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Frühzeitige Beurteilung der Effizienz systemisch- zytotoxischer Therapien bei Patienten mit Pankreaskarzinom durch onkologische Serum- Biomarker

Dissertation

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Einleitende Zusammenfassung
der schriftlichen, kumulativen Promotion

gemäß § 4a der Promotionsordnung der LMU vom 1. Juni 1983 in der achten Fassung der Änderungssatzung vom 1. April 2009

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1. Einleitung

1.1. Pankreaskarzinom

Das Pankreaskarzinom ist bekannt für seine sehr schlechte Prognose: mit einer durchschnittlichen 5-Jahres-Überlebensrate von 6% liegt es auf Platz 11 der weltweit führenden Ursachen für Krebstodesfälle, innerhalb der USA sogar auf Platz vier (WHO Datenbank 2008). Zum Zeitpunkt der Diagnosestellung besteht in 80% der Fälle bereits ein fortgeschrittenes Stadium, sodass eine potenziell kurative Operation oft nicht mehr möglich ist. (1, 2)

Ursache dafür sind zum einen das Fehlen charakteristischer Frühsymptome und zum anderen Schwierigkeiten bei Diagnose und Verlaufskontrollen mittels bildgebender Verfahren. Die Sonografie ist stark Untersucher- abhängig und in Computertomographie (CT) und Magnetresonanztomographie (MRT) können Umgebungsreaktionen wie z.B. die desmoplastische Stromareaktion oder Bestrahlungsnarben eine Tumorevaluation erschweren. (3, 4) Daher finden die allgemein anerkannten Evaluationskriterien für solide Tumoren nach RECIST (5) vor allem in Studien Anwendung, während in der Patientenversorgung klinische und laborchemische Parameter noch immer eine übergeordnete Rolle für Therapieentscheidungen und Therapiemonitoring spielen.

Da nur ein geringer Anteil der Pankreaskarzinome primär resektabel ist, haben palliative Behandlungsansätze mit dem Ziel eines verlängerten Überlebens und einer verbesserten Symptomkontrolle die größte Bedeutung im klinischen Alltag.

1.2. Molekularbiologische Zelltodmarker

Aufgrund der sehr ungünstigen Prognose und der diagnostischen Schwierigkeiten ist es wichtig, auf neuen Gebieten nach Möglichkeiten der Therapieevaluation zu suchen. Im Blutkreislauf zirkulierende Tumormarker, die minimalinvasiv über eine einfache Venenpunktion gewonnen werden können, wären so eine gut anwendbare Alternative. Für viele Tumorentitäten haben sich bereits bestimmte Tumormarker etabliert, so z.B. das Prostata- spezifische Antigen (PSA) für Prostatakarzinome, das carcinoembryonale Antigen (CEA) für kolorektale Karzinome und Carbohydrate-Antigen 19-9 (CA19-9) für Pankreaskarzinome. (6-8)

Im Idealfall könnte man anhand der Biomarker- Werte direkte Rückschlüsse auf die

Tumoraktivität bzw. den Tumorzelluntergang ziehen. „High molecular group box 1“ (HMGB1) und der „Receptor for advanced glycation endproducts“ (RAGE) als „Danger associated molecular patterns“ (DAMPs), die bei Zelluntergang regelmäßig freigesetzt werden und darüber hinaus die Immunantwort auf den Tumor modulieren, haben sich dafür als vielversprechend erwiesen.

HMGB1 ist ein Mitglied der HMG- Box- Proteinfamilie und kommt praktisch in jeder menschlichen Zelle vor. Es wird im Überfluss exprimiert und wird von sterbenden Zellen oder Zellen des angeborenen Immunsystems in den Extrazellularraum freigesetzt. Dort bindet es an viele verschiedene Rezeptoren, wie RAGE oder die Toll- like Rezeptoren (TLR) 2, 4 und 9 und induziert so T-Zell- Aktivierung, Antigenpräsentation und Zytokinausschüttung. (reviewed in 9) Mit einigen Bindungspartnern wird die immunstimulierende Wirkung von HMGB1 noch verstärkt, z.B. in Verbindung mit Nukleosomen an TLR 2 oder mit Einzelstrang- DNA an TLR 9. (10, 11)

RAGE ist der bevorzugte Rezeptor von HMGB1, er befindet sich als membranständiger Rezeptor auf den Oberflächen verschiedenster Zellen und in löslicher Form im Blutkreislauf („soluble“ oder sRAGE). Diese lösliche Form könnte auf zwei verschiedene Wege entstehen: zum einen durch alternatives Splicen der prä- m-RNA, zum anderen durch proteolytische Abspaltung vom membrangebundenen Rezeptor. SRAGE kann extrazellulär an HMGB1 binden und so seine Funktionen neutralisieren, also als Abbausignal oder “decoy receptor” fungieren. (12)

Nukleosomen, die bei Zelluntergang als Abbauprodukte der Kern-DNS freigesetzt werden, wurden von uns ebenfalls als Zelltodparameter untersucht, sowie die Aktivität der DNAse im Blutkreislauf, die mit dem Abbau der zirkulierenden Nukleosomen in Zusammenhang steht. (13)

1.3. Etablierte Tumormarker

Um die Effektivität der neuen molekularen Zelltodmarker im Einsatz als Tumormarker besser einschätzen zu können, wurden parallel auch die Messwerte dreier etablierter Tumormarker bestimmt und ausgewertet.

CA19-9 ist der gebräuchlichste Tumormarker des Pankreaskarzinoms, da es sich in einer Vielzahl von Studien als wertvoller Marker für Therapieansprechen und Prognose gezeigt hat. (14-16) Es entspricht einem sialynierten Hapten des Lewis- Blutgruppen- Antigens

und ist findet sich sowohl in fetalen als auch adulten menschlichen Schleimhautzellen. (17)

Von den weiteren Tumormarkern, die in klinischen Studien erfolgsversprechende Eigenschaften zeigten, jedoch in der klinischen Routine noch nicht in diesem Maße etabliert sind, wurden CEA und das Cytokeratinfragment 21-1 (CYFRA 21-1) untersucht.

