB Non-ICB: HR 0.35 (95% CI 0.16 to 0.75), p=0.007). In contrast, *CTLA4* promoter hypomethylation predicted response and, accordingly, favorable outcomes (PFS and OS) in patients with ICB-treated ccRCC, overcompensating the negative prognostic value of *CTLA4* hypomethylation at initial diagnosis. Moreover, in multivariable Cox regression, *CTLA4* promoter hypomethylation remained an independent predictor of improved outcome in ICB-treated ccRCC after co-adjustment of the International Metastatic Renal Cell Carcinoma Database Consortium score (HR 3.00 (95% CI 1.47 to 6.28), p=0.003).

**Conclusions** Our study suggests *CTLA4* methylation as a powerful predictive biomarker for immunotherapy response in metastatic RCC.

## BACKGROUND

In the era of cancer immunotherapy, application of immune checkpoint blockade (ICB) targeting cytotoxic T lymphocyte–associated protein 4 (CTLA-4) and/or the programmed cell death 1 (PD-1/PD-L1) axis led to improved clinical outcomes in advanced clear cell renal cell carcinoma (ccRCC).[1–3] Both combination of anti-PD-1 and anti-CTLA-4[4] as well as combined anti-PD-1/PD-L1 plus a tyrosine kinase inhibitor (TKI)[5–8] are currently applied as first-line therapy in metastatic ccRCC. As prospective clinical trials comparing these first-line therapies are still pending, both therapy combinations are currently considered equivalent in the intermediate and poor-risk groups defined by the International Metastatic Renal Cell Carcinoma Database Consortium (IMDC). In this context, a robust biomarker for an optimal upfront therapy decision and treatment sequencing in the clinical setting of metastatic ccRCC is missing.[1 9]

Tumor-intrinsic PD-L1 expression predicts response to anti-PD-1 ICB in various tumor entities, but in ccRCC it is of limited use and the European Association of Urology recommend not to consider this biomarker for patient stratification.[10–13] Furthermore, a predictive biomarker that evaluates the effectiveness of an anti-PD-1 blockade is also of limited relevance in ccRCC, as the PD-1/PD-L1 immune axis is targeted in both first-line therapies as the current backbone of first-line ccRCC therapy. Of note, in CheckMate214, the study that ultimately led to the approval of ICB +ICB in ccRCC, only PD-L1 expression was evaluated regarding response rates,[4] thereby excluding half of the biological mechanism of this therapy approach, precisely the blockade of the CTLA-4 immune checkpoint.[14] A robust predictive biomarker for anti-CTLA-4 monotherapy is also currently lacking despite its high clinical relevance, as several new antibodies and probodies, which promise reduced off-tumor toxicity, are being developed and are already tested in clinical trials (eg, ClinicalTrials.gov Identifier: NCT03369223).[15] In this context, we were recently able to provide strong evidence that the methylation status of the CTLA-4 encoding gene *CTLA4* predicts response to both anti-PD-1 and anti-CTLA-4 targeted ICB as well as anti-CTLA-4 monotherapy in patients with melanoma.[16 17] In the present study, we therefore comprehensively investigated the promoter DNA methylation status of *CTLA4* in ccRCC with regard to transcriptional activity, clinicopathological parameters (including survival and response to ICB and TKI), immune cell infiltrates, and an interferon-γ signature. Understanding the epigenetic regulation of *CTLA4* in ccRCC is of major interest, as it might be promising as a predictive biomarker to enable a more rational therapeutic decision in favor or against ICB +ICB in patients with ccRCC in the age of individualized therapy.

## METHODS

### Patient cohorts and clinical endpoints

#### TCGA cohort

Comprehensive methylation, expression, and immunogenomic data of the ccRCC TCGA dataset generated by *The Cancer Genome Atlas Research Network* (TCGA, http://cancergenome.nih.gov/) were used (n=533).[18–20] Event-free survival (EFS) was previously recommended as a meaningful clinical endpoint for the ccRCC TCGA cohort and defined as progression of disease, local or distant recurrence, or death due to any cause.[21]

#### UHB Non-ICB Cohort

For validation purposes, a second previously described ccRCC cohort of patients treated at the University Medical Center Bonn (n=116) was included.[22] According to the TCGA cohort, EFS was considered as a clinically meaningful endpoint in the UHB Non-ICB Cohort.

#### RCC-ICB Cohort

In addition, a multicenter ICB-treated RCC cohort was assembled (see table 1, n=71 also including n=4 non-ccRCC). The RCC-ICB Cohort included pre-treatment samples from patients who received either anti-PD-1 monotherapy second-line or later post-TKI (n=46) or first-line anti-PD-1 based combination therapy (n=25). Clinical endpoints were response to ICB according to RECIST V.1.1 and progression-free survival (PFS). PFS was defined as the time from ICB initiation until objective tumor progression or death. Overall survival (OS) was evaluated for all three cohorts.

### Transcriptome data assembly

Log2-transformed RSEM (RNA-Seq by Expectation Maximization) RNA sequencing data (RNA-Seq v2) of *CTLA4*, interferon-γ signature and cytolytic activity genes (*IFNG*, *STAT1*, *STAT2*, *JAK2*, *IRF9*, *GZMA*, *GZMB*, *PRF1*) generated by Illumina HiSeq (Illumina, San Diego, CA, USA) were downloaded from the UCSC Xena browser (http://xena.ucsc.edu) (ccRCC n=533, normal adjacent tissue (NAT) n=72).

Comprehensive immunogenomic data on the composition of the tumor microenvironment and the interferon-γ signature response of the ccRCC TCGA cohort were obtained from Thorsson *et al* and implemented.[20]

### *CTLA4* promoter methylation analysis

The ccRCC TCGA cohort contained comprehensive methylation data from n=318 ccRCC and n=160 NAT samples. The CpG sites cg08460026 (CpG1) and cg05074138 (CpG2) within the *CTLA4* promoter were probed by beads from the Infinium HumanMethylation450 BeadChip (Illumina). The genomic organization of *CTLA4* is illustrated in figure 1A. $\beta$ values, estimating the ratio of intensities between methylated and unmethylated alleles, were used for analyses.

In the UHB Non-ICB Cohort (n=116) and the RCC-ICB Cohort (n=71), we used a quantitative methylation-specific PCR (qMSP) assay in order to determine the methylation level of CpG1 within the *CTLA4* promoter. The qMSP assay contained primers that amplify methylation—unspecifically a 73 bp amplicon (forward primer: attcaattaaatacttaaaattatcttttc, reverse primer: tatatatgtgtatatatagaaggtatttg). The assay included two hydrolysis probes that specifically and competitively hybridize to methylated and unmethylated *CTLA4* sequences, respectively (methylated: 6-Fam-cccacgacttcctttctcgtaaa-BHQ-1, unmethylated: HEX-acccacaacttcctttctcataaaacc-BHQ-1). The assay probes CpG1 and an adjacent CpG site (genomic target sequence: CGGCTTCCTTTCTCG). We calculated Quantitative Methylation Scores (QMS) using the formula: $QMS=100/(1+2^{(CT_{methylated}-CT_{unmethylated})})$.[23 24] We used a PCR buffer composition as described earlier[25] and ran the PCR for 20 min at 95°C and 40 cycles with 15 s at 95°C, 15 s at 55°C, and 60 s at 52°C using a 7900HT Fast Real-Time PCR system (Applied Biosystems, Waltham, MA, USA).

**Table 1** Patient characteristics of n=71 patients with metastatic (stage IV) RCC treated with anti-PD-1 ICB and association with PFS, response, and *CTLA4* promoter methylation

| Characteristic | Total cohort (n=71) | PFS | | *CTLA4* methylation | | Response | | |
|---|---|---|---|---|---|---|---|---|
| | | HR (95% CI) | P value | Mean QMS (SD) | P value | ORR (n=20) | No ORR (n=51) | P value $\chi^2$ test |
| Median age (range) | 65 (44–79) | 1.03 (0.99 to 1.06) | 0.12 | | | | | |
| Sex—no. (%) | | | | | 0.52 | | | 0.58 |
| Male | 49 (69.9) | Ref group | | 73.8 (12.6) | | 14 (70.0) | 35 (68.6) | |
| Female | 22 (30.1) | 0.81 (0.50 to 1.71) | 0.81 | 71.4 (17.6) | | 6 (30.0) | 16 (31.4) | |
| Sample origin—no. (%) | | | | | 0.90 | | | 0.47 |
| Primary | 58 (81.7) | Ref group | | 73.1 (14.8) | | 17 (85.0) | 41 (80.4) | |
| Distant metastasis | 13 (13.8) | 0.49 (0.22 to 1.13) | 0.10 | 72.6 (12.1) | | 3 (15.0) | 10 (19.9) | |
| RCC histology—no. (%) | | | | | **0.010** | | | 0.31 |
| ccRCC | 67 (94.4) | Ref group | | 74.1 (12.8) | | 18 (90.0) | 49 (96.1) | |
| Non-ccRCC | 4 (5.6) | 0.63 (0.15 to 2.61) | 0.53 | 55.3 (25.5) | | 2 (10.0) | 2 (3.9) | |
| ICB response—no. (%) | | | | | **0.030** | | | ND |
| Objective response* | 20 (28.2) | 0.06 (0.02 to 0.17) | **<0.001** | 67.8 (18.5) | | 20 (100) | NA | |
| Stable disease | 17 (23.9) | 0.12 (0.05 to 0.27) | **<0.001** | 72.4 (10.7) | | NA | 17 (33.3) | |
| Progressive disease | 34 (47.9) | Ref group | | 79.8 (10.8) | | NA | 34 (66.7) | |

P values comparing response refer to $\chi^2$ test. Methylation levels between two or more groups were compared using Mann-Whitney *U* and Kruskal-Wallis tests, respectively. Cox proportional hazards were tested using Wald test.
*This category included patients with a complete response (n=4) and those with a partial response (n=16). Non-ccRCCs comprised two papillary, one chromophobe, and one medullary RCC. The origin of the included tissue of the distant metastases was lung (n=6), bone (n=4), and one each adrenal gland, skin, and gallbladder metastasis.
ccRCC, clear cell renal cell carcinoma; ICB, immune checkpoint blockade; NA, not applicable; ND, not determined; ORR, overall response rate; PFS, progression-free survival; QMS, Quantitative Methylation Score; RCC, renal cell carcinoma.

### Immunohistochemistry

Immune cell infiltrate scores of $CD4^+$ and $CD8^+$ T cells and pan-leukocytes ($CD45^+$) as quantified via immunohistochemistry (IHC) on whole slides were included from our previous work (UHB Non-ICB Cohort).[22] $CD8^+$ T cell infiltration in tumors from the multicenter RCC-ICB Cohort was evaluated accordingly.[22] PD-L1 IHC 22C3 pharmDx (Agilent Technologies, Santa Clara, CA, USA) was used for the assessment of PD-L1 Combined Positive Score (CPS) for the RCC-ICB Cohort following the manufacturer's instructions.

### Statistics

Microsoft Excel, GraphPad Prism, and SPSS V.25 were used for statistical analyses. Non-parametric Spearman's $\rho$ correlation coefficients were calculated. Group comparisons were made using parametric two-sided Student's t-test or nonparametric Mann-Whitney U or Kruskal-Wallis (>2 groups) test. Survival analyses of median dichotomized variables were performed using the log-rank test and visualized via Kaplan-Meier plots. Continuous log2-transformed variables were used for Cox proportional HR analyses with specified 95% CI.

## RESULTS

### *CTLA4* promoter is hypomethylated in ccRCC compared with normal adjacent renal tissue (NAT)

We investigated methylation of two CpG sites, referred to as CpG1 and CpG2, located in the central promoter region of *CTLA4* (figure 1A). Interestingly, both evaluated CpG sites showed significant hypomethylation in ccRCC compared with NAT ($p<0.001$) and, inversely, *CTLA4* mRNA expression was increased in ccRCC versus NAT (figure 1B–D). In addition, both analyzed CpG sites showed a high degree of co-methylation (Spearman's $\rho=0.68$, $p<0.001$).

### *CTLA4* transcriptional activity is associated with its promoter methylation

Next, we aimed to analyze to what extent the transcriptional activity of the *CTLA4* gene is associated with the methylation status of its promoter region. In ccRCC, hypomethylation of both CpG sites within the promoter was inversely correlated with *CTLA4* mRNA expression with significant Spearman's correlation coefficients: CpG1 $\rho=-0.54$; CpG2 $\rho=-0.51$ (both $p<0.001$, figure 1E,F). Thus, the transcriptional activity of the *CTLA4* gene strongly depends on its promoter methylation. In NAT, no significant correlation between the *CTLA4* promoter methylation and its mRNA expression was evident, which might be due to a low sample size (n=24) or indicates a tumor-specific methylation pattern.

### *CTLA4* promoter hypomethylation and *CTLA4* mRNA expression are associated with distinct immune cell infiltration and an interferon-γ expression signature

The tumor microenvironment is a complex assembly of different immunological cell types. Since tumor immunogenicity is an essential component for the success

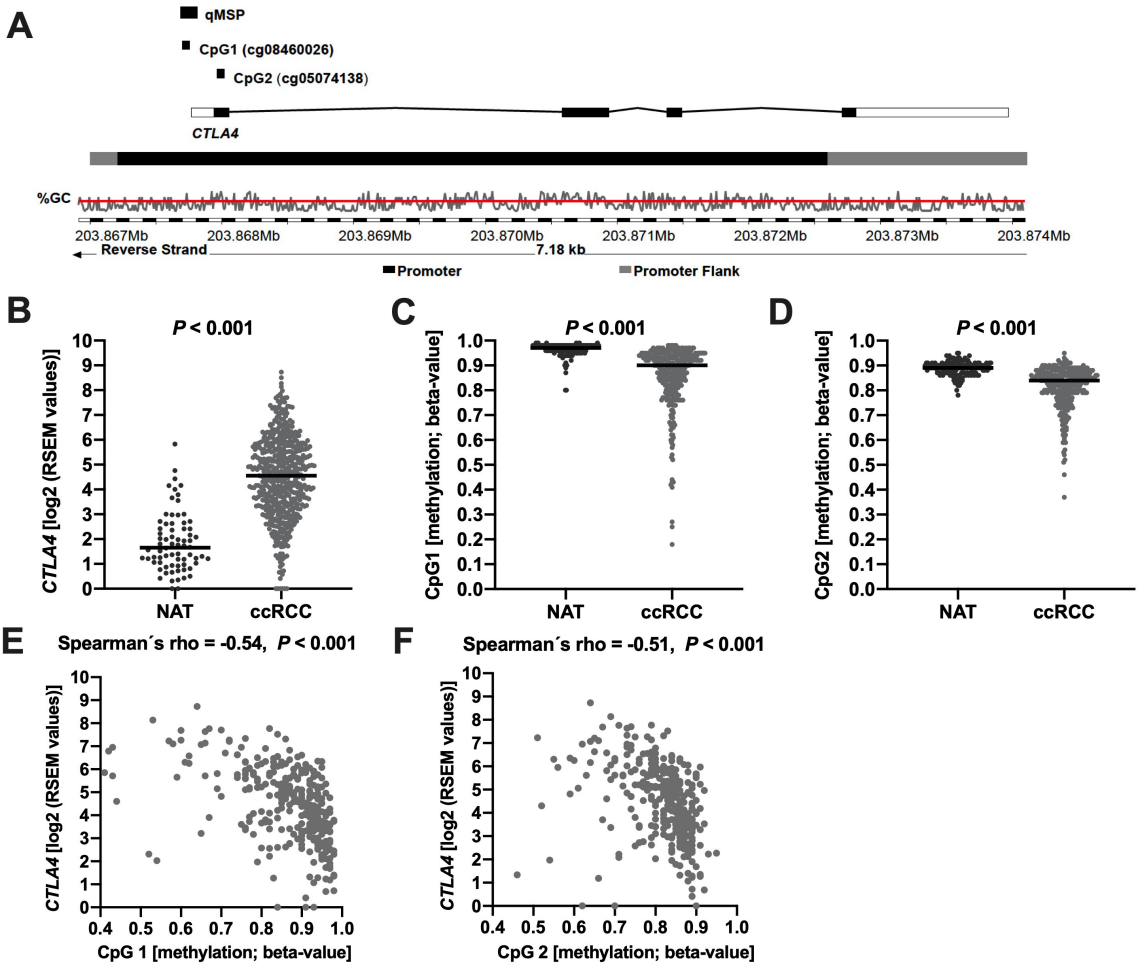


**Figure 1** (A) Genomic organization of the *CTLA4* gene and target sites of the Human Methylation450 BeadChip (CpG1: cg08460026 and CpG2: cg05074138) and of the quantitative methylation-specific PCR (qMSP). The illustration (modified) was taken from Ensembl release 104 and is based on Genome Reference Consortium Human Build 38 patch release 13 (GRCh38.p13).[34] (B–D) *CTLA4* mRNA expression and promoter methylation status of CpG1 and CpG2 in normal adjacent tissue (NAT) vs clear cell renal cell carcinoma (ccRCC). (E,F) Scatter plots representing *CTLA4* promoter methylation in relation to *CTLA4* mRNA expression.

of ICB,[14] we next aimed to investigate to what extent *CTLA4* methylation status is associated with the intratumoral immune cell composition. *CTLA4* promoter methylation, as well as mRNA expression, significantly correlated with the overall lymphocyte infiltration score (figure 2A). Considering the different subtypes of the lymphoid lineage, it is noteworthy that especially signatures of $CD8^+$ T cells, T follicular helper cells, regulatory T cells (Treg), and Th1 cells were associated with upregulated *CTLA4* mRNA expression and concurrent *CTLA4* promoter hypomethylation. In contrast, signatures of myeloid infiltration, especially monocytes and macrophages (in particular M2 macrophages), was correlated with low *CTLA4* mRNA expression and accompanying promoter hypermethylation. Next, we evaluated the relationship between *CTLA4* promoter methylation with the interferon-γ (IFN-γ) response signature and the cytolytic activity (*GZMA*, *GZMB*, *PRF1*), which are both well known to influence ICB efficacy.[11 26] Of note, both *CTLA4* promoter hypomethylation and mRNA expression were strongly associated with increased IFN-γ signatures and cytolytic activity (figure 2B).