CEA ist ein monomeres Glykoprotein, das in adulten Epithelzellen vorkommt und dessen prätherapeutische Werte bei kolorektalem Karzinom und Lungenkarzinom gut mit der Prognose korrelierten. (7, 18) Der zeitliche Verlauf der CEA- Werte gibt dagegen wertvolle Hinweise auf ein Tumorrezidiv, was insbesondere bei Patienten mit fehlender Expression des Lewis- Blutgruppen- Antigens (ca 5% der Patienten) von Bedeutung ist.

Nur wenige Studien untersuchten bisher den Nutzen von CYFRA 21-1 als Tumormarker bei Pankreaskarzinom. (19) Als Fragment des epithelialen Strukturproteins Cytokeratin 19 ist es ebenfalls in zahlreichen epithelialen Zellen vorhanden. Sein prognostischer Nutzen wurde bereits für Lungen- und Brustkrebs, sowie für Patienten nach Selektiver interner Radiotherapie (SIRT) bei Lebermetastasen eines kolorektalen Karzinoms gezeigt. (20-22)

2. Zielsetzung der klinisch- experimentellen Studie

2.1. Therapiemonitoring bei Pankreaskarzinom

Die Therapie maligner Erkrankungen ist komplex und sollte im Idealfall auf die speziellen Gegebenheiten jedes einzelnen Patienten abgestimmt werden. Dazu ist es notwendig, das Ansprechen auf mögliche Therapieoptionen im Vorfeld abzuschätzen und/oder nach Beginn der Therapie deren Wirksamkeit zu überprüfen und gegebenenfalls eine Umstellung vorzunehmen. Gerade bei dem bekannten schnellen Voranschreiten der Erkrankung und der geringen Aussagekraft der Bildgebung im Frühstadium des Pankreaskarzinoms könnte so wertvolle Lebenszeit und –qualität gewonnen werden.

In der den vorliegenden Veröffentlichungen zugrunde liegenden Forschungsarbeit sollte untersucht werden, ob die o.g. Auswahl neuer biologischer Zelltodmarker für die frühzeitige Vorhersage des Therapieansprechens geeignet sind. Außerdem wurde die Anwendbarkeit der bei anderen Tumorentitäten etablierten Tumormarker für das Pankreaskarzinom geprüft und vergleichend zu den „neuen“ Biomarkern untersucht.

2.2. Prognose des Krankheitsverlaufes

Ebenso wie das Ansprechen auf eine ausgewählte Therapie ist auch von Interesse, ob bereits vor Therapiebeginn oder im Verlauf der Behandlung anhand der Tumormarker eine Aussage zur Prognose getroffen werden kann. Dazu wurden alle Marker in regelmäßig abgenommenen Blutproben bestimmt und sowohl absolut als auch in ihrem Verlauf mit dem Überleben der Patienten korreliert. Wiederum wurden sowohl „neue“ als auch „alte“ Marker auf ihre prognostische Power überprüft, was außerdem auch einen unmittelbaren Vergleich zulässt.

3. Material und Methoden

3.1. Patientenkollektiv und Therapien

In diese prospektive Beobachtungsstudie wurden 83 Patienten mit fortgeschrittenem Pankreaskarzinom aufgenommen, die sich zwischen Mai 2006 und April 2010 in der Medizinischen Klinik III des Klinikums Großhadern in Behandlung befanden. Fünf Patienten mussten wegen fehlender prätherapeutischer Messwerte ausgeschlossen werden, sodass letztlich die Werte von 78 Patienten in die Auswertung eingingen.

Bei Eintritt in die Studie fand sich bei 9 Patienten ein lokal fortgeschrittener Tumor, bei 51 Patienten bereits eine Metastasierung und bei 18 Patienten ein Tumorrezidiv. Alle Patienten erhielten eine Erstlinientherapie, entweder mit Gemcitabin allein (n= 11), Gemcitabin plus Erlotinib (n= 43), Gemcitabin plus Everolimus (n=11), Capecitabine plus Erlotinib (n= 8), Gemcitabin plus Axitinib (n= 2), Capecitabin allein (n=2) oder nab-Paclitaxel (n= 1). Staginguntersuchungen mittels CT oder MRT wurden alle 8 Wochen nach RECIST (response evaluation criteria in solid tumors, version 1.0) durchgeführt. 35 der 78 Patienten wurden innerhalb von prospektiven Studien behandelt. Vor Beginn der Studie wurde die Genehmigung der lokalen Ethikkommission der LMU eingeholt und alle Patienten gaben schriftlich ihre Einwilligung zur Teilnahme.

Außerdem wurden als Referenzwerte für die neuen Biomarker die Blutproben von 30 gesunden Freiwilligen untersucht.

3.2. Probengewinnung und Messungen

Die Blutproben wurden mittels peripherer Venenpunktion vor Beginn der systemischen Therapie und danach in wöchentlichen Abständen abgenommen, bis zum Zeitpunkt des ersten radiologischen Stagings. Innerhalb von zwei Stunden nach Abnahme wurden die Proben für 15 Minuten bei 3000xG zentrifugiert, das Serum manuell abpipettiert und entweder ohne weitere Behandlung in Microtubes aliquotiert oder mit 10mM EDTA (pH8) stabilisiert, was für die Bestimmung der Nukleosomen notwendig war. Nach Beendigung der Probensammlung wurden die Proben (nach einer maximalen Lagerzeit von 3 Jahren) aufgetaut und die Parameter gemessen. Dabei wurde darauf geachtet, dass alle Proben eines Patienten in demselben Testdurchlauf gemessen wurden, um das Risiko einer verfälschten Verlaufsdarstellung durch eventuelle Interassay- Differenzen auszuschließen.

Die „neuen“ Biomarker HMGB1, RAGE, Nukleosomen und DNAse Aktivität wurden mittels manuell durchgeführtem ELISA gemessen.

Davor erfolgte die methodische Evaluation dieser ELISA- Tests auf Intra- und Interassay-Impräzision, Linearität, Stabilität und Probenstabilität vor und nach Zentrifugation unter verschiedensten Lagerungsbedingungen. Dazu wurden die Blutproben von sechs gesunden Freiwilligen zwei verschiedenen Prozeduren unterzogen: einmal wurden die Proben nach der Abnahme in Serum- Trennröhrchen (Sarstedt GmbH) bei Kühlschranktemperatur (4°C) und Raumtemperatur (25°C) wahlweise 0, 6 oder 24 Stunden gelagert und dann mit 3000xG für 15 Minuten zentrifugiert. Nach der Zentrifugation wurden die Serumproben aliquotiert und sofort bei -80°C tiefgefroren. In der zweiten Prozedur wurden die Proben sofort (innerhalb von 15-30 Minuten nach der Abnahme) zentrifugiert und danach die Serumproben für 0, 6, 24 und 48 Stunden sowie für 7 Tage bei 4°C und 25°C gelagert, bevor sie ebenfalls bei -80°C tiefgefroren wurden. Außerdem wurden einige Proben ein- bis dreimal aufgetaut und wieder eingefroren, um das multiple Auftauen bei verschiedenen Messungen oder das Unterbrechen der Kühlkette zu simulieren (23, 24)

Für die Messung von HMGB1 wurde das ELISA- Kit der IBL International GmbH (Hamburg, Deutschland) verwendet. Das vorhandene HMGB1 in der Patientenprobe bindet im Inkubationsschritt an fixierte HMGB1- spezifische Antikörper auf der Messplatte, sowie an Enzyme gebundene freie Antikörper („Sandwich- Technik“). Die Enzyme an diesen löslichen Antikörpern reagieren im zweiten Schritt mit dem zugegebenen Substrat, wobei es zum Farbumschlag kommt, der photometrisch gemessen bei Wellenlängen von 450nm und 540-570nm (Referenzwellenlänge) untersucht wird. Entsprechend den Angaben des Herstellers wurde eine „hoch sensitive“ Standardreihe mit folgenden Standards verwendet: 0,31; 0,63; 1,25; 2,5; 5; 10 und 20 ng/ml. (Katalog-Nr. ST51011)

RAGE wurde in derselben Sandwich- Technik mit dem Quantikine Human RAGE ELISA Testkit von R&D Systems (Abingdon, UK) gemessen. Der Test besteht aus denselben Schritten mit Bindung von RAGE an ortsständige und enzymgebundene polyklonale Antikörper und die darauf folgende photometrische Bestimmung der RAGE-Konzentration bei 450nm und 540- 570nm Wellenlänge. (Katalog-Nr. DRG00)

Für die Messung der Nukleosomen wurde der Cell Death Detection ELISAplus von Roche Diagnostics (Mannheim, Deutschland) verwendet, wie bereits in (25) beschrieben.

Hierbei werden die Proben auf die Streptavidin- beschichtete Platte pipettiert und mit Anti- Histon- Biotin und Anti- DNS- Peroxidase („horseradish peroxidase“/ HRP) beschichtet und inkubiert. Nach einem Waschschritt und der anschließenden Farbreaktion mit dem ABTS (2,2'-Azino-di-(3-ethylbenzthiazolin-6-sulfonsäure)- Substrat wird daraufhin wiederum im Photometer anhand der Kalibrierungskurve die Konzentration bestimmt. (Katalog-Nr. 11 774 425 001)

Zur Ermittlung der DNAse- Aktivität erfolgt ebenfalls in Sandwich- ELISA- Technik („solid phase ELISA“ von Orgentec Diagnostika GmbH, Mainz, Deutschland), bei der spezifisches DNAse- Substrat, wiederum in Verbindung mit HRP, zum Einsatz kommt. Nach Reaktion mit TMB (3,3’,5,5’- Tetramethylbenzidin) wird die Aktivität durch photometrische Messung bei 450nm und 600- 690nm ermittelt. (Katalog-Nr. ORG 590)

Die Messung der etablierten Tumormarker CA 19-9, CEA und CYFRA 21-1 erfolgte mittels ElektroChemoLumineszenz (ECLIA) automatisiert auf dem Elecsys® 2010-Analyzer von Roche Diagnostics.

3.3. Definitionen und Statistik

Die Zeit bis zum Fortschreiten der Erkrankung („time to progression“/ TTP) wurde definiert als die Zeitspanne zwischen Beginn der Chemotherapie und dem Auftreten von radiologischer oder klinischer Verschlechterung des Krankheitsbildes während der Erstlinientherapie. Als Gesamtüberleben („overall survival“/ OS) wird im Folgenden die Zeitspanne zwischen Beginn der Behandlung und Eintreten des Todesfalls bezeichnet, unabhängig von der Todesursache. Angesichts der fortgeschrittenen Erkrankung kann in den meisten Fällen allerdings von einem tumorbedingten Versterben ausgegangen werden.

Die Konzentrationen der einzelnen Marker vor Beginn der Therapie (Tag 0) und an den Tagen 7, 14, 21, 28 und 56 (entspricht dem Staging), sowie die Differenzwerte zu dem jeweiligen prätherapeutischen Wert waren Gegenstand der statistischen Auswertungen. Die Signifikanz der Wertunterschiede wurde dabei mit dem Wilcoxon- Test für gepaarte Stichproben ermittelt. Zur Auswertung des Therapieansprechens wurden zwei Patientengruppen gebildet, dabei wurden neben der Gruppe mit einer fortschreitenden Erkrankung („progressive disease“/ PD) die Patienten mit nicht- fortschreitenden Stadien (komplette Remission, Teilremission und stabiler Zustand) zu einer Gruppe

zusammengefasst. Die Signifikanztestung der Markerunterschiede in den beiden Gruppen erfolgte hier mithilfe des Wilcoxon- Mann- Whitney- Testes, die der Korrelation zwischen den Markerwerten mithilfe des Spearman Rang Koeffizienten, wobei p-Werte <0.05 als signifikant betrachtet wurden.

Für die Analyse der Zeit bis zur Progression (TTP) und des Gesamtüberlebens (OS) wurde die Anzahl der Patienten auf drei gleich große Tertile aufgeteilt und anschließend Kaplan-Meier- und Log-rank- Tests angewendet. Für die logarithmische Analyse der Markerwerte als kontinuierliche Variablen und in Tertilen als dichotome Variablen wurde jeweils ein univariates Cox- Modell erstellt. Außerdem wurden klinisch adjustierte Hazard Ratios unter Einbeziehung des Karnoffsky- Indexes und ein multivariates Cox-Regressionsmodell unter Einbeziehung aller im univariaten Modell guten (p- Wert < 0,1) Parameter erstellt. Die prognostische Stärke wurde mit dem C Index nach Harrell et al. bestimmt.