### *CTLA4* promoter hypomethylation is associated with an unfavorable clinical course in ccRCC

Next, we evaluated to what extent *CTLA4* promoter methylation and *CTLA4* transcriptional activity were associated with metastatic spread, the crucial step in ccRCC progression, and clinical outcomes. *CTLA4* overexpression and concurring hypomethylation in primary ccRCC (TCGA cohort) were strongly associated with metastatic spread (figure 3A,D). After dichotomization of the cohort by the median *CTLA4* mRNA expression, the overexpressing subgroup exhibited a worse clinical course regarding both event-free (EFS, HR 1.23 (95% CI 1.12 to 1.36), p<0.001) and overall survival (OS, HR 1.25 (95% CI 1.14

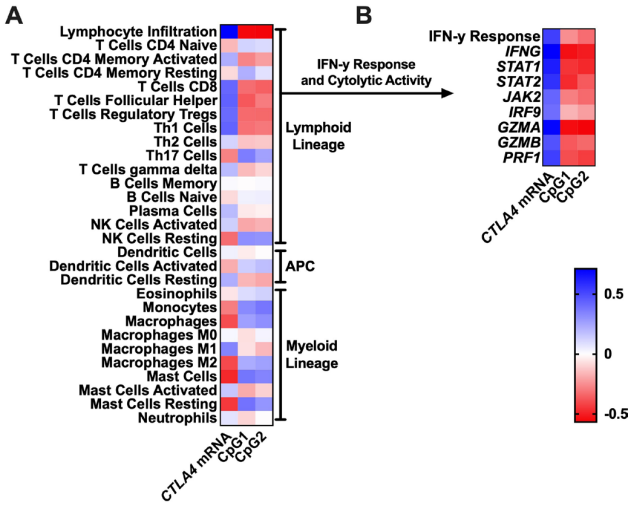


**Figure 2** Correlation heatmaps visualize Spearman's $\rho$ correlation coefficients of both the *CTLA4* mRNA expression and the promoter methylation status (CpG1 +2) with respect to the intratumoral immune cell composition (A), the IFN-γ response, and cytolytic activity (B), respectively. APC, antigen-presenting cell.

to 1.38), p<0.001, figure 3B,C). In accordance, promoter hypomethylation of CpG1 was associated with unfavorable EFS (HR 0.36 (95% CI 0.22 to 0.60), p<0.001) and OS (HR 0.30 (95% CI 0.18 to 0.49), p<0.001, figure 3E,F). The methylation status of CpG2 showed only a trend and no significant association with outcome (EFS: HR 0.20 (95% CI 0.03 to 1.57), p=0.13; OS: HR 0.15 (95% CI 0.02 to 1.13), p=0.065).

To validate the aforementioned results in an independent cohort, we evaluated *CTLA4* promoter methylation in our UHB Non-ICB Cohort (n=116) using a quantitative methylation-specific PCR assay. The qMSP targets CpG1 and one adjacent CpG site located 13 bp upstream from CpG1 (figure 1A). Of note, *CTLA4* promoter hypomethylation was correlated with an enriched immune cell infiltration pattern (IHC for $CD4^+$, $CD8^+$ T cells and pan-leukocytes ($CD45^+$)), which validates the aforementioned results that were based on immunogenomic RNA-Seq signatures (figure 4A). Confirming our results from the TCGA cohort, *CTLA4* hypomethylation in primary ccRCC tissue at initial diagnosis was significantly associated with unfavorable EFS (HR 0.36 (95% CI 0.17 to 0.78), p<0.010) and OS (HR 0.35 (95% CI 0.16 to 0.75), p=0.007) in the UHB Non-ICB Cohort (figure 4B,C).

### *CTLA4* promoter hypomethylation predicts response and outcome in metastatic RCC treated with anti-PD-1 immunotherapy

Since *CTLA4* promoter hypomethylation is associated with an enhanced immune infiltrate in ccRCC, assessment of *CTLA4* methylation status in treatment-naïve tissue samples prior to initiation of ICB therapy may have predictive value for ICB-treatment success. To evaluate the potential predictive value of *CTLA4* methylation prior to immunotherapy, we have assembled a multicenter ICB treated cohort (RCC-ICB Cohort, n=71) of patients treated at German tertiary referral centers. As shown in primary ccRCC earlier, *CTLA4* hypomethylation again correlated strongly with $CD8^+$ T cell infiltration in the RCC-ICB cohort (Spearman's $\rho$=−0.44, p<0.001).

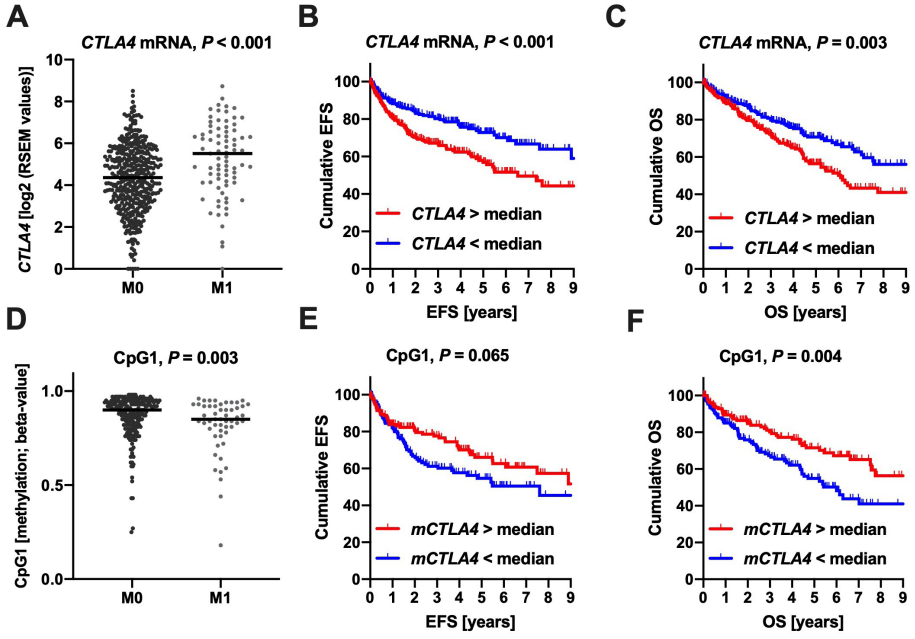


**Figure 3** Association of the *CTLA4* mRNA expression and promoter methylation of CpG1 with respect to the metastatic status (M stage, A,D) and the clinical endpoints event-free survival (EFS, B,E) and overall survival (OS, C,F) in the ccRCC TCGA cohort are depicted. ccRCC, clear cell renal cell carcinoma.

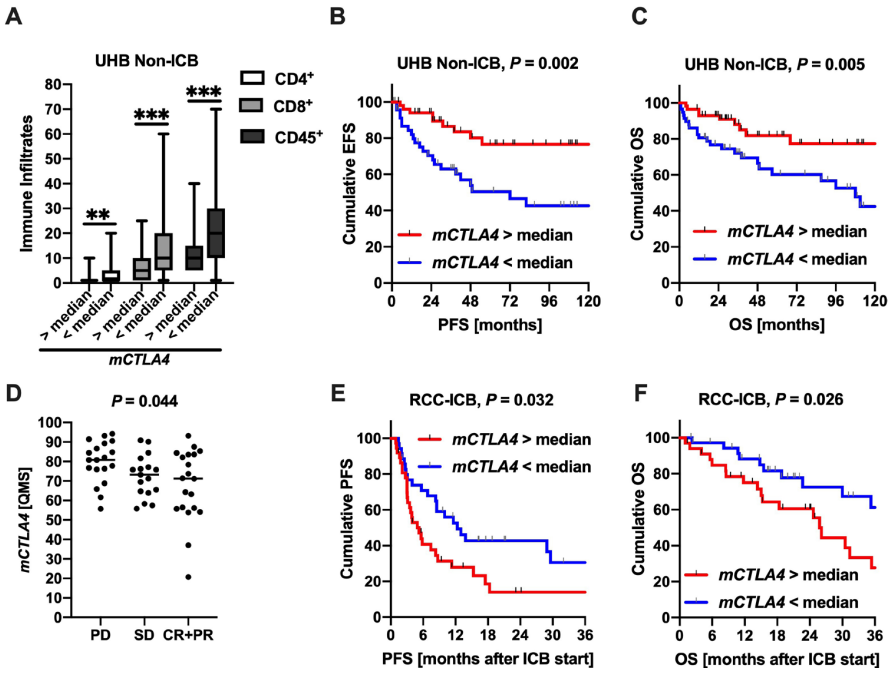


**Figure 4** (**A**) *CTLA4* promoter hypomethylation is associated with high lymphocyte infiltration, especially CD8+ T cells. (**B+C**) *CTLA4* promoter hypomethylation was associated with unfavorable event-free (EFS) and overall survival (OS). (D) In pre-treatment RCC samples, *CTLA4* promoter hypomethylation predicts immune checkpoint blockade (ICB)–treatment response and is associated with prolonged progression-free survival (PFS) after ICB-treatment initiation (E) and favorable OS (F).

Of note, *CTLA4* promoter hypomethylation in pre-treatment RCC samples predicted ICB-treatment response (figure 4D). In concordance with the better response of *CTLA4* hypomethylated tumors to immunotherapy, patients experienced a prolonged PFS and OS after ICB treatment initiation (PFS: HR 1.94 (95% CI 1.09 to 3.44), p=0.024; OS: HR 2.14 (95% CI 1.01 to 4.57), p=0.048; figure 4E,F). This finding is of particular importance because, in contrast, *CTLA4* hypomethylation at initial diagnosis was associated with worse survival. Therefore, the positive predictive value of *CTLA4* hypomethylation exceeded the negative prognostic value at initial diagnosis (TCGA, UHB Non-ICB). For a subgroup of n=44 patients in the RCC-ICB Cohort, response data on prior or subsequent TKI were available. In contrast to its predictive value on immunotherapy, *CTLA4* methylation status did not predict TKI-treatment success and outcome.

Of note, intratumoral PD-L1 expression (cut-off CPS>1) had no predictive value in our multicenter RCC-ICB cohort (PFS: HR 1.46 (95% CI 0.78 to 2.74), p=0.24). Furthermore, in multivariate Cox regression, *CTLA4* promoter hypomethylation remained an independent predictor of improved outcome following ICB-treatment initiation after co-adjusting the IMDC risk score (HR 3.00 (95% CI 1.47 to 6.28), p=0.003).

## DISCUSSION

An epigenetic regulation of the *CTLA4* gene via DNA methylation has already been observed in melanoma.[16] Interestingly, and of particular clinical interest, the *CTLA4* methylation status exhibited a predictive value in patients with melanoma treated with anti-PD-1 plus anti-CTLA-4 immune checkpoint therapy.[16] In the current clinical situation for metastatic ccRCC, a robust predictive biomarker for this particular ICB combination therapy, anti-PD-1 plus anti-CTLA-4, is urgently needed as the combination of anti-PD-1/PD-L1 plus TKI is currently considered equivalent and comparative studies are still pending.[14–69] In this study, we therefore comprehensively investigated *CTLA4* promoter methylation with regard to transcriptional activity, clinicopathological parameters, and the intratumoral microenvironment in ccRCC tissue. Of note, we observed a strong correlation between the transcriptional activity of *CTLA4* and its promoter methylation status in ccRCC. Moreover, *CTLA4* promoter methylation and its mRNA expression showed a significant association with the composition of the ccRCC tumor microenvironment: *CTLA4* overexpression and concomitant promoter hypomethylation were associated with particularly high lymphocyte infiltration and an increased interferon-γ signature as well as cytolytic activity. *CTLA4* hypomethylation thus appears to be a robust surrogate biomarker for an enriched tumor microenvironment. Further, an unfavorable clinical course was evident in primary RCC with hypomethylated *CTLA4* promoter and overexpression, respectively. These findings are in line with the literature describing increased immune cell infiltration and immune checkpoint expression in RCC as a negative prognostic marker.[26]

The transcriptomic and methylation data in the ccRCC TCGA dataset were obtained from whole tumor tissue samples of patients with ccRCC receiving nephrectomy[18 19] and are therefore based on the genomic signature of the tumor and its microenvironment, including tumor cells, stroma, infiltrating immune cells, and tumor-associated fibroblasts.[27] The complexity of epigenetics is highlighted by widespread tissue-specific and cell type–specific methylation patterns in diverse biological processes;[28] however, the characterization of an existing cell line–specific epigenetic regulation of *CTLA4* via DNA methylation patterns was not the focus of our study. The aim of our study was to investigate a predictive and whole-tissue based easy-to-implement biomarker for RCC, and excitingly, *CTLA4* promoter methylation seems to have cancer-independent predictive potential for ICB response in melanoma and RCC.[16 17]

As a chemically stable epigenetic modification that is not as dynamic as mRNA or protein expression, DNA methylation patterns represent particularly attractive biomarkers.[29] Furthermore, the fact that quantitative and investigator-independent measurement of DNA methylation is even possible in small samples (microdissected cells, liquid biopsies, circulating tumor cells) is a major advantage from the diagnostic point of view.[30 31] Basing the data on *CTLA4* DNA methylation shown here and the data on *CTLA4* methylation in melanoma[16 17] strengthen the rationale to test this particular methylation biomarker in clinical trials. In the present study, we have analyzed uncalibrated quantitative methylation levels by means of $\beta$ values (Illumina Infinium Technology) and QMS values (qMSP), respectively. These levels, however, do not necessarily reflect true percentage methylation levels. In order to determine percentage methylation, for example, for the transfer of clinically relevant cut-offs to different platforms and assay technologies, absolute methodologies, that is, bisulfite clone sequencing, could be applied.

The *CTLA4* promoter hypomethylated ccRCC subgroup was characterized by enhanced immune cell infiltration, in particular, $CD8^+$ T cell infiltration indicating these tumors as immunologically “hot tumors". Thus, we asked the question whether the *CTLA4* methylation status in treatment-naïve tissue samples prior to initiation of ICB therapy has predictive value to immunotherapy in RCC. Of note, in our multicenter RCC-ICB cohort, *CTLA4* promoter hypomethylation predicted ICB treatment success, which also translated into prolonged PFS and OS after ICB treatment initiation, thereby counteracting its negative prognostic value in primary ccRCC at initial diagnosis. *CTLA4* methylation status was not associated with TKI response, highlighting that *CTLA4* methylation appears to be predictive for immunotherapy only. At initial diagnosis (TCGA and UHB Non-ICB Cohorts), *CTLA4* promoter hypomethylation was a negative prognostic biomarker and associated with poor outcome, whereas in metastatic stage prior immunotherapy, it was a favorable biomarker. This is most likely due to its predictive value since the high response to ICB overcompensated the negative prognostic value at initial diagnosis. A similar phenomenon has already been described for melanoma. PD-L1 upregulation is associated with an aggressive subset of melanomas with unfavorable outcome at initial diagnosis but has predictive value for ICB response.[32 33] Thus, negative prognostic biomarkers at baseline with strong predictive value for immunotherapy response can overcome their initial negative prognostic value in advanced disease stages. This highlights the potential of *CTLA4* methylation as a promising predictive biomarker prior to ICB-treatment initiation in RCC, which has already been suggested for melanoma.[16 17]

In the current clinical setting of metastatic RCC with multiple first-line therapies, essentially either ICB+TKI or ICB+ICB, there is a tremendous clinical need for robust predictive biomarkers for rational upfront therapy selection, but despite significant efforts, no biomarker that can be easily implemented into clinical practice is available. PD-L1 expression is the only broadly used predictive biomarker, but in ccRCC it is of limited clinical use.[10–13] However, patients with ≥1% PD-L1 expression seem to benefit particularly from intensified immunotherapy with nivolumab plus ipilimumab.[3] In our multicenter RCC-ICB Cohort, *CTLA4* promoter hypomethylation outperformed PD-L1 CPS, which had no significant predictive value in our cohort. Thus, it remains to be prospectively elucidated whether the predictive potential of *CTLA4* promoter methylation status will lead to an improved stratification for rational upfront treatment decisions for either ICB+TKI or ICB+ICB.

The main limitations of our study are the retrospective design, the relative small sample size of our RCC-ICB cohort, the heterogeneity of included patients regarding histology (clear-cell and non-clear-cell RCC included), sample origin (primary tumor and distant metastases), and pre-treatment. In order to establish a robust biomarker in this clinical setting, prospective studies are needed to determine the clinical performance of *CTLA4* promoter hypomethylation as a predictive biomarker for ICB in patients with ccRCC.

## CONCLUSION

In ccRCC, the important immune checkpoint CTLA-4 is epigenetically regulated by promoter DNA methylation. *CTLA4* promoter hypomethylation is a strong biomarker for poor prognosis in patients with ccRCC at initial diagnosis. In contrast, *CTLA4* promoter hypomethylation predicted response and favorable outcome to immunotherapy in our multicenter ICB-treated RCC cohort. Thus, it represents a promising candidate for the urgently needed predictive biomarker for optimal upfront treatment decision in metastatic ccRCC.

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**Funding** This study was supported by a Ferdinand Eisenberger grant of the Deutsche Gesellschaft für Urologie (German Society of Urology), grant ID KIN1/FE-19 (NK), and junior research group funding by the BONFOR Program of the Medical Faculty of the University of Bonn, grant ID 2020-2A-12 (NK). The main funding source was the University Hospital Bonn.

**Competing interests** DD owns patents and patent applications on biomarker technologies and methylation of immune checkpoint genes as predictive and prognostic biomarkers (DE 10 2016 005 947.8, DE 10 2015 009 187.5, DE 10 2017 125 780.2, PCT/EP2016/001237). The patents are licensed to Qiagen GmbH (Hilden, Germany). DD is a consultant of Qiagen. The University Hospital Bonn (PI DD) received research funding from Qiagen.

**Patient consent for publication** Not required.

**Ethics approval** The study was approved by the Institutional Review Board (IRB, vote no. 187/16, 96/19). The IRB waived the requirement to obtain informed consent of this retrospective non-interventional study.

**Provenance and peer review** Not commissioned; externally peer reviewed.

**Data availability statement** Data are available on reasonable request. The results shown here are based on data generated by The Cancer Genome Atlas project (TCGA, http://cancergenome.nih.gov/) (18). Data based on the UHB Non-ICB cohort and the RCC-ICB cohort are available on reasonable request.

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### 3.3 Klümper, N. et al. (2021b). C-reactive protein flare-response predicts long-term efficacy to first-line anti-PD-1-based combination therapy in metastatic renal cell carcinoma. Clin Transl Immunol 10.