4. Ergebnisse

4.1. Allgemein

Das Durchschnittsalter der Patienten betrug 65 Jahre (Spannbreite 41-79 Jahre) und die meisten Patienten (65%) hatten bei Aufnahme in die Studie bereits Fernmetastasen. Bei Studienende war bei 74 von 78 Patienten ein Fortschreiten der Krankheit beobachtet worden und 73 von 78 Patienten waren verstorben. Von den 78 Patienten war bei 68 Patienten eine objektive Bildgebung mittels CT oder MRT möglich.

In die Auswertung von Gesamtüberleben und Zeit bis zur Progression wurden alle 78 Patienten eingeschlossen, von denen 74 Patienten im Verlauf eine fortschreitende Krankheit entwickelten und 73 bis zum Ende der Beobachtungszeit verstarben.

Das radiologische Staging nach durchschnittlich 57 Tagen wurde bei 68 Patienten durchgeführt und nach den RECIST 1.0- Kriterien bewertet. Danach wurde eine komplette Remission bei einem Patienten (2%), eine Teilremission bei 4 Patienten (6%), ein stabiles Krankheitsbild („stable disease“) bei 37 Patienten (54%) und progressives Tumorwachstum bei 26 Patienten (38%) festgestellt. Bei 10 Patienten war ein objektives, radiologisches Staging nicht möglich, sodass diese bei der statistischen Auswertung des Therapieansprechens nicht berücksichtigt werden konnten. Nur in Ausnahmefällen wich die Zeit bis zur Bildgebung deutlich vom Durchschnitt ab (Zeitspanne 9- 176 Tage), aufgrund von Begleiterkrankungen oder akut aufgetretenen Komplikationen.

Bei 26 Patienten war bereits im ersten Staging nach 8 Wochen eine Progression zu verzeichnen, wohingegen bei 42 Patienten eine vorübergehende Kontrolle der Krankheitsaktivität gelang. Das mediane progressionsfreie Überleben betrug 3,9 Monate, das mediane Gesamtüberleben 7,7 Monate. Der KRAS- Mutationsstatus (Exon 2, Codons 12 und 13) war bei 36 Patienten (davon 26 Patienten positiv), ein Diabetes mellitus bei 25 Patienten bekannt.

Von allen untersuchten klinischen Variablen hatte der Karnofsky- Index (KPS) den höchsten C- Wert (als Maß für die prognostische Stärke) bei progressionsfreiem und Gesamtüberleben. So hatten Patienten mit initial gutem klinischem Allgemeinzustand, d.h. hohem KPS, signifikant längere progressionsfreie Intervalle und Gesamtüberlebenszeiten. Patienten mit lokal fortgeschrittenem Tumor hatten mit durchschnittlich 14,2 Monaten ein deutlich längeres Gesamtüberleben als Patienten mit Rezidiv- Tumoren (8,3 Monate) oder synchron metastasiertem Pankreaskarzinom (6,6

Monate). Wie zu erwarten war, hatten auch Patienten ohne Progress im ersten Staging längere Gesamtüberlebenszeiten (10,4 Monate) als Patienten mit fortschreitender Erkrankung (6,3 Monate).

4.2. Relevanz von CYFRA 21-1 und den etablierten Markern CA 19-9 und CEA

Therapieansprechen

Die „alten", etablierten Tumormarker zeigten an fast allen erhobenen Zeitpunkten eine exzellente positive Korrelation der absoluten Messwerte mit dem Therapieansprechen. Für die absoluten Werte waren CYFRA 21-1 und CA 19-9 die stärksten Prädiktoren. Bei der Kinetik konnte diejenige von CEA an Tag 14 am besten zwischen den beiden Gruppen des Therapieansprechens unterscheiden (0% Abfall der Werte bei fortschreitender Krankheit vs. 22% bei stabiler Krankheit oder Remission, P- Wert 0,04), ansonsten waren die absoluten Werte der Kinetik klar überlegen.

Prognose

Die Absolutwerte aller etablierten Tumormarker bewiesen ihren Nutzen als signifikante prognostische Parameter für objektives Therapieansprechen vor Therapiebeginn und zum Zeitpunkt des Stagings. CYFRA 21-1 hatte die stärkste prätherapeutische Aussagekraft bezüglich progressionsfreiem Intervall und Gesamtüberleben in der univariaten Analyse. Prätherapeutische CYFRA 21-1- Werte konnten signifikant zwischen lokal fortgeschrittenen, metastasierten und Rezidiv- Tumoren unterscheiden und korrelierten ebenfalls mit dem Karnofsky- Index.

Zum Zeitpunkt des Stagings hatte allerdings CA 19-9 den größten Aussagewert für das Gesamtüberleben mit einem C- Wert von 0,69. In der Analyse als kontinuierliche Variablen zeigten die Absolutwerte von CYFRA 21-1 und CA 19-9 in etwa gleich gute Korrelationen mit progressionsfreiem Intervall und Gesamtüberleben, wohingegen CEA an einigen Messzeitpunkten keine Signifikanz aufwies. Im Allgemeinen spiegelten die Absolutwerte der Marker die Prognose um einiges besser wider als die entsprechenden Markerverläufe.

Auch in der multivariaten Analyse waren die absoluten CYFRA 21-1- Werte ein statistisch signifikanter, unabhängiger prognostischer Parameter. Für ein weiteres multivariates Cox- Modell wurden alle im univariaten Modell signifikanten Parameter eingeschlossen. In diesem Modell blieben nur der Karnofsky- Index und die prätherapeutischen Werte

von CYFRA 21-1 als unabhängige prognostische Marker übrig. Wurde berücksichtigt, dass nur Patienten mit dem Lewis- Antigen CA19-9 exprimieren können, war auch CA 19-9 ein unabhängiger prognostischer Marker für das Gesamtüberleben.

4.3. Relevanz der immunologischen Zelltod-Marker HMGB1, sRAGE, Nukleosomen und DNAse- Aktivität

Präanalytik

Am Beginn der Studie über die neuen immunologischen Zelltod (ICD)- Marker stand die methodische und präanalytische Evaluation der verwendeten Tests, für die teilweise noch keine Literaturdaten zur Verfügung standen. Im Folgenden werden stellvertretend die Ergebnisse der Evaluation des sRAGE- ELISA- Testkits von R&D Systems (Abingdon, UK) dargestellt, die Untersuchungen der anderen Tests z.B. für HMGB1 erfolgte nach einem ähnlichen Schema und sind u.a. in der Literaturstelle 23 dokumentiert. (23)

Die intraserielle Ungenauigkeit (intraassay imprecision) des sRAGE-ELISAs für fünf identischen Wiederholungen dreier Serumproben lag mit 6,0% in der niedrigkonzentrierten Probe, sowie 5,6% und 11,5% in den höher konzentrierten Proben im akzeptablen Bereich für eine manuelle Messung. Dasselbe gilt für die interserielle Ungenauigkeit (interassay imprecision), die anhand von 11 Wiederholungen zweier Proben getestet wurde (5,9% und 7,8% Ungenauigkeit).

In der Untersuchung der Verdünnungsreihen zeigte sich eine zufrieden stellende Wiederfindung von durchschnittlich 117% (Spannbreite 99,1- 131%).

Ob die Werte von sRAGE in Serum, EDTA- Plasma oder Heparin- Plasma gemessen wurden, erwies sich als nicht entscheidend für das Messergebnis, allerdings waren die Messwerte in Citratplasma entsprechend des Verdünnungsfaktors durch das vorgelegte Na-Citrat generell etwas niedriger als in den anderen drei Medien (durchschnittliche Wiederfindung 88,1% vs. 95,4% in EDTA-Plasma und 97,0% in Heparin- Plasma verglichen mit Serumproben).

Eine verlängerte Lagerung bei Raumtemperatur vor der Zentrifugation hatte keinen wesentlichen Einfluss auf die Messergebnisse, nach 6 Stunden betrug die Wiederfindung noch durchschnittlich 97,7% und nach 24 Stunden immer noch 93,6%. Ebenso zeigten sich keine nennenswerten Abweichungen der Messwerte bei verlängerten Lagerungsperioden nach der Zentrifugation, unabhängig ob bei Kühlschrank- oder

Raumtemperatur. Selbst nach zwei- oder dreimaligem Auftauen und erneutem Einfrieren war die Wiederfindung der Werte noch sehr gut (98,4% und 98,5%).

Die Messung von sRAGE in Blutproben von 30 gesunden Freiwilligen ergab eine gewisse Spannbreite an Werten mit einem Medianwert von 1,10 ng/ml, wobei 90% der Werte zwischen 0,52 und 1,49 ng/ml lagen.

Unterscheidung von Diagnosegruppen

In der klinischen Evaluation waren die prätherapeutischen Werte von sRAGE und DNAse bei Patienten mit einem Pankreaskarzinom signifikant niedriger als in der gesunden Kontrollgruppe, wohingegen die Werte von HMGB1 und Nukleosomen bei Karzinompatienten tendenziell erhöht waren (nicht statistisch signifikant).

Therapieansprechen

Bei den „neuen" immunogenen Biomarkern zeigten nur erhöhte absolute Nukleosomenwerte an Tag 28 und erniedrigte sRAGE- Werte zum Stagingzeitpunkt eine signifikante Korrelation mit schlechtem Therapieansprechen.

Prognose

In der univariaten und KPS- adjustierten Analyse der „neuen" Biomarker, in der die Werte als kontinuierliche Variablen behandelt wurden, zeigten Nukleosomen die beste Korrelation mit progressionsfreiem Intervall und Gesamtüberleben. Das galt sowohl für absolute Werte als auch für prozentuale Veränderungen an fast allen untersuchten Zeitpunkten. Daneben konnten auch für die anderen Biomarker signifikante Korrelationen an bestimmten Zeitpunkten gefunden werden, Näheres dazu in den u.g. Publikationen. Ganz allgemein lässt sich die Tendenz erkennen, dass HMGB1 und Nukleosomen bei Patienten mit fortschreitender Erkrankung höher sind als in der Vergleichsgruppe, wohingegen sRAGE und DNAse in der progressiven Gruppe erniedrigt sind. Auch in der Analyse der Tertilen zeigten sich erhöhte Nukleosomenwerte als bester prognostischer Marker für eine schlechte Prognose, insbesondere kürzeres progressionsfreies Intervall.

4.4. Diskussion des Studiensettings und Perspektiven

Die Stärken der vorliegenden Studie liegen zum Einen in der gründlichen Vorarbeit, die den Messungen vorausging: alle verwendeten Tests der „neuen" Marker wurden auf wichtige Qualitätskriterien wie Intra- und Interassay- Impräzision, Linearität in

Verdünnungsreihen und nicht zuletzt auf Stabilität der zu untersuchenden Parameter bei unterschiedlichster Lagerung der Proben getestet. (23, 24) Die Patientenproben wurden nach einem festen Schema wöchentlich gesammelt, nach einem standardisierten präanalytischen Protokoll behandelt und eingefroren, und schließlich als vollständige Verläufe der Patienten in den einzelnen ELISA-Testläufen vermessen (um eventuelle Interassay- Impräzisionen bei den Markerverläufen auszuschließen).

Die Kohorte war mit 78 Patienten repräsentativ für Pankreaskarzinome, zumal die Studie unizentrisch erfolgte. Außerdem war sie als sehr homogen anzusehen, da alle Patienten eine Erstlinien- Chemotherapie erhielten. Bisher gab es unseres Wissens keine so umfangreichen Daten zu derart engmaschig kontrollierten Verläufen etablierter und neuer Marker während einer systemischen Chemotherapie bei einer vergleichbaren Kohorte von Patienten mit einem Pankreaskarzinom. Insbesondere die hochfrequente Bestimmung von „altbekannten“ Markern wie CEA und CYFRA 21-1 sowie der neuen ICD-Marker im neuen Kontext des Pankreaskarzinoms, erlaubte einen Einblick in die Tumorbiologie, die Interaktion mit dem „Microenvironment“ der Tumorzellen sowie dem Immunsystem als Reaktion auf die applizierte Chemotherapie. Der Vergleich mit dem etablierten CA 19-9 schließlich zeigte den klinischen Nutzen der neuen Marker auf. Zukünftige Verbesserungsmöglichkeiten liegen im noch intensiveren Monitoring während der ersten Therapiewoche, da einige Studien schon die Bedeutung dieser ganz frühen Werte zeigen konnten. (26-28)

Die Qualität der Bewertung wird dadurch unterstrichen, dass die abschließende statistische Auswertung unabhängig von der Erhebung der klinischen Daten, des Ansprechens der Therapie sowie der Labordaten durch die Statistikerin des Instituts für Klinische Chemie erfolgte.