Die frühe longitudinale *on-treatment* CRP-Kinetik nach Start der Immuntherapie und insbesondere das kürzlich erstmalig beschriebene CRP-Flare-Response-Phänomen scheint das Immuntherapieansprechen bei Patienten mit mRCC vorherzusagen. In der primären Studie wurde das Phänomen jedoch nur für eine anti-PD-1-Monotherapie in der Zweitlinie oder später untersucht. Da aktuell anti-PD-1 basierte Kombinationstherapien, entweder plus anti-CTLA4 (ICI+CI) oder anti-VEGF-TKI (IO+TKI), als SOC in der Erstlinie des mRCC eingesetzt werden, wurde im Rahmen dieser Studie der prädiktive Wert der frühen CRP-Kinetik für diese Therapiekombinationen untersucht. Patienten mit mRCC aus sechs onkologischen Zentren, die in der Erstlinie entweder ICI+ICI (N=59) oder ICI+TKI (N=36) erhielten, wurden in die Kohorte eingeschlossen und untersucht. Die Patienten wurden anhand der beschriebenen Definition als CRP-Flare-Responder, CRP-Responder oder CRP Non-Responder klassifiziert. Das prädiktive Potenzial der frühen CRP-Kinetik konnte im Rahmen dieser Studie für die anti-PD-1-basierte Kombinationstherapie für Patienten mit mRCC bestätigt werden. CRP-Responder und insbesondere CRP-Flare-Responder zeigten eine signifikante Risikoreduktion von ~70-80% für Tumorprogression mit einem signifikant verlängerten PFS im Vergleich zu CRP Non-Respondern (medianes PFS: CRP-Flare-Responder: 19.2 versus Responder: 16.2 versus CRP Non-Responder: 5.6 Monate, log-rank $p<0.001$). Sowohl in der ICI+ICI als auch in der ICI+TKI-Subgruppe blieb die frühe longitudinale CRP-Kinetik signifikant mit einem verbesserten PFS assoziiert. Zudem war die CRP-Flare-Response Kinetik mit einem langfristigen Therapieansprechen ≥ 12 Monate assoziiert. Die frühe CRP-Kinetik scheint ein kostengünstiger und einfach in den klinischen Alltag zu implementierender *on-treatment* Biomarker für die Vorhersage des Ansprechens auf die anti-PD1-basierte Erstlinienkombinationstherapie für Patienten mit mRCC zu sein. Nach prospektiver Validierung könnte die frühe CRP-Kinetik als nicht-invasiver *on-treatment* Biomarker die Therapieüberwachung für Patienten mit mRCC unter Immuntherapie optimieren. Das frühzeitige Erkennen von Behandlungserfolg und -versagen hat das Potenzial ein therapeutisches Fenster für unmittelbare Therapieanpassungen zu öffnen und die Exposition gegenüber potenziell lebensbedrohlichen Immuntherapie-assoziierten Nebenwirkungen zu verhindern.

Clinical & Translational Immunology

Australian and New Zealand Society for Immunology Inc.

*Clinical & Translational Immunology* 2021; e1358. doi: 10.1002/cti2.1358
www.wileyonlinelibrary.com/journal/cti

ORIGINAL ARTICLE

# C-reactive protein flare-response predicts long-term efficacy to first-line anti-PD-1-based combination therapy in metastatic renal cell carcinoma

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Received 8 September 2021;
Revised 1 and 16 November 2021;
Accepted 17 November 2021

doi: 10.1002/cti2.1358

*Clinical & Translational Immunology*
2021; **10**: e1358

## Abstract

**Objectives.** Immune checkpoint blockade (IO) has revolutionised the treatment of metastatic renal cell carcinoma (mRCC). Early C-reactive protein (CRP) kinetics, especially the recently introduced CRP flare-response phenomenon, has shown promising results to predict IO efficacy in mRCC, but has only been studied in second line or later. Here, we aimed to validate the predictive value of early CRP kinetics for 1st-line treatment of mRCC with αPD-1 plus either αCTLA-4 (IO+IO) or tyrosine kinase inhibitor (IO+TKI). **Methods.** In this multicentre retrospective study, we investigated the predictive potential of early CRP kinetics during 1st-line IO therapy. Ninety-five patients with mRCC from six tertiary referral centres with either IO+IO ($N = 59$) or IO+TKI ($N = 36$) were included. Patients were classified as CRP flare-responders, CRP responders or non-CRP responders as previously described, and their oncological outcome was compared. **Results.** Our data validate the predictive potential of early CRP kinetics in 1st-line immunotherapy in mRCC. CRP responders, especially CRP flare-responders, had significantly prolonged progression-free survival (PFS) compared with non-CRP responders (median PFS: CRP flare-responder: 19.2 months vs. responders: 16.2 vs. non-CRP responders: 5.6, $P < 0.001$). In both the IO+IO and IO+TKI subgroups, early CRP kinetics remained significantly associated with improved PFS. CRP flare-response was also associated with

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long-term response ≥ 12 months. **Conclusions.** Early CRP kinetics appears to be a low-cost and easy-to-implement on-treatment biomarker to predict response to 1st-line IO combination therapy. It has potential to optimise therapy monitoring and might represent a new standard of care biomarker for immunotherapy in mRCC.

**Keywords:** biomarker, checkpoint inhibition, C-reactive protein, CRP flare-response, immunotherapy, metastatic renal cell carcinoma

## INTRODUCTION

First-line treatment of metastatic renal cell carcinoma (mRCC) has changed substantially in recent years because of the introduction of a new therapy regimen, mainly based on immune checkpoint inhibition (IO).[1–3] Currently, two different types of approved first-line combination therapies are applied equivalently for the treatment of intermediate and poor-risk metastatic mRCC according to IMDC (International Metastatic Renal Cell Carcinoma Database Consortium Score): (1) a combination of αPD-1 and αCTLA-4 immune checkpoint inhibitors as well as (2) a combination of αPD-1 (or αPD-L1) with small-molecule tyrosine kinase inhibitors (TKI) targeting the vascular endothelial growth factor receptor (VEGFR).[4–8] In essence, these two regimens can be classified as an intensified immune checkpoint inhibition (IO+IO) and a combination of immune checkpoint inhibition plus anti-angiogenic therapy (IO+TKI).

However, only a subset of patients responds to these first-line IO combination therapies. On the one hand, reliable predictive biomarkers could identify early therapy failure, which is of high clinical relevance. On the other hand, severe unnecessary side effects could be avoided, and the individual therapy regimen could be further optimised.

In general, IO treatment success is based on the induction of an antitumor immune response. C-reactive protein (CRP) is a serum acute-phase reactant and clinically widely used surrogate biomarker for the assessment of systemic inflammation. The occurrence and kinetics of systemic inflammatory response reflected by serum CRP has been implicated with clinical outcome and treatment response in diverse cancer entities, including urothelial cancer, non-small-cell lung cancer and mRCC.[9–14] Several studies investigated CRP levels at initial diagnosis or baseline before therapy initiation and associated increased systemic inflammation with poor oncologic prognosis. As cancers can also induce chronic inflammation, on-treatment CRP kinetics may have predictive value for immunotherapy treatment success.[15,16]

Just recently, Fukuda *et al*. described the CRP 'flare-response' phenomenon defined by an early CRP increase after IO treatment initiation with a subsequent drop below baseline. These early CRP changes appear to mirror the dynamic phase of systemic inflammation after inducing the desired antitumoral immune response on IO therapy.[15] Of note, this novel concept allowed an accurate prediction of therapy success in 42 mRCC patients treated with αPD-1. However, the investigated cohort only included a limited patient number and αPD-1 monotherapy was administered as 2nd-line (or later) post-TKI treatment. As IO monotherapy will occur less frequently in the future, our study aimed to investigate the emerging phenomenon of CRP flare-response in a multicentre mRCC cohort receiving either IO+IO or IO+TKI as 1st-line standard of care therapy.

## RESULTS

### Patient characteristics

Between November 2017 and April 2021, 95 were included in this study (for comprehensive patient characteristics, see Table 1). In brief, $N = 59$ patients (62.5%) received IO+IO and $N = 36$ (37.5%) IO+TKI. The median patient age was 67 (interquartile range, IQR 57.5–75.0) years, and 64 (67.4%) patients were male. Most patients had been diagnosed with clear cell RCC (71.6%), had an Eastern Co-operative of Oncology Group (ECOG) score ≤ 1 (91.6%) and were IMDC intermediate risk (65.3%). The median follow-up was 11.1 (5.6–17.3) months.


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**Table 1.** Comparison of baseline patient and tumor demographics between CRP flare-responders, CRP responders and non-CRP responders

| | | Early CRP kinetics | | | |
|---|---|---|---|---|---|
| | Total cohort | Non-CRP responder | CRP responder | CRP flare-responder | *P*-value |
| No. of patients | 95 | 48 (50.1%) | 34 (35.8%) | 13 (13.7%) | |
| Age | 67.0 (57.50–75.0) | 67.5 (54.8–77.0) | 68.0 (58.3–72.8) | 67.0 (64.0–72.0) | 0.987 |
| Male gender | 64 (67.4%) | 32 (66.7%) | 23 (67.6%) | 9 (69.2%) | 1 |
| ECOG | | | | | |
| 0 | 42 (44.2%) | 19 (39.6%) | 17 (50.0%) | 6 (46.2%) | 0.886 |
| 1 | 45 (47.4%) | 24 (50.0%) | 14 (41.2%) | 7 (53.8%) | |
| 2 | 6 (6.3%) | 4 (8.3%) | 2 (5.9%) | 0 (0%) | |
| 3 | 1 (1.1%) | 1 (2.1%) | 0 (0%) | 0 (0%) | |
| IMDC | | | | | |
| Favorable | 16 (16.8%) | 9 (18.8%) | 3 (8.8%) | 4 (30.8%) | 0.352 |
| Intermediate | 62 (65.3%) | 31 (64.6%) | 23 (67.6%) | 8 (61.5%) | |
| Poor | 16 (16.8%) | 7 (14.6%) | 8 (23.5%) | 1 (7.7%) | |
| Synchronous metastasis | 65 (68.4%) | 33 (68.8%) | 24 (70.6%) | 8 (61.5%) | 0.805 |
| Prior nephrectomy | 75 (78.9%) | 39 (81.3%) | 25 (73.5%) | 11 (84.6%) | 0.668 |
| Clear cell histology | 68 (71.6%) | 35 (72.9%) | 23 (67.6%) | 10 (76.9%) | 1 |
| Tumor stage | | | | | |
| T1 | 32 (33.7%) | 16 (33.3%) | 12 (35.3%) | 4 (30.8%) | 0.916 |
| T2 | 8 (8.4%) | 4 (8.3%) | 3 (8.8%) | 1 (7.7%) | |
| T3 | 33 (34.7%) | 18 (37.5%) | 9 (26.5%) | 6 (46.2%) | |
| T4 | 7 (7.4%) | 5 (10.4%) | 1 (2.9%) | 1 (7.7%) | |
| Lymph nodes | | | | | |
| N0 | 44 (46.3%) | 22 (45.8%) | 16 (47.1%) | 6 (46.2%) | 0.834 |
| N1 | 21 (22.1%) | 10 (20.8%) | 9 (26.5%) | 2 (15.4%) | |
| NX | 22 (23.2%) | 12 (25.0%) | 6 (17.6%) | 4 (30.8%) | |
| Grade | | | | | |
| 1 | 4 (4.2%) | 3 (6.3%) | 0 (0%) | 1 (7.7%) | 0.063 |
| 2 | 27 (28.4%) | 18 (37.5%) | 4 (11.8%) | 5 (38.5%) | |
| 3 | 32 (33.7%) | 16 (33.3%) | 13 (38.2%) | 3 (23.1%) | |
| 4 | 14 (14.7%) | 4 (8.3%) | 7 (20.6%) | 3 (23.3%) | |
| Positive margins | 6 (6.3%) | 3 (6.3%) | 2 (5.9%) | 1 (7.7%) | 0.668 |
| Radiotherapy | 29 (30.5%) | 15 (31.3%) | 11 (32.4%) | 3 (23.1%) | 0.881 |
| Time to therapy | 7.0 (1.0–25.5) | 10.0 (2.0–32.3) | 3.0 (1.0–10.8) | 10.0 (4.0–33.0) | **0.025** |
| 1st-line therapy | | | | | |
| IO+IO | 59 (62.1%) | 32 (66.7%) | 22 (64.7%) | 5 (38.5%) | 0.185 |
| IO+TKI | 36 (37.9%) | 16 (33.3%) | 12 (35.3%) | 8 (61.5%) | |
| Baseline CRP in mg $dL^{-1}$ | 2.21 (0.50–14.86) | 0.87 (0.23–2.35) | 8.87 (3.94–58.42) | 1.73 (0.78–9.17) | < **0.001** |
| No. of CRP measurements first 3 months | 5.0 (3.0–8.0) | 5.0 (3.0–8.0) | 4.0 (3.0–7.75) | 7.0 (5.0–8.0) | 0.232 |

Significant *P*-values are displayed in bold.

## Response and outcomes by early CRP kinetics

Thirteen (13.7%) patients were classified as CRP flare-responders, 34 (35.8%) as CRP responders and 48 (50.5%) as non-CRP responders (Figure 1). There were no significant differences in baseline characteristics (Table 1), except median time from initial diagnosis to start of systemic therapy and median baseline CRP values, as CRP non-responders had significantly lower CRP values than CRP (flare) responders ($P < 0.001$). The median follow-up length did not differ between CRP dynamic groups ($P = 0.292$). A median of 6.0 doses (4.0–14.5) of intravenous IO therapy was administered in the whole study population and the amount differed significantly between the three subgroups ($P = 0.016$). CRP flare-responders, CRP responders and non-CRP responders had a median maximum target lesion change of −16.3% (IQR −32.5% to −1.0%), −31.7% (IQR −38.7% to −12.5%) and 6.8% (IQR −7.8% to 40.8%), correspondingly (Figure 2a, $P < 0.001$). Five (5/12 = 41.7%) patients in the CRP flare-responder, 14 (14/31 = 45.2%) in the CRP responder and 8 (8/48 = 17.0%) in the non-CRP responder group had

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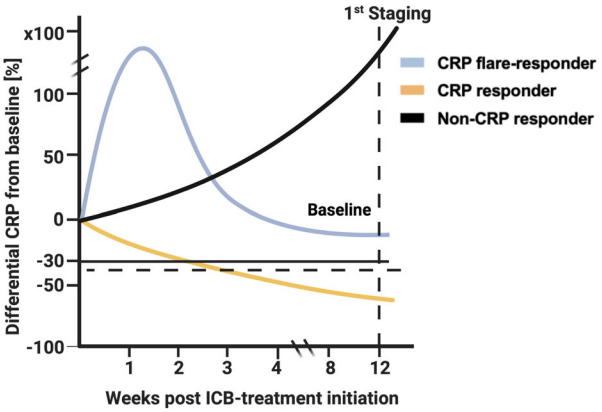


**Figure 1.** Model of early C-reactive protein (CRP) kinetics with the CRP flare-response phenomenon, CRP response and non-CRP response after IO therapy initiation up to 1st staging. Adapted from Fukuda *et al.*,[15] created with BioRender.com.

an objective therapy response, which differed significantly ($P = 0.019$).

### Survival analysis by early CRP kinetics

The median progression-free survival (PFS) after initiation of IO treatment was 5.6 months (95% CI 3.4–12.2 months) for non-CRP responders, 16.2 months for CRP responders (95% CI 10.9 months – not reached) and 19.2 months for CRP flare-responders (95% CI 17.4 months – not reached) and differed significantly (Figure 2b). When the overall cohort was divided into subgroups of patients receiving IO+IO or IO+TKI, early CRP kinetics remained significantly associated with PFS on immunotherapy in both groups (Supplementary figure 1). Of note, the majority of CRP flare-responders (8/10 = 80.0%) showed long-term therapy response lasting ≥ 12 months. Thus, the mean duration of IO response differed significantly between the groups ($P = 0.001$; Figure 3a).

### Cox regression

In the univariate Cox regression, early CRP dynamics was the only factor that was significantly associated with the PFS, besides ECOG score (Table 2). Of note, baseline CRP level was not associated with PFS, but highest in the CRP response group (Figure 3b, $P < 0.001$). Compared to Non-CRP responders, CRP responders had a risk reduction for progression of 68% [hazard ratio HR 0.32, 95% confidence interval (CI) 0.17–0.62, $P = 0.001$] and CRP flare-responders of 73% (HR 0.27 95% CI 0.11–0.66, $P = 0.004$). No other patient or tumor-related factor had an impact on the PFS after IO treatment initiation. In the multivariate Cox regression model, the impact of CRP dynamics and ECOG score remained significant (Table 2). Additionally, the therapy regimen, baseline CRP (HR 1.01, $P = 0.021$) and T stadium (T2 vs. T1: OR 7.56, $P = 0.004$) now also had a significant impact on progression.

Regarding OS, only ECOG had a significant impact in the univariate Cox regression, as patients with worse performance status had an increased risk for death from any cause (Supplementary table 1). However, this association did not remain significant in the multivariate Cox regression model.

## DISCUSSION

In this retrospective multicentre study, we validate that early CRP kinetics on immunotherapy is a promising predictive biomarker in mRCC. Because of its low cost and wide clinical availability, the CRP kinetic assessment is easy to implement into daily clinical practice and may prove to be a valuable tool for IO therapy monitoring in the future.