Die einzelnen Schlussfolgerungen der vorliegenden Studie bezüglich Prognose, Therapieansprechen und Vorhersage von TTP und OS sind natürlich nicht völlig unabhängig voneinander, allerdings beeinflussen sie verschiedene Prozesse der Entscheidungsfindung im klinischen Alltag:

- Prätherapeutische Vorhersagen des Therapieansprechens führen zur frühzeitigen Auswahl der zur Verfügung stehenden Therapiemöglichkeiten.

- Das Therapiemonitoring während der laufenden Therapie entscheidet über Fortführung oder Abbruch, ggf. auch Umstellung auf erfolgsversprechendere Verfahren.
- Die Vorhersage von TTP und OS, unabhängig von radiologischer Diagnostik und auch frühzeitiger als diese, kann über rechtzeitige Therapieeskalation entscheidend für die verbleibende Lebenszeit und –qualität der Patienten sein.

Die Erforschung der einzelnen Marker und ihrer optimalen Einsatzgebiete innerhalb dieser Subgruppen steht noch am Anfang. In dieser Studie zeigten die untersuchten Marker die größten Stärken auf dem Gebiet der prätherapeutischen Prädiktion des Therapieansprechens bzw. der Abschätzung der Prognose. Gerade für die neuen Marker ist jedoch noch nicht klar, ob eine Messung an anderen Zeitpunkten während der Therapie eventuell nützlichere Resultate erzielt oder ob absolute oder relative Markerveränderungen aussagekräftiger sind. Die hier untersuchten neuen ICD-Marker sind ebenfalls nur eine kleine Auswahl aller zur Verfügung stehender, bereits bekannter Schlüsselmoleküle des immunogenen Zelltodes. Um dieser Vielfalt an Möglichkeiten Rechnung zu tragen, wurden im Rahmen dieser Dissertation eine Vielzahl von Untersuchungen durchgeführt, die deutlich explorativen Charakter aufweisen und nur die vielzitierte „Spitze des Eisberges“ beleuchten können. Für die Erforschung des restlichen „Eisberges“ werden in den nächsten Jahren weitere konfirmative Studien nötig sein.

5. Zusammenfassung

5.1. Neue molekulare Zelltodmarker im Therapiemonitoring bei fortgeschrittenem Pankreaskarzinom

Das Pankreaskarzinom ist bekannt für seine schlechte Prognose und fehlende Möglichkeiten der Früherkennung. Gerade bei dieser Tumorentität werden neue Methoden für Therapiemonitoring und Rezidiv- Früherkennung dringend benötigt.

Im Rahmen dieser Dissertation wurden die immunogenen Zelltodmarker HMGB1, sRAGE, DNAse und zirkulierende Nukleosomen auf ihre mögliche Verwendung als neue Tumormarker für Prognose und Therapiemonitoring hin untersucht. Außerdem wurden CA19-9, ein klinisch bereits weit verbreitet eingesetzter Tumormarker für das Pankreaskarzinom, sowie CYFRA 21-1 und CEA, zwei bei anderen Tumorentitäten etablierte Marker, mitbestimmt. Vor Beginn der Messungen wurden die manuellen ELISA-Tests der neuen Zelltodmarker auf ihre methodische Qualität hin untersucht.

In dieser prospektiven Single Center- Studie der Abteilung für Onkologie, Med. Klinik III am Klinikum Großhadern (Universität München, LMU) wurden Blutproben von 78 Patienten mit fortgeschrittenem Pankreaskarzinom vor und wöchentlich nach Beginn der palliativen systemischen Chemotherapie bis zum radiologischen Staging nach ca. zwei Monaten (Median 56 Tage) gesammelt. Alle Parameter wurden anschließend gemessen und mit dem radiologischen Staging (nach RECIST- Kriterien), der Klinik (KPS), der Zeit bis zur Progression (TTP) und dem Gesamtüberleben (OS) korreliert. Die mediane TTP betrug 3,9 Monate, das mediane Gesamtüberleben 7,7 Monate. Die prätherapeutischen Werte von sRAGE und DNAse- Aktivität waren signifikant niedriger bei Tumorpatienten als in der gesunden Kontrollgruppe. Jedoch konnte keiner der „neuen“ Biomarker vor Beginn der Therapie signifikant zwischen lokal fortgeschrittenen Tumoren, primärer Metastasierung und Tumorrezidiven unterscheiden. Im Verlauf der Therapie zeigten Nukleosomen von allen „neuen“ Markern die beste Korrelation mit der Prognose, sowohl in absolut gemessenen Werten als auch in der Kinetik an bestimmten Zeitpunkten. Dabei korrelierten hohe Nukleosomenwerte mit kurzem progressionsfreiem Intervall und schlechtem Gesamtüberleben. Die Absolutwerte von Nukleosomen und HMGB1 zum Zeitpunkt des Stagings sowie die Kinetik der DNAse- Aktivität kurz nach Beginn der Therapie blieben auch als unabhängige prognostische Faktoren für das Gesamtüberleben bestehen, wenn die Daten auf den KPS korrigiert wurden.

Von den etablierten Markern zeigten CYFRA 21-1 und CA 19-9 in der univariaten Analyse eine hoch signifikante Korrelation mit TTP und OS an fast allen gemessenen Zeitpunkten. In der multivariaten Analyse blieb nur CYFRA 21-1 als unabhängiger prognostischer Parameter für das Gesamtüberleben bestehen, zusammen mit dem KPS.

Zusammenfassend lässt sich festhalten, dass sich Nukleosomen, HMGB1 und sRAGE als sinnvolle neue Tumormarker für Prognose und Therapiemonitoring bei fortgeschrittenem Pankreaskarzinom bewährt haben. Zudem erwies sich CYFRA 21-1 neben dem etablierten CA19-9 ebenfalls als starker und unabhängiger prognostischer Marker bei Pankreaskarzinomen.

5.2. New markers of molecular cell-death for therapy monitoring in advanced pancreatic cancer

Pancreatic cancer is a disease known for its unfavorable prognosis and missing early symptoms. Especially in this tumor entity new means of therapy monitoring and recurrence detection are desperately needed.

In this doctoral thesis the biomarkers of immunogenic cell death HMGB1, sRAGE, DNAse and circulating nucleosomes have been investigated for possible use as new promising tools in prognosis and evaluation of therapy. Moreover CA19-9, commonly used as tumormarker in pancreatic cancer, as well as CYFRA 21-1 and CEA, established markers for other tumor entities, have been evaluated. Before initiation of this study the methodical characteristics of the manually performed ELISA- testkits for new cell death markers were investigated.

Within a prospective single- center study conducted at the Department of Medical Oncology (Campus Großhadern, University of Munich) blood samples were obtained from 78 patients with advanced pancreatic cancer before and weekly during the course of palliative systemic chemotherapy until radiologic staging after a median of 56 days. All parameters were assessed and correlated with radiologic therapy response (according to RECIST criteria), clinical performance (KPS), time to progression (TTP) and overall survival (OS). Median TTP was 3.9 months, median OS 7.7 months. Pretherapeutic values of sRAGE and DNAse activity were significantly lower in tumor patients than in healthy control. However, none of the “new” biomarkers could distinguish significantly between locally advanced, metastatic and recurrent disease before initiation of therapy.

During the course of therapy levels of circulating nucleosomes showed best correlation with prognosis of all “newly assessed” biomarkers, in absolute levels as well as in percentual changes at certain timepoints. Here, high levels of nucleosomes correlated with short TTP and poor OS. Absolute nucleosome and HMGB1 levels at staging and early DNAse kinetics also remained independent prognostic factors for OS when adjusted to KPS. Of the established markers, CYFRA 21-1 and CA 19-9 showed a highly significant correlation with TTP and OS at almost all timepoints assessed in univariate analysis. In multivariate analysis, only CYFRA 21-1 remained an independent predictor for OS, together with KPS.

All in all the new biomarkers circulating nucleosomes, HMGB1 and sRAGE proved to be useful tools for prognosis and monitoring of therapy in pancreatic cancer at certain timepoints during therapy. In addition, CYFRA 21-1, besides the established marker CA19-9, was also shown to be a strong and independent prognostic marker for use in pancreatic cancer.

6. Literaturverzeichnis

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7. Eigenanteil an den vorgelegten Arbeiten

Die der Doktorarbeit zugrunde liegenden Arbeiten gliederten sich in eine Hauptstudie über die Wertigkeit von Biomarkern beim Pankreaskarzinom, die insbesondere die Biomarker CYFRA 21-1, CEA, CA 19-9 und Nukleosomen untersuchte, und eine Substudie über die Marker HMGB1, sRAGE und DNAse Aktivität.

An der Konzeption der Hauptstudie, der Erstellung des Studienprotokolls und Einholung des Ethikvotums waren Herr PD Dr. Stefan Holdenrieder (SH) und Frau Dr. Petra Stieber (PS) vom Institut für Klinische Chemie sowie Herr PD Dr. Stefan Böck (SB) und Herr Prof. Dr. Volker Heinemann (VH) von der Medizinischen Klinik III des Universitätsklinikums München beteiligt. An der Konzeption der Substudie war SH, SB sowie die Doktorandin Frau Christin Wittwer (CW) beteiligt.
Die Patientenrekrutierung und Erhebung der klinischen Daten erfolgte durch SB, VH und Herrn Michael Haas (MH) in der Medizinischen Klinik III.
Die standardisierte präanalytische Bearbeitung und Asservierung der Proben wurde durch PS und SH im Institut für Klinische Chemie gewährleistet.
Die Messung der etablierten Marker CA 19-9, CYFRA 21-1 und CEA wurde durch PS und ihre Mitarbeiterinnen im Institut für Klinische Chemie organisiert und durchgeführt.
Die Messung der neuen Marker Nukleosomen, HMGB1, sRAGE und DNAse Aktivität wurde durch CW, SH und Frau Siegele im Institut für Klinische Chemie durchgeführt.
Die Dokumentation und Aufarbeitung der „neuen“ Labordaten erfolgt durch CW, die Bearbeitung der klinischen Daten und des Therapieansprechens erfolgte durch CW und SB.
Die methodische und präanalytische Austestung der „neuen“ Marker wurden von CW und Julia Lehner vorgenommen.
Die statistische Auswertung der Daten wurde von Frau Dr. rer. nat. Dorothea Nagel (DN) durchgeführt.
Die Ergebnisse der Hauptstudie wurden von SH, PS, SB, VH und DN, die Ergebnisse der Substudie von CW, SH, SB und DN diskutiert und bewertet.
Das Manuskript für die Hauptstudie erstellten SB, SH, CW, PS, VH und DN, für die Substudie war dies federführend CW mit SH, SB und DN.

Zusammenfassend war die Doktorandin CW an der Konzeption der Substudie beteiligt, führte nach Einweisung durch eine Fachkraft (medizinisch- technische Assistentin Fr. Siegele) alle manuellen ELISA- Messungen an allen Patientenproben selbst durch und war eigenständig für den fachgerechten Umgang mit den Proben und den Laborgeräten verantwortlich. Daneben war sie für die Erhebung von klinischen Patientendaten und deren Eingabe in das für die statistische Auswertung vorgesehene Computersystem zuständig. Nach der statistischen Analyse durch die Statistikerin des Institutes Fr. Dr. Nagel, war die Doktorandin aktiv in die Diskussion und Interpretation der Daten eingebunden, fertigte selbständig die Hauptpublikation der Substudie über die neuen Marker an und war maßgeblich an der Erstellung der zweiten Hauptpublikation über die etablierten Marker beteiligt.

8. Originalarbeiten

8.1. Hauptpublikationen der kumulativen Dissertation

Die oben aufgeführten Ergebnisse waren die Grundlager mehrerer Publikationen, von denen die zwei Hauptartikel dieser Dissertation im Folgenden näher erläutert werden:

- Boeck S, Wittwer C, Heinemann V, Haas M, Kern C, Stieber P, Nagel D, Holdenrieder S. Cytokeratin 19-fragments (CYFRA 21-1) as a novel serum biomarker for response and survival in patients with advanced pancreatic cancer. Br J Cancer 2013; 108: 1684- 1694.