In our cohort consisting of 95 patients with either IO+IO- or IO+TKI-based first-line therapy, CRP flare-response was associated with long-term response and improved PFS in the αPD-1-based first-line setting of mRCC. However, in our mRCC cohort, early CRP kinetics showed no significant association with OS, which is most likely attributed to the relatively low number of events in the cohort. Since the new 1st-line combination therapies in mRCC remarkably prolong OS, we plan to reanalyse this cohort after extending the follow-up period. Further, early CRP kinetics was significantly associated with improved PFS in both subgroups (IO+IO and IO+TKI), leading us to conclude that early CRP kinetics is a robust predictive biomarker in mRCC independent of the chosen first-line treatment combination. Since Fukuda *et al*. described the predictive value of early CRP kinetics for nivolumab monotherapy in 2nd line or later, it appears that early CRP kinetics can therefore be used to optimise treatment monitoring for all αPD-1-based therapies in mRCC.[15] We consider this to be a particularly important information for the daily clinical routine, as early CRP kinetics could be used as a


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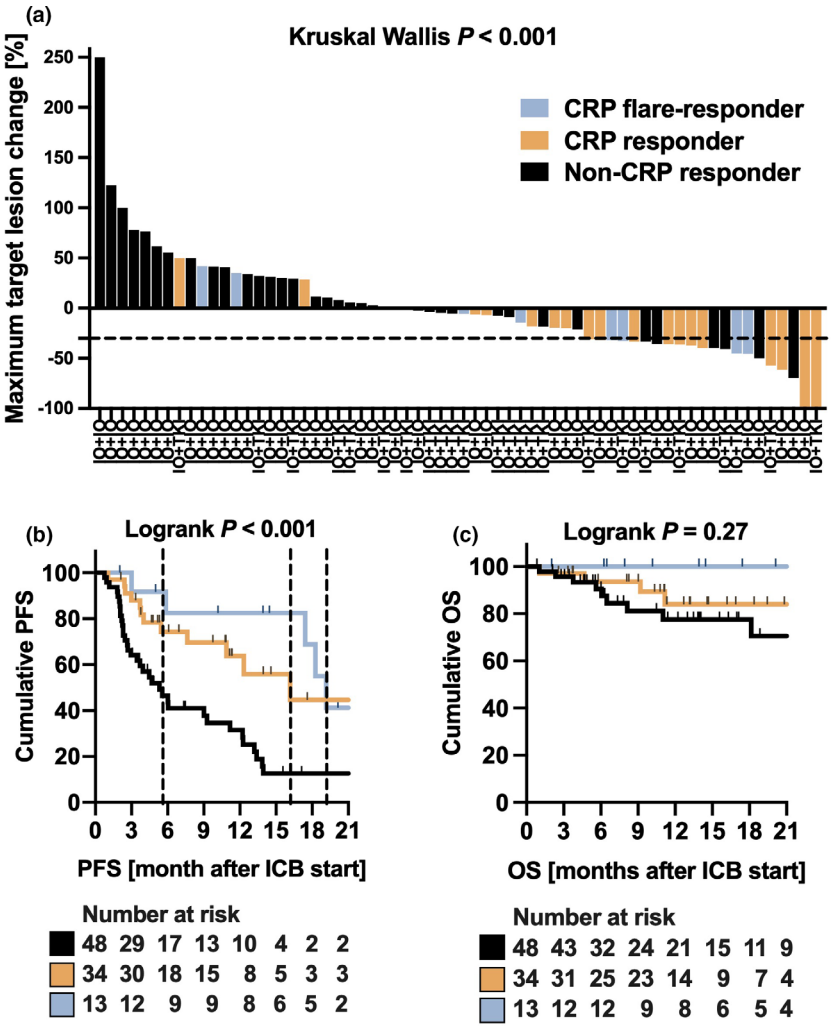


**Figure 2. (a)** Waterfall plot depicting the maximum target lesion change in the three defined CRP kinetic subgroups of the multicentre mRCC cohort ($N$ = 64; no RECIST data available for $N$ = 31 patients). **(b, c)** Progression-free (PFS) and overall survival (OS) after IO treatment initiation for CRP flare-responder ($N$ = 13), CRP responder ($N$ = 34) or non-CRP responder ($N$ = 48). Median PFS is depicted as a dotted line, median OS not reached.

simple and cost-effective biomarker for all immunotherapy regimen in mRCC. Non-CRP response would lead to earlier staging, and in the event of tumor progression, allow clinicians to administer alternative and more effective therapies while preventing exposure to potentially life-threatening toxic effects of immunotherapy.[17] In our analysis, early CRP kinetics appear to have the potential to predict treatment response before initial staging and thus lead to earlier treatment modification, which could ultimately improve the clinical course of mRCC patients.

In addition, it appears to be highly relevant to sensitise clinicians to the characteristic CRP flare-response phenomenon, as a rapid increase in CRP could be the result of a desirable antitumor immune response. CRP flare-response should, in the absence of other clinical symptoms, thus not be misinterpreted as a bacterial infection or another side effect after IO therapy initiation especially since antibiotic-induced dysbiosis can compromise the clinical activity of immunotherapy by modulating, for example the gut microbiome.[18]

Exploring the tumor immunologic basis of the differential CRP kinetics after initiation of immunotherapy might further enhance our understanding of the interplay between the RCC tumor cells and its tumor microenvironment (TME).[19–21] Baseline serum CRP concentration, which may reflect the baseline RCC

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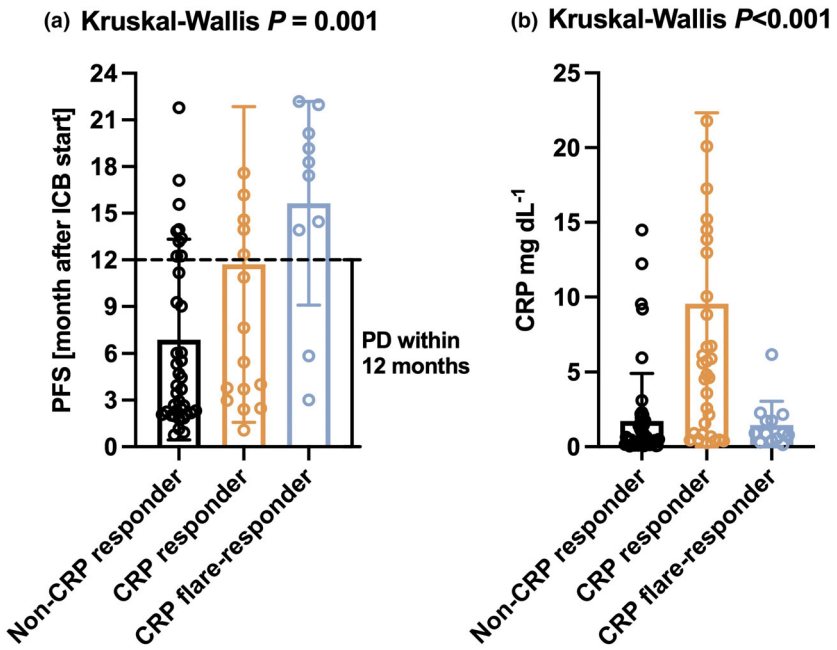


**Figure 3. (a)** Duration of immunotherapy depending on the CRP dynamic subgroups is shown. Long-term IO response was defined as ≥ 12 months. Patients with ongoing IO therapy but follow-up less than 12 months were excluded for this analysis because achievement of long-term response cannot be stratified. **(b)** Boxplot depicting baseline CRP serum concentration stratified by CRP dynamic groups (mean with SD).

immunogenicity, differs significantly between the CRP response groups. The low baseline CRP level in flare-responders could be an indirect surrogate for low or absent chronic inflammation caused by the tumor burden. Thus, we hypothesise that in treatment-naïve RCC tissue, differential immune phenotypes may predict early CRP kinetics as IO treatment triggers distinct immune cell infiltration patterns to enrich the TME. Thereafter, the induction of an antitumor immune response leads to systemic inflammation through the release of inflammatory mediators, which can ultimately be measured by serum CRP. To address this hypothesis, future studies will need to perform comprehensive phenotyping of treatment-naïve tumor tissue, followed by integration of the early CRP kinetic subset. From a clinical point of view, the identification of specific TME patterns in treatment-naïve RCC tissue that robustly predict early CRP kinetics and response would be of high relevance to stratify our patients before therapy, especially since currently available predictive tools such as PD(L)-1 immunohistochemistry (IHC) only play a minor role in mRCC.[22] From a cancer-immunologic point of view, it would be of high relevance to identify the distinct immune signatures associated with non-CRP response and IO treatment failure to identify potential targets for tailored combination therapy in this immunotherapy-unresponsive RCC subgroup.

Increased baseline concentration of inflammation markers such as CRP or IL-8 before oncological treatment has also been associated with worse clinical outcome in mRCC patients treated with immunotherapy elsewhere, but the dynamic and early change in systematic inflammation after therapeutic intervention was mostly neglected.[9,10,12,23,24] In mRCC, an early decrease in CRP after initiation of TKI therapy has already been associated with improved response and survival.[25] Only recently, the predictive potential of characteristic longitudinal changes in CRP, especially the newly described flare-response, during the first 3 months of αPD-1 monotherapy in the post-TKI setting has been highlighted. We evaluated the predictive value of early CRP dynamics in a larger, multicentre and more clinically relevant cohort in the first-line setting in mRCC and demonstrated that CRP responders and particularly CRP flare-responders showed favorable progression-free survival (PFS) and mostly durable treatment response. Further studies will have to clarify whether the flare-response kinetics of systemic inflammation can be sharpened by replacing the relatively nonspecific CRP with other acute-phase reactants or immune


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**Table 2.** Uni- and multivariable Cox regression analyses for progression-free survival

| | Univariable | | Multivariate | |
|---|---|---|---|---|
| | HR (95% CI) | *P*-value | HR (95% CI) | *P*-value |
| Therapy | | | | |
| IO+IO | ref. | 0.059 | ref. | 0.030 |
| IO+TKI | 0.58 (0.33; 1.02) | | 0.29 (0.1; 0.89) | |
| CRP dynamics | | | | |
| No response | ref. | < 0.001 | ref. | 0.002 |
| Responder | 0.32 (0.17–0.62) | 0.001 | 0.22 (0.06; 0.76) | 0.017 |
| Flare-responder | 0.27 (0.11–0.66) | 0.004 | 0.19 (0.06; 0.60) | 0.005 |
| Baseline CRP | 1.00 (0.97; 1.04) | 0.893 | 1.01 (1.00; 1.02) | 0.021 |
| Age | 0.98 (0.96; 1.00) | 0.094 | 0.98 (0.95; 1.01) | 0.246 |
| Gender | | | | |
| Male | ref. | 0.545 | ref. | 0.987 |
| Female | 1.19 (0.68; 2.10) | | 0.63 (0.28; 1.43) | |
| ECOG | | | | |
| 0 | ref. | 0.010 | ref. | 0.001 |
| 1 | 1.74 (0.98–3.09) | 0.058 | 3.40 (1.57; 7.36) | 0.002 |
| 2 | 6.46 (2.1–19.90) | 0.001 | 23.60 (4.1; 136.03) | < 0.001 |
| 3 | 0.0 | 0.980 | 0.0 | 0.978 |
| IMDC | | | | |
| Favorable | ref. | 0.620 | ref. | 0.330 |
| Intermediate | 1.40 (0.67; 2.94) | 0.372 | 0.47 (0.13; 1.70) | 0.249 |
| Poor | 1.51 (0.60; 3.83) | 0.382 | 0.84 (0.17; 4.05) | 0.826 |
| Histology | | | | |
| Clear cell | ref. | 0.233 | ref. | 0.108 |
| Non-clear cell | 1.54 (0.76; 3.11) | | 2.40 (0.83; 7.00) | |
| pT stadium | | | | |
| pT1 | ref. | 0.500 | ref. | 0.022 |
| pT2 | 1.69 (0.65; 4.38) | 0.280 | 7.56 (1.91; 29.87) | 0.004 |
| pT3 | 1.57 (0.81; 3.05) | 0.182 | 3.04 (1.14; 8.08) | 0.026 |
| pT4 | 1.76 (0.58; 5.39) | 0.320 | 5.66 (1.31; 24.47) | 0.020 |

mediators. After prospective validation of the predictive potential of early CRP kinetics in mRCC and possibly in additional tumor subtypes, we propose early CRP kinetics as a promising on-treatment biomarker for stratifying our patients in the era of immuno-oncology.

Despite noteworthy strengths, such as the multicentre approach and the comparably large study cohort, our study also has several limitations. First and foremost, we acknowledge that the study is limited by its observational nature and the relatively short follow-up time, especially for the meaningful endpoint OS. Moreover, our results should be interpreted within the limitations of the retrospective design. CRP was measured in different routine clinical laboratories at the study centres and without a standardised scheme, so some CRP flare-responses may have been missed. In addition, modification of the new and not prospectively validated early CRP kinetic concept might increase its predictive value. Nevertheless, we propose a prospective evaluation of our results in future studies, based on our promising retrospective data.

If prospectively validated, we propagate that early CRP kinetics should be assessed as an easy-to-implement, non-invasive biomarker during IO combination therapy in mRCC as the new standard of care, as early detection of treatment success and failure might have the potential to optimise treatment monitoring and adjustment and to prevent exposure to potentially life-threatening side effects of IO therapy.

## METHODS

In this retrospective multicentre study, $N = 118$ consecutive mRCC patients from six German tertiary referral centres receiving either first-line IO+IO (αPD-1/nivolumab + αCTLA4/ipilimumab) or IO+TKI (α PD-1/pembrolizumab + VEGFR-TKI/axitinib) were screened. Patients with CRP measurements at baseline (closest to treatment initiation, maximum 6 weeks before), at least once within the first month of treatment

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and at least one further CRP at the time of first staging or clinical progression were included in the study. Of the total $N = 118$ patients initially studied, $N = 23$ were excluded due to missing CRP values, resulting in a study cohort of $N = 95$ patients.

This study was conducted according to the Declaration of Helsinki and approved by the responsible ethical review board (reference #20201211-01).

The patient demographics and baseline parameters including IMDC risk criteria were obtained. Tumor response was graded according to response evaluation criteria in solid tumors (RECIST v1.1).[26] Therapy outcomes were compared among the three characteristic therapy groups, defined by diverging CRP dynamics. According to the earlier definition by Fukuda *et al*., 'CRP flare-responders' were defined as an early increase in CRP levels to more than double from baseline within 1 month after therapy initiation and a subsequent decrease below the baseline within 3 months. Patients with a decrease by $\geq 30\%$ from baseline within 3 months without flare-response were classified as 'CRP responders', all other patients as 'non-CRP responders' (Figure 1).[15] To define these CRP dynamic groups, CRP at baseline, during the first month after treatment initiation and follow-up visits was obtained. Serum CRP concentration was measured in accredited routine laboratories in each participating centre and is given in mg $dL^{-1}$ (clinical reference $< 0.5$ mg $dL^{-1}$).

Categorical variables were reported as frequencies and proportions, continuous data as the median and range. Fisher's exact tests, Mann–Whitney *U*-tests and Kruskal–Wallis tests were applied to perform intergroup comparisons. The PFS and OS, including 95% confidence intervals, were estimated from the day of treatment initiation until the respective event using the Kaplan–Meier method and compared with log-rank tests. Progression was defined according to the RECIST v1.1 criteria including death from any cause. To compare the impact of the therapy regimen (IO+IO vs. IO+TKI), CRP dynamics (CRP flare-responder, CRP responder vs. non-CRP responder), baseline patient (age, gender, ECOG) and tumor-related parameters (e.g. IMDC, histology, pT-stage) on OS and PFS, univariate and multiple Cox regressions were conducted. Patient age and CRP baseline were defined as continuous, all others as categorical variables. In the event of missing data, cases were excluded from the analysis. Statistical analyses were performed with SPSS version 25 (IBM, Armonk, NY, USA), R (version x64 4.0.3) and GraphPad Prism 9 (GraphPad Software Inc, CA, USA). All statistical tests were two-sided, and *P*-values $< 0.05$ were considered significant.

## ACKNOWLEDGMENTS

This study was supported by the junior research group funding by the BONFOR Program of the Medical Faculty of the University of Bonn, grant ID 2020-2A-12 (NK). Open Access funding enabled and organized by Projekt DEAL.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

**Niklas Klümper:** Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Visualization; Writing – original draft. **Philipp Schmucker:** Formal analysis; Investigation; Methodology; Project administration; Visualization; Writing – original draft. **Oliver Hahn:** Data curation; Investigation; Writing – review & editing. **Benedikt Höh:** Formal analysis; Investigation; Writing – review & editing. **Angelika Mattigk:** Formal analysis; Investigation; Writing – review & editing. **Severine Banek:** Resources; Supervision; Writing – review & editing. **Jörg Ellinger:** Resources; Supervision; Writing – review & editing. **Julia Heinzelbecker:** Resources; Supervision; Writing – review & editing. **Danijel Sikic:** Formal analysis; Investigation; Writing – review & editing. **Markus Eckstein:** Formal analysis; Investigation; Writing – review & editing. **Arne Strauß:** Resources; Supervision; Writing – review & editing. **Friedemann Zengerling:** Resources; Supervision; Writing – review & editing. **Michael Hölzel:** Resources; Supervision; Writing – review & editing. **Philip Zeuschner:** Formal analysis; Investigation; Methodology; Project administration; Validation; Writing – original draft. **Charis Kalogirou:** Conceptualization; Investigation; Project administration; Resources; Supervision; Validation; Writing – review & editing.

## ETHICS APPROVAL

This study was approved by the responsible ethical review board (20201211-01).

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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### 3.4 Klümper, N. et al., (2022). C reactive protein flare predicts response to checkpoint inhibitor treatment in non-small cell lung cancer. J Immunother Cancer 10, e004024.

Die frühe longitudinale *on-treatment* CRP-Kinetik nach Beginn der Immuntherapie wurde in einer retrospektiven Entdeckungs- (N=105) und prospektiven Validierungskohorte (N=108) von Patienten mit ICI-behandelten NSCLC untersucht. Die Patienten wurden, wie zuvor für das mRCC beschrieben, als CRP-Flare-Responder, CRP-Responder oder CRP Non-Responder klassifiziert und ihr onkologischer Verlauf unter Immuntherapie wurde miteinander verglichen. In dieser Studie konnte erstmalig auch für Patienten mit NSCLC die gleiche charakteristische longitudinale CRP-Flare Kinetik, die bereits für das ICI-behandelte mRCC beschrieben wurde, beobachtet werden. In der prospektiven Kohorte wurden N=40 Patienten als CRP-Non-Responder, N=39 als CRP-Responder und N=29 als CRP-Flare-Responder mit einem medianen PFS von 2.4, 8.1 bzw. 14.3 Monaten und einem medianen OS von 6.6, 18.6 bzw. 32.9 Monaten definiert (beide log-rank $p<0,001$). Cox-Regressionsanalysen zeigten, dass CRP-Flare-Responder im Vergleich zu CRP-Non-Respondern eine multivariabel adjustierte Risikoreduktion von 78% für das Versterben aufwiesen. Die frühe CRP-Kinetik zeigte keinen prädiktiven Wert für Patienten unter Chemoimmuntherapie oder bei gleichzeitiger Verabreichung von Steroiden in äquivalenter Dosis zu 10mg Prednisolon. Die *on-treatment* CRP-Kinetik wies unabhängig von der Histologie für die beiden häufigsten NSCLC-Subtypen, dem pulmonalen Adeno- und Plattenepithelkarzinom, einen prädiktiven Wert auf. In einem explorativen Ansatz konnte die ursprüngliche CRP-Kinetik-Definition von Fukuda et al. durch Anpassung des Beobachtungsintervalls optimiert werden: Nach der neuen Definition wies die frühe longitudinale CRP-Kinetik bereits vier Wochen nach Therapiebeginn einen robusten prädiktiven Wert auf. Da das erste Routine-Staging in der Regel 8-12 Wochen nach Therapiestart durchgeführt wird, öffnet sich durch Anwendung der modifizierten CRP-Kinetik Definition ein großes therapeutisches Fenster. Zusammenfassend lässt sich sagen, dass in zwei unabhängigen Kohorten mit insgesamt N=213 Patienten mit NSCLC bewiesen werden konnte, dass CRP-Flare das Ansprechen auf eine anti-PD-1-Monotherapie unabhängig von der Histologie vorhersagt. Aufgrund seiner breiten klinischen Verfügbarkeit könnte die frühe CRP-Kinetik zu einem einfach zu bestimmenden, kosteneffektiven und nicht-invasiven *on-treatment* Biomarker werden, welcher das Immuntherapieansprechen für Patienten mit NSCLC innerhalb des ersten Therapiemonats robust vorhersagen kann.

Journal for ImmunoTherapy of Cancer

# C reactive protein flare predicts response to checkpoint inhibitor treatment in non-small cell lung cancer

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**To cite:** Klümper N, Saal J, Berner F, *et al*. C reactive protein flare predicts response to checkpoint inhibitor treatment in non-small cell lung cancer. *Journal for ImmunoTherapy of Cancer* 2022;**10**:e004024. doi:10.1136/jitc-2021-004024

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/jitc-2021-004024).

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Accepted 14 February 2022

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## ABSTRACT

Biomarkers for predicting response to anti-programmed death-1 (PD-1) immune checkpoint blockade (ICB) in non-small cell lung cancer (NSCLC) remain in demand. Since anti-tumor immune activation is a process, early dynamic changes of the acute-phase reactant C reactive protein (CRP) may serve as a predictive on-treatment biomarker. In a retrospective (N=105) and prospective (N=108) ICB-treated NSCLC cohort, early CRP kinetics were stratified after the start of immunotherapy until weeks 4, 6, and 12 as follows: an early doubling of baseline CRP followed by a drop below baseline (CRP flare-responder), a drop of at least 30% below baseline without prior flare (CRP responders), or those who remained as CRP non-responders. In our study, we observed characteristic longitudinal changes of serum CRP concentration after the initiation of ICB. In the prospective cohort, N=40 patients were defined as CRP non-responders, N=39 as CRP responders, and N=29 as CRP flare-responders with a median progression-free survival (PFS) of 2.4, 8.1, and 14.3 months, respectively, and overall survival (OS) of 6.6, 18.6, and 32.9 months (both log-rank p<0.001). Of note, CRP flare-responses, characterized by a sharp on-treatment CRP increase in the first weeks after therapy initiation, followed by a decrease of CRP serum level below baseline, predict ICB response as early as 4 weeks after therapy initiation. Of note, early CRP kinetics showed no predictive value for chemoimmunotherapy or when steroids were administered concurrently. On-treatment CRP kinetics had a predictive value for both major histological NSCLC subtypes, adenocarcinoma and squamous cell carcinoma. The results were verified in an independent retrospective cohort of 105 patients. In conclusion, CRP flare predicted anti-PD-1 monotherapy response and survival in two independent cohorts including a total of 213 patients with NSCLC, regardless of histology. Due to its wide clinical availability, early CRP kinetics could become an easily determined, cost-efficient, and non-invasive biomarker to predict response to checkpoint inhibitors in NSCLC within the first month.

## INTRODUCTION

The treatment landscape for advanced non-small cell lung cancer (NSCLC) has been changing rapidly in recent years. The introduction of immunotherapy with blockade of the programmed death-1 (PD-1)/programmed death ligand-1 (PD-L1) axis (immune checkpoint blockade (ICB)) constituted a major breakthrough, prolonging survival and offering treatment options beyond cytostatic chemotherapy and targeted therapy. For the first time, long-term survival in metastatic NSCLC has been observed in a significant percentage of patients. However, not all patients benefit from ICB and immune-related adverse events (irAEs) can be life threatening.[1 2]

Robust predictive biomarkers are of great clinical interest to maintain the balance between potential irAE and therapeutic benefit.[3] Since anti-tumor immune activation is a dynamic process, on-treatment inflammatory biomarkers such as acute-phase reactants have great potential to capture the precise tumor-immune interplay and might serve as accurate prediction tools.

Previous studies have demonstrated the use of different liquid biopsy-based biomarkers to predict ICB response in NSCLC and other tumors.[4–6] An early increase in proinflammatory cytokines such as interleukin 6 (IL-6) or tumor necrosis factor α (TNF-α) after the onset of ICB correlated with response in NSCLC.[4] After initiation of ICB treatment, characteristic longitudinal CRP kinetics were associated with response to anti-PD-1 monotherapy and combination therapy in two independent retrospective cohorts of metastatic renal cell carcinomas (RCCs), but without prospective validation.[6 7] Patients were divided into three groups based on their on-treatment CRP levels as defined by Fukuda *et al*.[6] ICB treatment was most effective in patients with a so-called CRP flare-response meaning an early CRP increase after ICB initiation ('flare') and a subsequent drop of serum CRP level below baseline. The early increase of proinflammatory cytokines, for example, IL-6

is the main stimulus for hepatic CRP production, after the onset of the antitumor immune response seems to be the immunological basis for this early CRP kinetics. Due to its broad availability and relatively low cost, CRP kinetics appears to be an excellent easy-to-implement biomarker to predict immunotherapy response. Here, we assessed whether early CRP kinetics predicts response to immunotherapy and treatment outcomes in NSCLC using a representative retrospective discovery and a prospective validation cohort, each including N>100 patients.

## MATERIALS AND METHODS

### Clinical sample collection

Two independent NSCLC cohorts receiving (chemo) immunotherapy were analyzed: Discovery Cohort: a retrospective cohort of patients with NSCLC treated at the University Medical Center Bonn (UKB) and Center for Integrated Oncology, Germany (CIO NSCLC, N=105). Inclusion into the retrospective CIO NSCLC cohort required measurement of CRP at baseline (maximum 30 days before first ICB application), at least once within 30 days after the start of ICB treatment and at the time of first staging or clinical progression. Validation Cohort: patients with NSCLC receiving anti-PD-1/PD-L1 treatment within the prospective immune monitoring of immune therapy study (IMIT NSCLC, N=108), which was conducted in four Swiss centers (Kantonsspital St Gallen, Spital Grabs, Spital Wil, and Spital Flawil) from July 1, 2016, to January 15, 2021.[8] Response data according to RECIST criteria at first staging were available for N=88 patients. In both cohorts, relevant steroid comedication was defined as prednisolone 10 mg or equivalent dosage of other steroids. Patients who only received steroids as an antiemetic agent in combination with chemotherapy were not included in the 'concurrent steroid medication' subgroup.

### Measuring serum parameters in blood samples

Baseline and longitudinal serum CRP concentrations were measured in accredited routine laboratories for both cohorts. A CRP value of above 5 mg/L was considered as elevated. For the validation cohort, lactate dehydrogenase (LDH) was measured in accredited routine laboratories, and values above 250 U/L were considered elevated.

### Neutrophil-to-lymphocyte ratio

The neutrophil-to-lymphocyte ratio (NLR) was calculated on the basis of the differential blood count as neutrophils divided by lymphocytes. The median NLR of the IMIT NSCLC validation cohort was 4.7 and considered as the threshold for elevated NLR.

### Definition of early CRP kinetics

Patients were classified according to the CRP kinetics definition as previously described.[6] CRP flare-response was defined as at least twofold increase of baseline CRP within 30 days after ICB treatment followed by a decrease of serum CRP level below baseline. CRP response was defined as serum CRP level falling 30% below baseline within 12 weeks in at least one measurement. All other patients were classified as CRP non-responders. In an exploratory analysis, early CRP kinetics definition was applied at 4 or 6 weeks after initiation of immunotherapy in the prospective IMIT NSCLC validation cohort.

### Statistical analysis

R studio (V.1.4.1106) using the 'survminer' package was used to perform statistical analyses. Kruskal–Wallis rank-sum test, Pearson's $\chi^2$ test, and Fisher's exact test were applied to perform intergroup comparisons. Progression was defined according to the RECIST V.1.1 criteria including death from any cause. Progression-free survival (PFS) and overall survival (OS) after ICB initiation were estimated by univariable Kaplan–Meier regression and tested with the log-rank test. Univariable and multivariable Cox regression analyses were performed to compare the prognostic value of early CRP kinetics (CRP flare-responders, CRP responders vs CRP non-responders) with baseline characteristics with respect to PFS and OS after ICB initiation. Variables were only included in multivariable Cox regression models if survival effects were significant in univariable analyses. All tests were two-sided, and p values <0.05 were considered significant.

## RESULTS

### Retrospective CIO NSCLC discovery cohort

To determine the relevance of serum CRP kinetics for the efficacy of ICB in NSCLC, we retrospectively analyzed N=105 patients receiving (chemo)immunotherapy for advanced NSCLC at the UKB between 2005 and 2020. Using previously described criteria for serum CRP kinetics,[6] we determined a CRP flare-response (twofold increase of baseline CRP within 30 days after ICB and drop of serum CRP below baseline within 12 weeks on-treatment) in 29.5% (N=31) of the patients, 39.0% (N=41) showed a CRP response (CRP level falling 30% below baseline at least once within 12 weeks on-treatment), and 31.4% (N=33) were classified as CRP non-responder (patients that did not meet the above-mentioned criteria) (online supplemental figure 1A). Among CRP non-responders, median PFS and OS were 2.6 and 11.8 months compared with 12.1 and 28.2 months for CRP responder and 9.2 and 21.5 months for CRP flare-responder (online supplemental figure 1B). Baseline characteristics were similar with regard to age, gender, and PD-L1 tumor proportion score (TPS) across all three CRP kinetics groups (online supplemental table 1). Results for univariable Cox regression are summarized in online supplemental table 2. In conclusion, the CRP (flare)-response in our discovery cohort was associated with prolonged PFS and OS in patients with NSCLC treated with ICB.

### Prospective IMIT NSCLC validation cohort

To validate our findings, we examined early CRP kinetics in N=108 patients with NSCLC (stage IIIb–IV)

**Table 1** Comparison of baseline parameters between CRP flare-responders, CRP responders, and CRP non-responders in the IMIT NSCLC validation cohort

| | Baseline characteristics of the validation cohort | | | | |
|---|---|---|---|---|---|
| **Characteristic** | **Overall, N=107*** | **Non-responder, N=39** | **Responder, N=40** | **Flare-responder, N=28** | **P value†** |
| Age | | | | | >0.9 |
| Median (IQR) | 67 (61–73) | 67 (61–73) | 67 (59–74) | 66 (62–73) | |
| Range | 33–84 | 44–80 | 33–83 | 54–84 | |
| Sex | | | | | 0.074 |
| Male | 61 (57%) | 26 (67%) | 24 (60%) | 11 (39%) | |
| Female | 46 (43%) | 13 (33%) | 16 (40%) | 17 (61%) | |
| Histology | | | | | 0.8 |
| Adenocarcinoma | 78 (76%) | 28 (74%) | 27 (73%) | 23 (82%) | |
| SCC | 21 (20%) | 8 (21%) | 8 (22%) | 5 (18%) | |
| Mixed histology | 4 (3.9%) | 2 (5.3%) | 2 (5.4%) | 0 (0%) | |
| Unknown | 4 | 1 | 3 | 0 | |
| Number of pack-years | | | | | 0.8 |
| Median (IQR) | 40 (30–60) | 50 (30–60) | 40 (30–55) | 40 (30–50) | |
| Range | 0–99 | 0–99 | 12–99 | 0–90 | |
| Unknown | 12 | 4 | 5 | 3 | |
| Presence of cerebral metastasis | | | | | 0.2 |
| Yes | 35 (34%) | 16 (44%) | 12 (31%) | 7 (25%) | |
| No | 68 (66%) | 20 (56%) | 27 (69%) | 21 (75%) | |
| Unknown | 4 | 3 | 1 | 0 | |
| Line of therapy | | | | | 0.6 |
| First line | 42 (39%) | 13 (33%) | 16 (40%) | 13 (46%) | |
| Higher line | 65 (61%) | 26 (67%) | 24 (60%) | 15 (54%) | |
| PD-L1 expression (%) | | | | | 0.5 |
| Median (IQR) | 10 (0–60) | 1 (0–30) | 30 (0–70) | 20 (0–75) | |
| Range | 0–100 | 0–100 | 0–90 | 0–100 | |
| Unknown | 35 | 10 | 16 | 9 | |
| PD-L1 expression | | | | | 0.081 |
| TPS ≤50% | 46 (64%) | 23 (79%) | 13 (54%) | 10 (53%) | |
| TPS >50% | 26 (36%) | 6 (21%) | 11 (46%) | 9 (47%) | |
| Unknown | 35 | 10 | 16 | 9 | |
| Concurrent cytostatic therapy | | | | | 0.6 |
| No | 84 (79%) | 32 (82%) | 32 (80%) | 20 (71%) | |
| Yes | 23 (21%) | 7 (18%) | 8 (20%) | 8 (29%) | |
| Concurrent steroid medication | | | | | >0.9 |
| No | 90 (84%) | 33 (85%) | 34 (85%) | 23 (82%) | |
| Yes | 17 (16%) | 6 (15%) | 6 (15%) | 5 (18%) | |
| Baseline CRP | | | | | **0.004** |
| Baseline CRP ≤5 mg/L | 23 (23%) | 8 (21%) | 4 (10%) | 11 (46%) | |
| Baseline CRP >5 mg/L | 79 (77%) | 30 (79%) | 36 (90%) | 13 (54%) | |
| Unknown | 5 | 1 | 0 | 4 | |
| Baseline LDH | | | | | **0.018** |
| Baseline LDH ≤260 U/L | 49 (49%) | 18 (50%) | 12 (32%) | 19 (68%) | |
| Baseline LDH >260 U/L | 52 (51%) | 18 (50%) | 25 (68%) | 9 (32%) | |
| Unknown | 6 | 3 | 3 | 0 | |
| Baseline NLR | | | | | 0.9 |

Continued

**Table 1** Continued

| Baseline characteristics of the validation cohort | | | | | |
|---|---|---|---|---|---|
| **Characteristic** | **Overall, N=107*** | **Non-responder, N=39** | **Responder, N=40** | **Flare-responder, N=28** | **P value†** |
| Baseline NLR ≤4.7 | 51 (50%) | 19 (50%) | 17 (47%) | 15 (54%) | |
| Baseline NLR >4.7 | 51 (50%) | 19 (50%) | 19 (53%) | 13 (46%) | |
| Unknown | 5 | 1 | 4 | 0 | |

*c('Median (IQR)', 'range'); n (%).
†Kruskal–Wallis rank-sum test; Pearson's $\chi^2$ test; Fisher's exact test.
CRP, C reactive protein; IMIT, immune monitoring of immune therapy; LDH, lactate dehydrogenase; NLR, neutrophil-to-lymphocyte ratio; NSCLC, non-small cell lung cancer; PD-L1, programmed death-ligand 1; SCC, squamous cell carcinoma; TPS, tumor proportion score.

treated with ICB within the prospective IMIT observational study (baseline characteristics are shown in table 1). Similar to our discovery cohort, 37.0% (N=40) were classified as CRP non-responders, 36.1% (N=39) as CRP responder, and 26.9% (N=29) as CRP flare-responder (figure 1A). Median PFS for CRP non-responders, CRP responders, and CRP flare-responders was 2.4, 8.1, and 14.3 months; OS was 6.6, 18.6, and 32.9 months, respectively (figure 1B,C). Cox regression analyses showed that CRP flare-responders have a multivariable adjusted risk reduction of 78% (HR=0.22, 95% CI 0.10 to 0.48, p<0.001) for death compared with CRP non-responders (table 2). The objective response rate (ORR) differed significantly for the CRP kinetics groups and was 22.2% for CRP non-responders, 44.4% for CRP responders, and

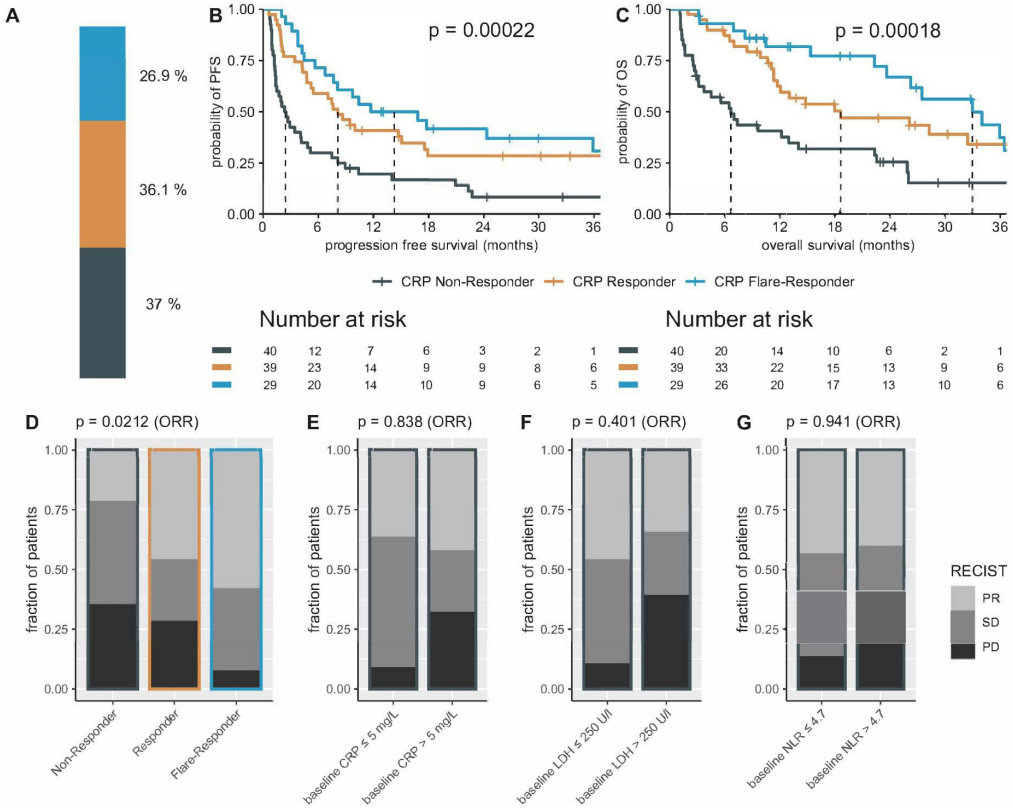


**Figure 1** Distinct early on-treatment C reactive protein (CRP) kinetics correlates with progression-free survival (PFS) and overall survival (OS) in immune checkpoint blockade-treated patients with NSCLC. (A) The bar plot shows the frequency of patients categorized into three CRP kinetic subgroups in the immune monitoring of immune therapy NSCLC validation cohort. (B, C) Kaplan–Meier survival curves showing the PFS and OS after ICB initiation stratified according to CRP kinetics groups. Median PFS/OS is depicted as dotted lines. Distribution of response at first staging according to RECIST among the different CRP kinetics groups (D), as well as for patients with non-elevated or elevated CRP (E), lactate dehydrogenase (F), and neutrophil-to-lymphocyte ratio (G) at baseline. P values in (D–G) are calculated using the $\chi^2$ test for the objective response rate (ORR). NLR, neutrophil-to-lymphocyte ratio; NSCLC, non-small cell lung cancer; PD, progressive disease; PR, partial remission; SD, stable disease.

**Table 2** Univariable and multivariable Cox regression analysis regarding progression-free and overall survival after immunotherapy start in the IMIT NSCLC validation cohort

| | PFS | | | | OS | | | |
|---|---|---|---|---|---|---|---|---|
| **Characteristic** | **N** | **HR** | **95% CI** | **P value** | **N** | **HR** | **95% CI** | **P value** |
| Univariate Cox regression | | | | | | | | |
| CRP kinetics | 107 | | | | 107 | | | |
| Non-responder | | — | — | | | — | — | |
| Responder | | 0.42 | 0.25 to 0.69 | **<0.001** | | 0.37 | 0.21 to 0.65 | **<0.001** |
| Flare | | 0.32 | 0.18 to 0.56 | **<0.001** | | 0.26 | 0.13 to 0.50 | **<0.001** |
| PD-L1 expression (TPS) | 72 | | | | 72 | | | |
| PDL <50% | | — | — | | | — | — | |
| PDL≥50% | | 0.62 | 0.35 to 1.10 | 0.10 | | 0.60 | 0.32 to 1.12 | 0.11 |
| Baseline CRP | 103 | | | | 103 | | | |
| Baseline CRP ≤5 mg/L | | — | — | | | — | — | |
| Baseline CRP >5 mg/L | | 1.89 | 1.09 to 3.27 | **0.024** | | 1.89 | 1.01 to 3.54 | **0.047** |
| Baseline LDH | 102 | | | | 102 | | | |
| Baseline LDH ≤250 U/L | | — | — | | | — | — | |
| Baseline LDH >250 U/L | | 1.97 | 1.25 to 3.10 | **0.004** | | 1.92 | 1.16 to 3.20 | **0.011** |
| Baseline NLR | 103 | | | | 103 | | | |
| Baseline NLR ≤4.7 | | — | — | | | — | — | |
| Baseline NLR >4.7 | | 1.34 | 0.86 to 2.09 | 0.2 | | 1.66 | 1.00 to 2.74 | **0.049** |
| Presence of cerebral metastasis | 104 | | | | 104 | | | |
| No | | — | — | | | — | — | |
| Yes | | 2.13 | 1.35 to 3.36 | **0.001** | | 2.39 | 1.45 to 3.93 | **<0.001** |
| Multivariate Cox regression | | | | | | | | |
| CRP kinetics | 91 | | | | 91 | | | |
| Non-responder | | — | — | | | — | — | |
| Responder | | 0.20 | 0.10 to 0.39 | **<0.001** | | 0.20 | 0.10 to 0.42 | **<0.001** |
| Flare | | 0.27 | 0.14 to 0.52 | **<0.001** | | 0.22 | 0.10 to 0.48 | **<0.001** |
| Baseline CRP | 91 | | | | 91 | | | |
| Baseline CRP ≤5 mg/L | | — | — | | | — | — | |
| Baseline CRP >5 mg/L | | 2.03 | 1.04 to 3.97 | **0.038** | | 1.91 | 0.91 to 4.01 | 0.088 |
| Baseline LDH | 91 | | | | 91 | | | |
| Baseline LDH ≤250 U/L | | — | — | | | — | — | |
| Baseline LDH >250 U/L | | 2.93 | 1.68 to 5.11 | **<0.001** | | 2.38 | 1.34 to 4.24 | **0.003** |
| Baseline NLR | 91 | | | | 91 | | | |
| Baseline NLR ≤4.7 | | — | — | | | — | — | |
| Baseline NLR >4.7 | | 0.85 | 0.50 to 1.44 | 0.5 | | 1.43 | 0.78 to 2.59 | 0.2 |
| Presence of cerebral metastasis | 91 | | | | 91 | | | |
| No | | — | — | | | — | — | |
| Yes | | 1.45 | 0.88 to 2.40 | 0.15 | | 1.47 | 0.85 to 2.55 | 0.2 |

CRP, C reactive protein; IMIT, immune monitoring of immune therapy; LDH, lactate dehydrogenase; NLR, neutrophil-to-lymphocyte ratio; NSCLC, non-small cell lung cancer; OS, overall survival; PD-L1, programmed death-ligand 1; PFS, progression-free survival; TPS, tumor proportion score.

60.0% for CRP flare-responders (figure 1D). In line with previous data,[9] we could show that baseline CRP, LDH, and NLR have a prognostic value in ICB-treated patients with NSCLC, but of those, only baseline LDH remained a significant predictor of OS in multivariable Cox analysis (table 2). In addition, the static CRP, LDH, or NLR did not predict ORR at the first staging (figure 1E–G). Thus, our data obtained from two independent cohorts demonstrate that early CRP kinetics predicts immunotherapy response and is associated with improved survival in NSCLC.

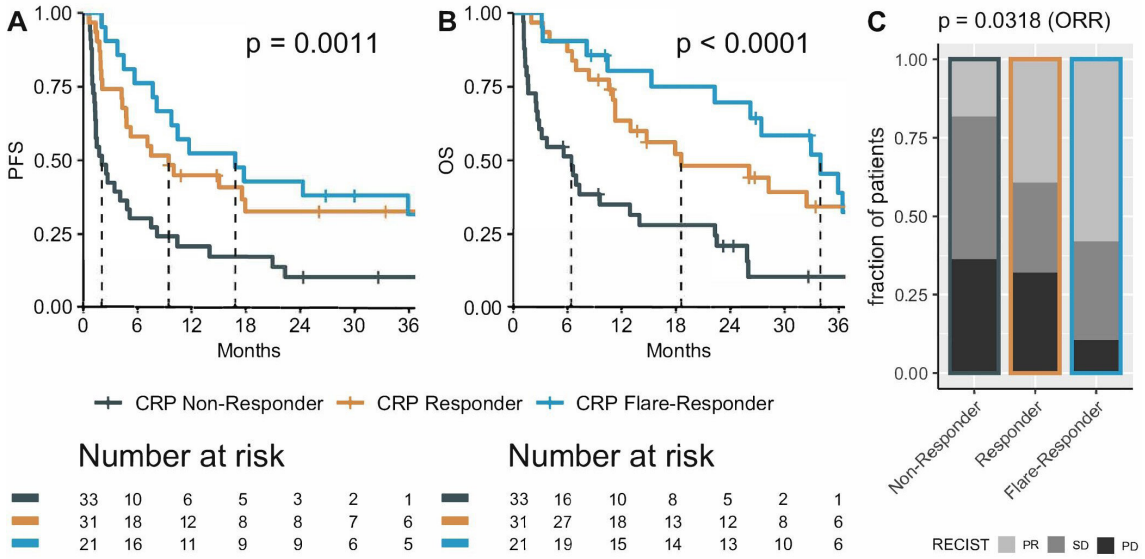


**Figure 2** Early on-treatment CRP kinetics predicts treatment response and outcome of anti-PD-1 monotherapy. Kaplan–Meier survival curves showing the progression-free survival (PFS) (A) and overall survival (OS) (B) after immune checkpoint blockade initiation stratified according to CRP kinetics groups for the anti-PD-1 monotherapy subgroup of the immune monitoring of immune therapy non-small cell lung cancer validation cohort. (C) Distribution of response at first staging according to RECIST among the different CRP kinetics groups. p value based on $\chi^2$ test for ORR. CRP, C reactive protein; ORR, objective response rate; PD, progressive disease; PR, partial remission; SD, stable disease.

### Association of early CRP kinetics with NSCLC histology

In both the discovery and the validation cohorts, early CRP kinetics were significantly associated with prolonged PFS and OS for the two major histological subtypes adenocarcinoma and squamous cell carcinoma (online supplemental figure 2).

### Effect of concurrent chemotherapy and steroid medication on early CRP kinetics

Both the discovery and the validation cohorts included patients receiving anti-PD-1 monotherapy and chemo-immunotherapy in first or later lines (table 1 and online supplemental table 1). In our validation cohort, the predictive value of CRP kinetics was found to be independent of the line of therapy (online supplemental table 3). However, the impact of CRP flare-response was more pronounced in the first-line setting, with a ~90% risk reduction for progression and death compared with the CRP non-responders (online supplemental table 3). Most patients receiving chemoimmunotherapy were treated with a platinum-containing doublet chemotherapy and only a minority of N=8 patients in our discovery cohort received atezolizumab in combination with bevacizumab, carboplatin, and paclitaxel. Of note, in both cohorts early on-treatment CRP kinetics predicted ICB response, which was associated with PFS and OS solely in the patient subgroup without concurrent chemotherapy (figure 2, online supplemental tables 3 and 4). Furthermore, the predictive value of CRP kinetics was not evident in the patient subgroup with relevant concomitant steroid medication defined as prednisolone 10 mg or equivalent dose (online supplemental tables 3 and 4).

### Early CRP kinetics predicts response to ICB at 4 weeks on-treatment

In the study protocol of our prospective validation cohort, CRP levels were measured at baseline and weeks 1, 2, 4, 6, 10 after the initiation of ICB, which allowed a more detailed analysis of early on-treatment CRP kinetics (figure 3). While the initial definition of CRP kinetics allows identification of responders or non-responders until the first radiological assessment after 12 weeks, individual CRP dynamics suggest that refined criteria may allow differentiation between responders and non-responders at an earlier stage. Indeed, two-third of the CRP flare-responders and >90% of the CRP responders could be correctly classified 4 weeks after the start of ICB therapy (figure 3B,C). After 6 weeks, >90% of CRP flare-responders had dropped below baseline CRP levels and thus met the criteria for a CRP flare-response (figure 3C). Of note, the predictive value of early CRP kinetics remained stable in both the overall cohort and the anti-PD-1 monotherapy subgroup when the definition was changed to an observation interval of 4 weeks (figure 4). Considering only the subgroup that received first-line anti-PD-1 monotherapy, early on-treatment CRP kinetics stratified until week 4 showed a strong association with outcome despite the relatively small subgroup (online supplemental figure 3). Since the majority of CRP (flare)-responders were already identified as early as 4 weeks on-treatment, while routine staging is usually performed after 12 weeks, a large therapeutic window (on anti-PD-1 monotherapy) opens for early response evaluation and the chance for therapy adjustments.

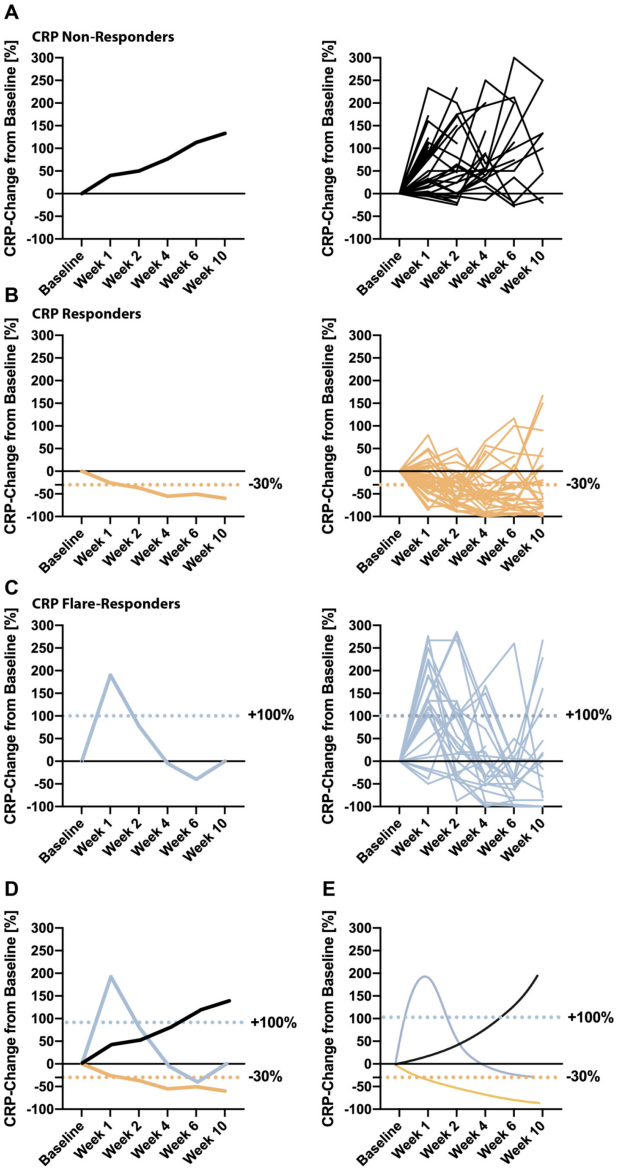


**Figure 3** Longitudinal CRP changes from baseline after initiation of immunotherapy in the three early on-treatment CRP kinetics subgroups. (A) For CRP non-responders, (B) for CRP responders, and (C) for CRP flare-responders: median CRP change from baseline in per cent is shown in the left panel; the CRP changes of the individual patients are shown in the right panel. The dashed lines indicate the thresholds for CRP responder and CRP flare-responder subgroups. (D) Integration of median change in baseline CRP of the three CRP kinetics subgroups in the immune monitoring of immune therapy non-small cell lung cancer validation cohort and (E) conceptual representation of early on-treatment CRP kinetics. CRP, C reactive protein.

## DISCUSSION

In our study, in both a retrospective discovery and a prospective validation cohort, we were able to demonstrate that early CRP kinetics can robustly predict immunotherapy response in NSCLC regardless of histological subtype. Early on-treatment CRP kinetics is an easily determined cost-effective non-invasive biomarker for predicting response to immunotherapy as early as 4 weeks after therapy initiation and has the potential to optimize therapy monitoring. We therefore propose that evaluation of early CRP kinetics should be used as a standard-of-care tool for patients with NSCLC (both adenocarcinoma and squamous cell carcinoma) undergoing immunotherapy.

The original definition of CRP flare-response is based on a retrospective cohort of 42 patients with metastatic RCC and assumed a treatment interval of 3 months for 'early' CRP kinetics assessment.[6] Using a retrospective discovery and prospective validation cohort including a total of 213 patients, we were able to demonstrate great predictive potential of CRP flare-response kinetics in ICB-treated patients with NSCLC. Furthermore, we were able to refine the definition of on-treatment CRP kinetics to an observation interval of 4 weeks, thus significantly increasing its clinical value for early therapy adjustments, for example, in the context of biomarker-stratified intervention studies. Our data suggest that early CRP kinetics is a biological cancer-independent phenomenon and thus, early CRP kinetics may represent an interesting tool for improved therapy monitoring in the era of immuno-oncology and warrants further investigation in other cancer types.

Currently, ICB is used as monotherapy or in combination with chemotherapy for metastatic NSCLC. In both cohorts, we found that early CRP kinetics predicted treatment response and outcome only in the setting of anti-PD-1 monotherapy without concurrent chemotherapy or steroid medication. This could be due to the immunomodulatory effect of concomitant chemotherapy or steroids. However, further studies are needed to clarify the role of early CRP kinetics in patients receiving chemo-immunotherapy or concomitant steroids. Recently, for the first-line treatment of metastatic RCC, we have shown that early CRP kinetics predicts response and is associated with improved PFS also for the immunotherapy combinations, anti-PD-1 either plus anti-CTLA-4 or tyrosine kinase inhibitor, suggesting that early CRP kinetics seems to be relevant not only for anti-PD-1 monotherapy.[7]

Further, within the framework of the prospective validation cohort, we were able to closely examine early CRP kinetics through the standardized study visits. Our data demonstrate that CRP kinetics can stratify patients as early as 4 weeks after the onset of ICB, a significant period before the first radiological assessment which is usually performed at week 8–12. The majority of CRP (flare)-responders can be correctly stratified at 4 weeks after immunotherapy start. This easy-to-implement on-treatment biomarker therefore opens a therapeutic window for earlier therapy adjustments. Of note, in patients who received first-line anti-PD-1 monotherapy, CRP kinetics showed a strong association with PFS and OS, even in a small subgroup of N=21 patients (online supplemental figure 3). Early CRP kinetics could thus identify the vulnerable CRP non-responder subgroup on (first-line)

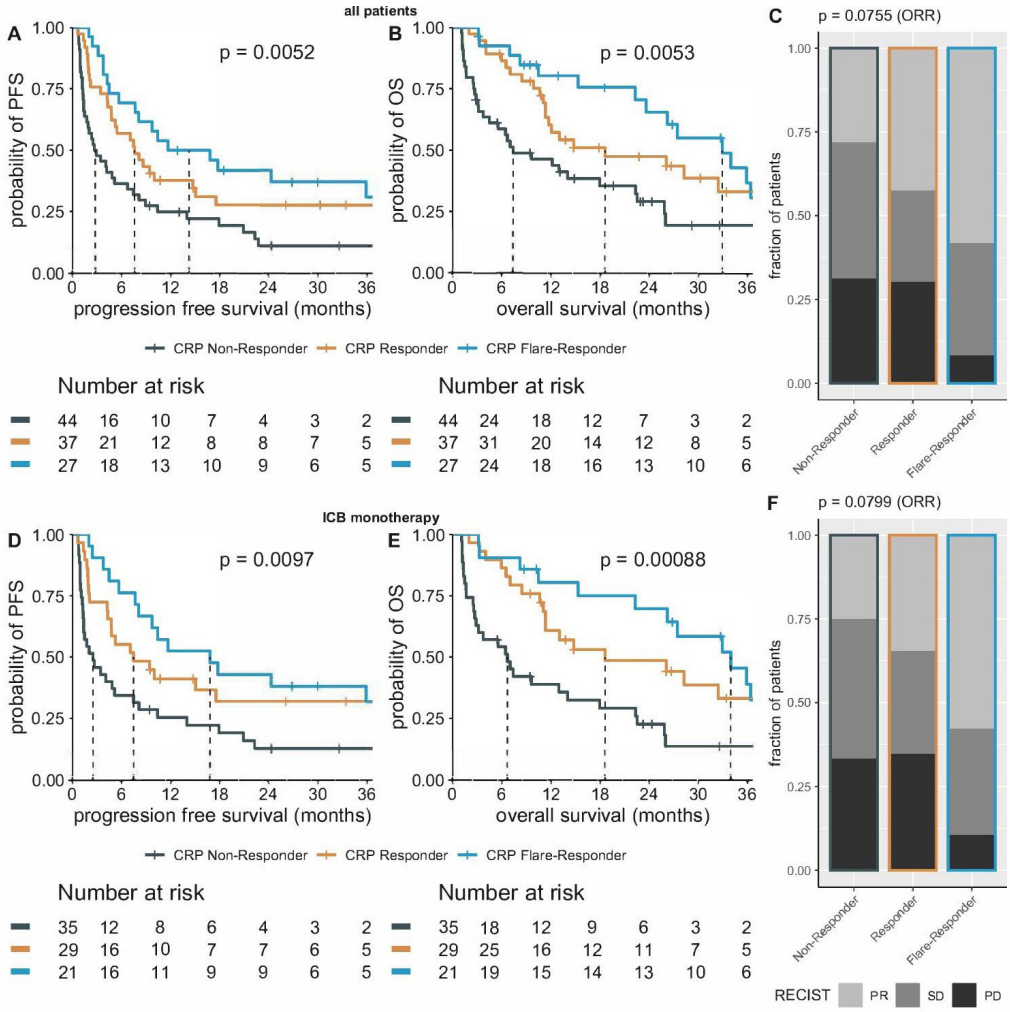


**Figure 4** Refined early on-treatment CRP kinetics criteria lead to robust prediction of immunotherapy response in NSCLC as early as week 4. Application of early CRP kinetics definition until week 4 after immunotherapy start for all patients included in the immune monitoring of immune therapy NSCLC validation cohort (A, B) and the ICB monotherapy subgroup (D, E). Progression-free survival (PFS) after ICB initiation stratified according to CRP kinetics groups is depicted in A+D, overall survival (OS) in B+E. CRP kinetics stratification predicts response to ICB, but this does not reach statistical significance based on $\chi^2$ test for ORR (C, F). P value based on $\chi^2$ test for ORR. CRP, C reactive protein; ICB, immune checkpoint blockade; ORR, objective response rate; PD, progressive disease; PR, partial remission; SD, stable disease.

anti-PD1 monotherapy, who could benefit from early therapy switch or escalation, for example, in the context of biomarker-stratified studies, well before the first routine staging. We therefore suggest future CRP kinetics-stratified study protocols, which could lead to therapy escalation (eg, radiation, chemotherapy, additional ICB such as LAG3 or TIGIT, immune-activating drugs) in the CRP non-responder arm as early as 4 weeks after anti-PD-1/PD-L1 monotherapy. In addition to therapy escalation for CRP non-responders, therapy de-escalation, for example, early cessation of concomitant chemotherapy (for PD-L1 low/negative NSCLC) or drug holiday for CRP flare-responders, also seems a rational concept for this well-performing patient subgroup.

Anti-tumor immune infiltration after the start of immunotherapy can lead to pseudoprogression via inflammatory tissue edema with subsequent enlargement of the tumor lesions.[10 11] CRP kinetics could potentially help to distinguish more accurately between true disease progression and pseudoprogression. However, a prospective radiological assessment using iRECIST criteria with simultaneous assessment of early CRP kinetics is required to address this hypothesis in the future.[11]

PD-L1 expression is the only widely used predictive biomarker in patients with NSCLC to date, although it has numerous limitations.[3] We did not observe significant differences in PD-L1 TPS within our CRP subgroups. Importantly, early CRP kinetics can identify patients

who will respond to ICB independent of the static tissue biomarker PD-L1. In our examined NSCLC cohorts, PD-L1 TPS had no prognostic value in univariable Cox regression models, and therefore, early CRP kinetics may even outperform static pretreatment PD-L1 expression in the future. If early on-treatment CRP kinetics would prospectively prove to be a more robust tool for evaluating immunotherapy response compared with PD-L1 expression in NSCLC or other tumor types, this would further emphasize its potential for future biomarker-stratified intervention studies.

Elevated CRP or LDH level at baseline has been correlated with inferior survival in NSCLC and other tumors, partially due to its role as a measure of tumor burden.[9 12] We found that baseline CRP, LDH, and NLR had prognostic value in univariable Cox regression analysis, but none of them predicted objective immunotherapy response measured by RECIST (figure 1E–G, table 2). In multivariable Cox regression analysis, apart from early CRP kinetics, only baseline LDH remained a significant predictor for OS (table 2). Low NLR and early on-treatment reduction of NLR have recently been associated with prolonged survival in NSCLC and metastatic RCC under immunotherapy.[13 14] Our data confirm this observation, but an early decline in NLR on ICB within the first month was not predictive of response to ICB in our prospective validation cohort (data not shown). Given the urgent need for a predictive biomarker for the response to immunotherapy, we propose to consider implementing early CRP kinetics into clinical practice, as it yields information about immunotherapy response instead of only being a prognostic parameter.

The immunological basis of our observed CRP kinetics is not clear. Immunogenicity of the tumor as well as immunological factors such as pre-existing shared tumor antigens of the individual patient might contribute to the observed CRP kinetics. Elucidating the molecular basis of this phenomenon will help to further improve our understanding of ICB response heterogeneity and develop rational strategies to overcome some shortcomings in the immuno-oncology era.

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**Funding** This study was supported by a Ferdinand Eisenberger grant of the Deutsche Gesellschaft für Urologie (German Society of Urology), grant ID KIN1/FE-19 (NK), and junior research group funding by the BONFOR Program of the Medical Faculty of the University of Bonn, grant ID 2020-2A-12 (NK). This study was funded in part by grant PP00P3_157448 from the Swiss National Science Foundation and by grant KLS-4409-02-2018 from the Swiss Cancer League (to LF). The study coordination of the IMIT NSCLC cohort was supported by a grant from the Forschungsförderung of the Kantonsspital St Gallen. MH and TB are funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany's Excellence Strategy – EXC2151–390873048.

**Competing interests** None declared.

**Patient consent for publication** Not applicable.

**Ethics approval** This study involves human participants and the IMIT study protocol was approved by the Ethikkommission Ostschweiz. Reference #232/21. Participants gave informed consent to participate in the study before taking part.

**Provenance and peer review** Not commissioned; externally peer reviewed.

**Data availability statement** Data are available upon reasonable request.

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# 4. Diskussion

Da in der Erstlinie für das mRCC aktuell unterschiedliche, aber als gleichwertig eingestufte anti-PD-1 basierte Kombinationstherapien (ICI+ICI versus ICI+TKI) eingesetzt werden (Bedke et al., 2021; Xu et al., 2020), ergibt sich aufgrund fehlender vergleichender prospektiver Studien für den klinischen Kontext ein Dilemma. Trotz intensiver Bemühungen in der translationalen Forschung gibt es aktuell keinen robusten prädiktiven Biomarker für eine rationale Erstlinientherapieentscheidung für Patienten mit mRCC.

Der einzige im klinischen Alltag eingesetzte prädiktive Biomarker im Zeitalter der Immunonkologie ist der im therapienaiven Tumorgewebe immunhistochemisch bestimmte PD-L1-Rezeptorstatus, der jedoch für das mRCC aufgrund diverser Limitationen wie einer uneindeutigen Studienlage hinsichtlich der Endpunkte PFS und OS (Carretero-González et al., 2020) eine untergeordnete Rolle spielt.

Ein vielversprechender Ansatz zur Identifizierung robuster prädiktiver Biomarker für die Immunonkologie ist die Verwendung von Methylierungssignaturen, welche im Vergleich zum immunhistochemisch gemessenen PD-L1 Status einige Vorteile aufweisen: Die Methylgruppen sind kovalent an die DNA gebunden und stellen somit eine stabile epigenetische Modifikation dar, die weniger dynamische Schwankungen wie die mRNA- oder Proteinexpression aufweist (Luo et al., 2018). Es ist aus diversen, teils eigenen Arbeiten bekannt, dass die Expression von Immuncheckpoint-Genen wie PD-L1 in verschiedenen Tumorentitäten epigenetisch durch DNA-Methylierung reguliert wird und prädiktives Potenzial zur Vorhersage des Immuntherapieansprechen aufweisen kann (Franzen et al., 2018; Gevensleben et al., 2016, 2016; Goltz et al., 2017a, 2017b; Klümper et al., 2020, 2021a; Micevic et al., 2019; Newell et al., 2021; Ralser et al., 2021).

Die Identifizierung eines robusten Biomarkers zur Vorhersage des Ansprechens auf dem anti-CTLA4 Antikörper Ipilimumab könnte die unselektive Erstlinientherapieentscheidung für Patienten mit mRCC (ICI+ICI versus ICI+TKI) vereinfachen. Ein vielversprechener Kandidat für die Vorhersage des Ansprechens auf eine anti-PD-1 und anti-CTLA4-gerichtete Kombinationstherapie sowie anti-CTLA4-Monotherapie für Patienten mit Melanom ist die Promotorhypomethylierung von *CTLA4* (mCTLA4) (Fietz et al., 2020; Goltz et al., 2018). Dieser Biomarkertest wurde mittels mCTLA4-qMSP auf DNA

aus archivierten FFPE-Tumorgewebe durchgeführt. Der Einsatz von FFPE-Material für molekularpathologische Analysen wird in der Klinik standardmäßig eingesetzt und somit scheint dieser mCTLA4-qMSP ein vielversprechender prädiktiver Biomarkertest zu sein, der rasch in den klinischen Alltag implementiert werden könnte. Ein wichtiges Ziel dieser kumulativen Habilitationsschrift ist es daher, die epigenetische Regulierung von *CTLA4* sowie den prädiktiven Wert von mCTLA4 für Patienten mit mRCC unter Immuntherapie zu untersuchen. Es konnte im Rahmen unser Studie gezeigt werden, dass mCTLA4 den prädiktiven Wert der PD-L1 Expression übertrifft, der in der multizentrischen RCC-ICI Kohorte keinen prädiktiven Wert aufwies (Klümper et al., 2021a). In der multizentrischen RCC-ICI-Kohorte wurden Patienten, die entweder mit einer anti-PD-1-Kombinationstherapie in der Erstlinie oder mit einer ICI-Monotherapie nach einer vorherigen TKI-Behandlung behandelt wurden, eingeschlossen. Diese retrospektive Kohorte inkludiert daher insgesamt ein heterogenes Patientengut, aber zeigt nichtsdestotrotz, dass die *CTLA4*-Promotorhypomethylierung das Immuntherapieansprechen und ein verlängertes PFS und OS vorhersagen kann. In einem multivariablen Cox-Regressionsmodell bleibt mCTLA4 ein unabhängiger prognostischer Faktor und somit kann geschlussfolgert werden, dass mCTLA4 ein vielversprechender prädiktiver Biomarker für die Vorhersage des Ansprechens auf Immuntherapie bei Patienten mit mRCC zu sein scheint, der jedoch prospektiv validiert werden muss. Zukünftige Studien sollten insbesondere untersuchen, ob das prädiktive Potenzial von mCTLA4 zu einer verbesserten Stratifizierung für eine rationale Erstlinientherapieentscheidung (ICI+TKI oder ICI+ICI) bei Patienten mit mRCC führen kann.

Neben den klinisch bereits breit eingesetzten ICI zur Blockade von CTLA4 und der PD-1/ PD-L1 Achse rücken weitere Immuncheckpoints in den Fokus der Immunonkologie. Zuletzt erreichte erstmalig eine neue immunonkologische Kombinationstherapie, der anti-LAG3 Antikörper Relatlimab plus anti-PD-1, in der klinischen Phase II-III RELATIVITY-047-Studie bei Patienten mit einem unbehandelten, metastasierten oder inoperablen Melanom den klinischen Endpunkt eines verbesserten PFS (Tawbi et al., 2022). LAG3 scheint auch für Patienten mit mRCC ein vielversprechendes Angriffsziel für zukünftige ICI Kombinationstherapien zu sein – derzeit laufen auch klinische Studien zu anti-LAG3 Antikörpern für Patienten mit mRCC (FRACTION-RCC: NCT02996110, voraussichtliches Datum der Fertigstellung der Studie ist Januar 2023). Im Rahmen dieser kumulativen Habilitationsschrift soll daher die epigenetische Regulierung von

LAG3 im ccRCC untersucht werden, um im Falle positiver Studiendaten ebenfalls einen vielversprechenden prädiktiven Biomarker vorschlagen zu können. Unsere Ergebnisse deuten darauf hin, dass die LAG3-Expression in Tumor- und Immunzellen durch *LAG3* Promotormethylierung (mLAG3) epigenetisch reguliert wird (Klümper et al., 2020). Die Hypomethylierung des *LAG3*-Promotors im therapienaiven Gewebe identifiziert eine Patientengruppe mit starker intratumoraler Inflammation und eingeschränkter Prognose. Es ist bekannt, dass primäre ccRCC mit hoher Lymphozyteninfiltration im Gegensatz zu einem kolorektalen Karzinom, Melanom und Mamma-Karzinom eine schlechtere Prognose aufweisen (Fridman et al., 2017). Passend dazu zeigte der *LAG3*-Methylierungsstatus im Melanom eine inverse Korrelation zum Überleben im Vergleich zum ccRCC (Fröhlich et al., 2020). Es kann aus den erhobenen Daten geschlussfolgert werden, dass diese die Initiierung weiterer Untersuchungen zu mLAG3 als prädiktiven Biomarker für das Ansprechen auf anti-LAG3-ICI stützen.

Bislang stand die Identifizierung eines robusten statischen Biomarkers vor der Therapieeinleitung im Mittelpunkt der Wissenschaft für das mRCC. Die vorgeschlagenen statischen Biomarker basierten häufig auf teuren Gewebeanalysen wie RNAseq-basierten Transkriptionsprofilen oder TMB (McDermott et al., 2018; Motzer et al., 2020a, 2020b; Samstein et al., 2019). Eine rationale Erklärung dafür, dass bisher kein robuster prädiktiver statischer Biomarker für Patienten mit mRCC identifiziert wurde, könnte die ausgeprägte interläsionale und intratumorale Heterogenität und der daraus resultierende *Sampling Bias* sein (Callea et al., 2015; Gerlinger et al., 2012, 2014; Raimondi et al., 2020). Darüber hinaus scheinen statische Biomarker aufgrund der komplexen Wechselwirkung zwischen Tumor und Immunsystem nicht ausreichend, um das Ansprechen genau vorhersagen zu können (Bai et al., 2020; Havel et al., 2019; Lesterhuis et al., 2017). Die Messung dynamischer longitudinaler Veränderungen nach Therapieeinleitung scheint somit insbesondere für die Immunonkologie ein attraktiver Ansatz zu sein (Lesterhuis et al., 2017). Ein großer klinischer Vorteil vieler *on-treatment* Biomarker ist zudem, dass sie häufig blutbasierte Analysen darstellen und somit ohne zusätzliche Gewebeentnahme mit potenziellen Risiken für den Patienten erhoben werden können.

Für die unselektive Erstlinientherapieentscheidung für das mRCC muss jedoch konstatiert werden, dass die Bestimmung von *on-treatment* Biomarkern den Therapiestart voraussetzt. Dementsprechend steht keine Rationale vor Einleitung einer definitiven

Therapie zur Verfügung, sodass die Anwendung prädiktiver *on-treatment* Biomarker vielmehr ein optimiertes Therapiemonitoring mit der Möglichkeit auf eine rasche Therapieadjustierung ermöglichen könnte.

Der frühe Anstieg inflammatorischer Zytokine wie CRP oder IL-6, welcher auf eine Immunaktivierung hindeutet, sagte nach Immuntherapieeinleitung das Therapieansprechen bei Patienten NSCLC voraus (Ozawa et al., 2019). Aufgrund seiner breiten Verfügbarkeit und der relativ geringen Kosten scheint insbesondere die Messung der longitudinalen CRP-Kinetik als Surrogat für das Inflammationsniveau ein leicht in den klinischen Alltag zu implementierender Biomarker für die Vorhersage des Ansprechens auf eine Immuntherapie zu sein (Fukuda et al., 2021; Ishihara et al., 2019; Kijima et al., 2021; Saito and Kihara, 2011; Saito et al., 2009; Tachibana et al., 2021; Tomisaki et al., 2021).

Zuletzt wurde erstmalig nach Immuntherapieeinleitung eine charakteristische longitudinale CRP-Kinetik mit prädiktivem Wert für Patienten mit mRCC beschrieben (Fukuda et al., 2021). Das Auftreten der sogenannten CRP-Flare-Response, welches durch einen raschen CRP-Anstieg innerhalb des ersten Monats nach ICI-Therapiestart mit anschließendem Abfall unter das Baseline-Niveau gekennzeichnet ist, sagte in einer relativ kleinen retrospektiven Kohorte von N=42 Patienten mit mRCC, die eine anti-PD-1-Monotherapie nach antiangiogenetischer TKI-Therapie in der Zweitlinie oder später erhielten, das Therapieansprechen und ein verbessertes Langzeitüberleben voraus (Fukuda et al., 2021). Da jedoch in der Erstlinie für das mRCC vorrangig ICI-Kombinationstherapien eingesetzt werden (Bedke et al., 2021), ist ein weiteres Ziel dieser kumulativen Habilitationsarbeit den prädiktiven Wert dieser frühen *on-treatment* CRP Kinetik für die ICI+ICI oder ICI+TKI (N=95) Erstlinienkombinationstherapie für Patienten mit mRCC zu untersuchen (Klümper et al., 2021b). Unsere Daten validieren das prädiktive Potenzial der frühen CRP-Flare-Response Kinetik für das Erstliniensetting des mRCC. Patienten mit charakteristischer CRP-Flare-Response zeigen eine multivariabel adjustierte Risikoreduktion von ~80% für Tumorprogression im Vergleich zur CRP Non-Responder Gruppe (medianes PFS: CRP-Flare-Responder: 19.2 versus CRP Non-Responder: 5.6 Monate, log-rank $p<0.001$). Interessanterweise blieb die frühe longitudinale CRP-Kinetik sowohl in der Subgruppe der mit ICI+ICI als auch mit ICI+TKI behandelten Patienten, trotz der relativ kleinen Fallzahlen (ICI+ICI N=59, ICI+TKI N=36), ein signifikanter Prädiktor für ein besseres PFS. Neben den Stärken

dieser Studie, wie dem multizentrischen Ansatz und der vergleichsweise großen Studienkohorte), weist unsere Studie auch einige Limitationen auf. In erster Linie sollten die Ergebnisse unter Berücksichtigung der Einschränkungen des retrospektiven Designs interpretiert werden. Darüber hinaus wurde das CRP in verschiedenen klinischen Routinelabors und ohne ein standardisiertes Schema an den Studienstandorten gemessen, sodass einige CRP-Flare-Responses möglicherweise nicht erfasst wurden. Zusätzlich könnte eine Modifizierung des neuen und noch nicht prospektiv validierten Konzepts der frühen CRP-Kinetik von Fukuda et al. (Fukuda et al., 2021) den prädiktiven Wert weiter erhöhen. Es kann dennoch geschlussfolgert werden, dass die frühe CRP-Kinetik nach prospektiver Validierung ein leicht zu implementierender, nicht-invasiver *on-treatment* Biomarker wäre, der einen frühen Behandlungs(miss)erfolg vorhersagen könnte um dadurch eine verbesserte Therapieüberwachung, -steuerung und -anpassung für Patienten mit mRCC zu ermöglichen.

Spannend ist, dass die Gruppe um Tomisaki et al. in einer kleinen Kohorte von N=31 Patienten mit metastasiertem UC (mUC) den prädiktiven Wert der frühen CRP-Kinetik auch für eine weitere Tumorentität bestätigen konnte (Tomisaki et al., 2021). Der prädiktive Wert der frühen *on-treatment* CRP-Kinetik wurde in einer eigenen multizentrischen, retrospektiven Beobachtungsstudie bestätigt. Diese Studie schloss N=154 Patienten mit mUC ein, die ICI in der Erstlinie (N=33, Cisplatin-unfit) oder nach einer platinhaltigen Chemotherapie (N=121) erhielten (Klümper et al., 2022*, European Journal of Cancer, accepted (25.02.2022), in press*).

Um die Relevanz der frühen CRP-Kinetik für Patienten mit mRCC und mUC zu bestätigen, ist eine prospektive, multizentrische Beobachtungsstudie im Rahmen des Centrums für Integrierte Onkologie Aachen-Bonn-Köln-Düsseldorf (CIO ABCD) mit dem Titel „Prospective Evaluation of the CRP-FLAre phenomenon on Immunotherapy REsponse in Urooncology (FLAIRE)“ geplant. Über das Förderinstrument für klinische Studien des Studienzentrums Bonn (FKS) konnte erfolgreich eine Studienfinanzierung eingeworben werden (Principal Investigator und Antragsteller Dr. Niklas Klümper).

Die frühe *on-treatment* CRP-Kinetik könnte möglicherweise auch bei weiteren Tumorentitäten ein prädiktives Potenzial aufweisen. Im Rahmen dieser kumulativen Habilitationsarbeit ist daher auch der prädiktive Wert der CRP-Flare Kinetik für das ICI-behandelte NSCLC untersucht worden, da dort ebenfalls die Immuntherapie als SOC

eingesetzt wird. In dieser Studie wurde für Patienten mit NSCLC die gleiche charakteristische longitudinale CRP-Flare Kinetik beobachtet, die auch im ICI-behandelten mRCC und mUC auftritt (Fukuda et al., 2021; Klümper et al., 2021b). So konnte in zwei unabhängigen Kohorten – einer retrospektiven Entdeckungs- und einer prospektiven Validierungskohorte von je N>100 Patienten mit NSCLC – erstmalig auch prospektiv bewiesen werden, dass die CRP-Flare-Response das Ansprechen auf anti-PD-1-Monotherapie im NSCLC vorhersagt (Klümper et al., 2022). Aufgrund seiner breiten klinischen Verfügbarkeit könnte die frühe CRP-Kinetik somit ein einfach zu bestimmender, kosteneffektiver und nicht-invasiver Biomarker für die Vorhersage des Ansprechens auf ICI bei Patienten mit NSCLC werden.

Im Studienprotokoll unserer prospektiven Validierungskohorte (IMIT NSCLC (Berner et al., 2019)) wurde die CRP-Konzentration nach Therapiestart sehr regelmäßig in den Wochen 1, 2, 4, 6 und 10 gemessen, was eine detailliertere und strukturiertere Analyse der frühen *on-treatment* CRP-Kinetik ermöglichte. Während die ursprüngliche Definition der CRP-Kinetik die Identifizierung von Therapieansprechen und -versagen bis zur ersten radiologischen Beurteilung nach 8-12 Wochen ermöglicht, deutet die individuelle CRP-Dynamik darauf hin, dass verfeinerte Kriterien eine Prädiktion des Therapieansprechens zu einem früheren Zeitpunkt ermöglichen könnte. In der Tat konnten zwei Drittel der CRP-Flare-Responder und >90% der CRP-Responder bereits vier Wochen nach Beginn der ICI-Therapie korrekt klassifiziert werden. In einem explorativen Ansatz konnte die ursprüngliche CRP-Kinetik-Definition von Fukuda et al. (Fukuda et al., 2021) durch Änderung des Beobachtungsintervalls geschärft werden, sodass unsere neue Definition der frühen longitudinalen CRP-Kinetik bereits vier Wochen nach Therapiebeginn einen robusten prädiktiven Wert aufwies. Da das erste Routine-Staging in der Regel 8-12 Wochen nach Therapiestart durchgeführt wird, öffnet sich durch die Anwendung unserer modifizierten CRP-Kinetik Definition ein großes therapeutisches Fenster für rasche Therapieadjustierung. Auf Grundlage der neuen CRP-Kinetik Definition scheinen daher zukünftige CRP-Kinetik-stratifizierte Studienprotokolle denkbar (Dancey et al., 2010; Hu and Dignam, 2019), die im CRP-Non-Responder-Arm bereits vier Wochen nach Start der anti-PD-1/PD-L1-Monotherapie zu einer Therapieeskalation (z.B. zusätzliche Bestrahlung, Chemotherapie, Blockade neuer Immuncheckpoints wie LAG3, TIGIT [...], immunaktivierende Medikamente) führen könnten. Bei den CRP-Flare-Respondern, also der klinisch sehr gut laufenden Subgruppe,

scheint auch eine Therapiedeeskalation, z.B. frühzeitiges Absetzen begleitender Therapie (z.B. TKI, anti-CTLA4, Chemotherapie) oder *Drug Holiday* ein potenziell vielversprechendes Konzept zur Einsparung von Therapie-vermittelter Toxizität (Puzanov et al., 2017). Die Einbeziehung von Biomarkern in klinische Interventionsstudien ist jedoch komplex (Hu and Dignam, 2019). Wenn Biomarker zur Stratifizierung einer therapeutischen Intervention verwendet werden, werden sie als integrale Biomarker bezeichnet und die Tests müssen in einem CLIA (*Clinical Laboratory Improvement Amendment*)-zertifizierten Labor durchgeführt werden (Dancey et al., 2010; Hu and Dignam, 2019). Da es sich bei der CRP-Messung um ein klinisch etabliertes Instrument handelt, scheint der Transfer der Methodik in die klinische Praxis nicht limitiert. Prospektive Studien müssen jedoch prüfen, ob durch die Integration zusätzlicher Marker (z.B. statische Gewebemarker, zusätzliche dynamische Biomarker) der Wert und die Robustheit der bisherigen *on-treatment* CRP-Kinetik Definition noch gesteigert werden kann. Insbesondere muss berücksichtigt werden, dass CRP ein relativ unspezifischer Marker ist, der lediglich das systemische Inflammationsniveau misst. Akute Infektionen oder Nebenwirkungen, die ebenfalls häufig eine systemische Inflammation induzieren, können daher als CRP-Flare-Response fehlinterpretiert werden. Wenn keine klinischen Symptome für akute Infektionen oder Nebenwirkungen vorliegen, deuten unsere Daten zur frühen CRP-Kinetik jedoch darauf hin, dass ein rascher CRP-Anstieg nach Immuntherapieeinleitung nicht als Zeichen einer systemischen Infektion fehlinterpretiert werden sollte. Es ist daher von großer Bedeutung, Urologen und Onkologen für diese charakteristische frühe CRP-Kinetik nach Start der Immuntherapie zu sensibilisieren. Es sind weitere Studien erforderlich, um festzustellen, ob eine Erweiterung der reinen CRP-Kinetik Definition durch zusätzliche Marker, wie beispielsweise den relativ spezifischen bakteriellen Sepsismarker Procalcitonin, die Unterscheidung zwischen antitumoraler Immunaktivierung gegenüber einer akuten systemischen (bakteriellen) Infektion erleichtern könnte. Die korrekte Unterscheidung zwischen ICI-induzierter antitumoraler Immunantwort, Infektion und autoimmunologischen Nebenwirkung, die allesamt zu CRP-Erhöhungen führen können, ist zudem von besonderer klinischer Relevanz, um den unangemessenen Einsatz von Antibiotika zu vermeiden. Insbesondere muss berücksichtig werden, dass der Einsatz von Antibiotika mit einem verminderten Ansprechen auf Immuntherapie in Verbindung gebracht wurde, was höchstwahrscheinlich durch Veränderung des Darmmikrobioms zu erklären ist (Elkrief et al., 2019; Hopkins et al., 2020).

Eine weitere wichtige Variable, die umfassend charakterisiert werden muss, bevor die frühe CRP-Kinetik als integraler Biomarker für die Stratifizierung von Therapiemaßnahmen verwendet werden kann, ist die Berücksichtigung von (immunmodulierender) Begleitmedikation. In unserer Studie zur Untersuchung der Relevanz der frühen CRP-Kinetik bei Patienten mit NSCLC, die mit (Chemo-)Immuntherapie behandelt wurden, wurde die gleichzeitige Einnahme relevanter Mengen von Kortikosteroiden (definiert als äquivalente Dosis zu 10mg Prednisolon) sowie der Wert der frühen CRP-Kinetik auf die Subgruppe der Patienten, die eine reine ICI-Therapie im Vergleich zur Chemoimmuntherapie erhielten, untersucht. In beiden Kohorten wurde festgestellt, dass die frühe CRP-Kinetik nur das Ansprechen auf eine reine anti-PD-1-Monotherapie ohne gleichzeitige Chemotherapie oder relevante Steroidmedikation vorhersagte. Diese Beobachtung könnte auf die immunmodulatorische Wirkung der begleitenden Chemotherapie oder Steroide zurückzuführen sein. Es sind jedoch weitere Studien erforderlich, um die Rolle der frühen CRP-Kinetik bei Patienten zu klären, die eine Chemoimmuntherapie oder begleitende Steroide erhalten. Zum jetzigen Zeitpunkt scheint die frühe CRP-Kinetik jedoch insbesondere für Patienten unter reiner anti-PD-1 Monotherapie (NSCLC, mRCC, mUC) oder unter Kombinationstherapien ohne relevante Immunsuppression, z.B. die Erstlinientherapie ICI+ICI oder ICI+TKI für Patienten mit mRCC (Klümper et al., 2021b), zur Vorhersage des Therapieansprechens besonders vielversprechend zu sein.

Die molekularen und immunologischen Grundlagen des CRP-Flare-Phänomens sind bisher nicht untersucht worden. Die weitere Erforschung der molekularen Grundlagen dieses Phänomens könnte dazu beitragen, unser Verständnis im Zeitalter der Immunonkologie zu verbessern und rationale Strategien zu entwickeln, um in Zukunft bei einem größeren Teil unserer Patienten eine robuste Immunantwort gegen den Tumor zu induzieren.

Da die frühe *on-treatment* CRP-Kinetik interessanterweise ein biologisches, vom Tumortyp unabhängiges Phänomen zu sein scheint (Fukuda et al., 2021; Klümper et al., 2021b; Tomisaki et al., 2021, Klümper et al., 2022), könnten die im Rahmen dieser kumulativen Habilitationsarbeit erhobenen Daten einen wichtigen Ausgangspunkt für die Untersuchung weiterer prädiktiver *on-treatment* Biomarker-Modelle auf der Grundlage von longitudinalen inflammatorischen Markern für die Immunonkologie darstellen. Dies könnte zukünftig zu einer Verbesserung der Therapieüberwachung für Patienten

unter Immuntherapie führen. Eine rasche Identifizierung von Therapieerfolg- und misserfolg könnte so eine unmittelbare Therapieadjustierung ermöglichen, um zum einen die Therapie-vermittelte Toxizität einzusparen und um zum anderen frühzeitig effektivere Therapien verabreichen zu können.

# 5. Zusammenfassung

Das übergeordnete Ziel dieser kumulativen Habilitationsschrift ist die Identifizierung von vielversprechenden prädiktiven Biomarkern, die das Ansprechen auf Immuntherapie für Patienten mit metastasiertem Nierenzellkarzinom (mRCC) vorhersagen können. Diese Zielsetzung ist von besonderer klinischer Relevanz, da aktuell unterschiedliche, aber als gleichwertig eingestufte anti-PD-1 basierte Kombinationstherapien (ICI+ICI versus ICI+TKI) in der Erstlinientherapie des mRCC eingesetzt werden. Da vergleichende, prospektive Studien noch ausstehend sind, ergibt sich für den klinischen Alltag daher zum jetzigen Zeitpunkt eine weitgehend unselektierte Erstlinientherapieentscheidung. Trotz intensiver Bemühungen in der translationalen Forschung gibt es bisher keinen robusten, prädiktiven, im klinischen Alltag eingesetzten Biomarker, der eine rationale Therapieentscheidungsfindung ermöglicht.

In zwei der in dieser Habilitationsschrift zugrunde liegenden wissenschaftlichen Arbeiten wurden Methylierungssignaturen im therapienaiven Tumorgewebe untersucht, um das Therapieansprechen auf Immuntherapie vorherzusagen. So konnte eine Rationale für die Messung des *LAG3*-Promotormethylierungsstatus als möglicher prädiktiver Biomarker für eine anti-LAG3 Immuntherapie geschaffen werden. Zudem wurde der Promotormethylierungsstatus von *CTLA4* (mCTLA4) untersucht, der das Ansprechen auf Immuntherapie bei Patienten mit mRCC zuverlässig vorhersagen konnte und hinsichtlich des prädiktiven Potenzials der PD-L1-Expression überlegen war. Inwieweit mCTLA4 zu einer verbesserten Erstlinientherapieentscheidung für Patienten mit mRCC beitragen kann, muss im Rahmen prospektiver Validierungsstudien untersucht werden.

Um die Komplexität der Tumor-Immun-Interaktion genauer vorhersagen zu können, wurden zudem dynamische *on-treatment* Biomarker untersucht. Im Rahmen dieser Habilitationsschrift ist es gelungen, den prädiktiven Wert einer charakteristischen frühen *on-treatment* CRP-Kinetik nach Einleitung der Immuntherapie, welche im Januar 2021 durch Fukuda et al. erstbeschrieben wurde, sowohl für das mRCC und metastasierte Urothelkarzinom (mUC) zu bestätigen als auch erstmalig für das NSCLC mittels retrospektiver Entdeckungs- und einer prospektiver Validierungskohorte zu beweisen.

Unserer Ergebnisse deuten darauf hin, dass die frühe *on-treatment* CRP-Kinetik ein biologisches Tumortyp-unabhängiges Phänomen zu sein scheint und somit in der Ära

der Immunonkologie auch für weitere Tumorentitäten ein prädiktives Potenzial aufweisen könnte. Ein besonders hervorzuhebender Vorteil für die Anwendung der frühen CRP-Kinetik als neuen prädiktiven *on-treatment* Biomarker ist, dass die Bestimmung der Serum CRP-Konzentration ein klinisch etablierter, kosteneffizienter und nicht-invasiver Biomarker ist, der somit rasch in den klinischen Alltag integriert werden könnte.

Ein wesentliches Ergebnis dieser kumulativen Habilitationsarbeit ist, dass durch eine Modifikation der ursprünglichen CRP-Kinetik-Definition der klinische Wert dieses *on-treatment* Biomarkers signifikant gesteigert werden konnte. Durch Anwendung unserer modifizierten CRP-Kinetik Definition kann bereits vier Wochen nach Start der Immuntherapie das Therapieansprechen robust vorausgesagt werden. Da das erste Routine-Staging in der Regel 8-12 Wochen nach Therapiestart durchgeführt wird, öffnet sich durch unsere Anpassung der CRP-Kinetik Definition ein großes therapeutisches Fenster für rasche Therapieadjustierungen. Basierend auf unserer neuen CRP-Kinetik Definition könnten zukünftig CRP-Kinetik-stratifizierte Studien initiiert werden, in denen bereits vier Wochen nach Einleitung der Immuntherapie für den CRP-Non-Responder-Arm eine Therapieeskalation folgen könnte, wohingegen für den CRP-Flare-Responder-Arm auch eine Therapiedeeskalation ein rationales Therapiekonzept darstellen könnte.

Zusammenfassend lässt sich sagen, dass die frühe *on-treatment* CRP-Kinetik ein biologisches Tumortyp-unabhängiges Phänomen zu sein scheint, welches ein großes Potenzial hat, das Therapieansprechen auf eine Immuntherapie vorherzusagen. Ob die frühe longitudinale CRP-Kinetik Definition den für diese Anwendung optimalen prädiktiven *on-treatment* Biomarker darstellt, müssen prospektive Untersuchungen zeigen. Die im Rahmen dieser Habilitationsarbeit erhobenen Daten stellen jedoch einen wichtigen Ausgangspunkt für die Untersuchung weiterer prädiktiver *on-treatment* Biomarkermodelle dar, welche zukünftig das Therapiemonitoring und damit auch die Therapieadjustierung einer Vielzahl von Tumorpatienten unter Immuntherapie verbessern könnte.

## 6. Erklärung zur kumulativen Habilitationsschrift

Die vorliegende Habilitationsschrift hat vier publizierte Originalarbeiten zur Grundlage. In dieser kumulativen Habilitationsarbeit fließen Forschungsarbeiten ein, in denen der Habilitand als geteilter Erstautor gelistet ist. Eine Überlappung mit anderen Habilitationsschriften ist nicht gegeben.

Der Verfasser möchte an dieser Stelle herausstellen, dass die Untersuchung des CRP-Flare-Response Phänomens durch seine Studienkonzeption initiiert wurde. In enger Kooperation mit weiteren Wissenschaftlerinnen und Wissenschaftlern und Zentren konnte die Relevanz dieses vielversprechenden prädiktiven *on-treatment* Biomarkers suffizient ausgearbeitet werden.

Die jeweiligen Eigenbeiträge der geteilten Erstautorenschaften sind im Folgenden für die unter 1. Grundlage der kumulativen Habilitationsschrift aufgeführten Originalarbeiten in Anlehnung zu dem *authors contributions* Teil dargestellt:

I. **N Klümper*, DJ Ralser*** et al. LAG3 (LAG-3, CD223) DNA methylation correlates with LAG3 expression by tumor and immune cells, immune cell infiltration, and overall survival in clear cell renal cell carcinoma. *J. Immunother. Cancer*, e000552 (2020).

   Dr. Klümper (NK) und Dr. Ralser (DJR) sind geteilte Erstautoren:
   NK: Planung und Konzeption, Durchführung der Experimente; Statistische Auswertung; Schreiben des Manuskriptes.
   DJR: Planung und Konzeption; Durchführung der Experimente; Schreiben des Manuskriptes.

II. **N. Klümper** et al. CTLA4 promoter hypomethylation is a negative prognostic biomarker at initial diagnosis but predicts response and favorable outcome to anti-PD-1 based immunotherapy in clear cell renal cell carcinoma. *J. Immunother. Cancer*, e002949 (2021).

   Dr. Klümper ist alleiniger Erstautor.

III. **N. Klümper*, P. Schmucker*** et al. C-reactive protein flare-response predicts long-term efficacy to first-line anti-PD-1-based combination therapy in metastatic renal cell carcinoma. *Clin. Transl. Immunol*. 10, (2021).

Dr. Klümper (NK) und Herr Schmucker (PS) sind geteilte Erstautoren:
NK: Konzeptualisierung; Formale Analyse; Fördermittelakquise; Recherche; Methodik; Projektadministration; Ressourcen; Supervision; Visualisierung; Schreiben des Manuskriptes.
PS: Formale Analyse; Untersuchung; Methodik; Projektverwaltung; Visualisierung; Schreiben des Manuskriptes.

IV. **N. Klümper*, J. Saal*** et al. C-reactive protein flare predicts response to checkpoint inhibitor treatment in non-small cell lung cancer. *J Immunother. Cancer,* e004024 (2022).

Dr. Klümper (NK) und Dr. Saal (JS) sind geteilte Erstautoren:
NK: Konzept und Gestaltung; Beschaffung, Analyse und Interpretation der Daten; Schreiben des Manuskriptes; Ressourcen
JS: Beschaffung, Analyse und Interpretation der Daten; Schreiben des Manuskriptes; Statistische Auswertung

# 7. Literaturverzeichnis


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