In diesem Artikel werden die ausgezeichneten Ergebnisse der etablierten Tumormarker, insbesondere CYFRA 21-1, für Prognose und Therapiemonitoring bei Pankreaskarzinomen beschrieben.

Das „British Journal of Cancer“ gehört zur Nature Publishing Group des Vereinigten Königreiches. Es erscheint seit 1947 in 24 Ausgaben jährlich in englischer Sprache. Herausgeber ist Prof. Adrian L. Harris vom University College London Cancer Institute (Vereinigtes Königreich). Alle veröffentlichten Artikel unterliegen bestimmten Auswahlkriterien und der Annahme durch ein Auswahlkommitee. Der Impactfactor lag 2012 bei 5, 082 (2012 Journal Citation Reports® Science Edition; Thomson Reuters, 2013). In der Kategorie “Onkologie” des ISI Web of Knowledge ist es damit an Platz 35 von 197 gelistet.

- Wittwer C, Boeck S, Heinemann V, Haas M, Stieber P, Nagel D, Holdenrieder S. Circulating nucleosomes and immunogenic cell death markers HMGB1, sRAGE and DNAse in patients with advanced pancreatic cancer undergoing chemotherapy. Int. J. Cancer 2013; Int. J. Cancer: 133: 2619–2630

In diesem Artikel werden die Ergebnisse der Untersuchungen der „neuen“ biologischen Zelltodmarker HMGB1, sRAGE, DNAse und Nukleosomen dargelegt. Das “International Journal of Cancer” wird vom Wiley- Verlag veröffentlicht. Es erscheint zweiwöchentlich in englischer Sprache. Chefherausgeber ist Prof. Peter Lichter vom Deutschen Krebsforschungszentrum (DKFZ) in Heidelberg. Auch bei dieser Fachzeitschrift unterliegen alle angenommenen Artikel einem Auswahl- und Reviewprozess. Der Impactfactor lag 2012 bei 6,198, entsprechend Platz 23 von 197 in der Liste des ISI Web of Knowledge.

8.2. Weitere Publikationen

Originalarbeiten

- Wittwer C, Lehner J, Fersching D, Siegele B, Stoetzer OJ, Holdenrieder S.

Methodical and preanalytical evaluation of a RAGE immunoassay.
Anticancer Res 2012; 32: 2075-2078.

- Lehner J, Wittwer C, Fersching D, Siegele B, Holdenrieder S, Stoetzer OJ.

Methodical and preanalytical evaluation of an HMGB1 immunoassay.
Anticancer Res 2012; 32: 2059-2062.

- Stoetzer OJ, Wittwer C, Lehner J, Fahmueller Y, Kohles N, Fersching D, Leszinski G, Roessner J, Holdenrieder S.

Circulating nucleosomes and biomarkers of immunogenic cell death as predictive and prognostic markers in cancer patients undergoing cytotoxic therapy.
Exp Opin Biol Ther 2012; 12 (S1): 217-224.

- Wittwer C, Boeck S, Heinemann V, Haas M, Nagel D, Stieber P, Holdenrieder S.

Soluble receptor of advanced glycation end products (sRAGE) indicates response to chemotherapy in pancreatic cancer patients.
Int J Clin Pharmacol Ther. 2013 Jan; 51(1):67-9

- Wittwer C, Holdenrieder S.

Immunogene Zelltodmarker HMGB1 und sRAGE als neue prädiktive und prognostische Serum Biomarker bei Tumorerkrankungen.
Laboratoriumsmedizin 2013; 37(1): 29-52

Abstracts von Kongressbeiträgen

- Wittwer C, Lehner J, Fersching D, Siegele B, Holdenrieder S.

Methodical validation of an immunoassay for the determination of soluble receptors of advanced glycation end products (sRAGE).

Tumor Biol 2010; 31 (S1): S75-76.

Conference of the International Society of Oncology and BioMarkers (ISBOM) 2010 in München, Poster

- Lehner J, Wittwer C, Fersching D, Stötzer OJ, Siegele B, Holdenrieder S.

Methodical validation of an immunoassay for the determination of soluble high mobility group box 1 (sHMGB1).

Tumor Biol 2010; 31 (S1): S69.

Conference of the International Society of Oncology and BioMarkers (ISBOM) 2010 in München, Poster

- Holdenrieder S, Roessner J, Hoecherl EF, Wolf K, Durner J, Hofmann W, Fersching D, Stoetzer OJ, Lehner J, Wittwer C, Siegele B, Nagel D.

Clinical relevance of soluble HMGB1, RAGE and nucleosomes in trauma and cancer patients

Clin Chem Lab Med 2010; 48: A106-107.

Jahrestagung der Deutschen Gesellschaft für Klinsiche Chemie und Laboratoriumsmedizin (DGKL) 2010 in Mannheim, Poster

- Wittwer C, Lehner J, Fersching D, Siegele B, Stötzer OJ, Holdenrieder S.

Methodical and preanalytical evaluation of a RAGE immunoassay.

Anticancer Res 2011; 31: 2008.

Hamburg Symposium on Tumor Markers (HSTM) 2011 in Hamburg, Poster

- Lehner J, Wittwer C, Fersching D, Siegele B, Stötzer OJ, Holdenrieder S.

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FULL PAPER

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Cytokeratin 19-fragments (CYFRA 21-1) as a novel serum biomarker for response and survival in patients with advanced pancreatic cancer

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Background: CYFRA 21 1 serves as biomarker in several epithelial malignancies. However, its role in pancreatic cancer (PC) has not yet been investigated.

Methods: Within a prospective single centre study serial blood samples were collected from patients with confirmed advanced PC. Pre treatment values and weekly measurements of CYFRA 21 1, carbohydrate antigen 19 9 (CA 19 9) and carcinoembryonic antigen (assessed by Elecsys 2010, Roche Diagnostics) during palliative first line chemotherapy were obtained. Biomarker data were correlated with objective response (determined by RECIST) as well as time to progression (TTP) and overall survival (OS) using uni and multivariate analyses.

Results: Seventy eight patients were included, 45% of these received treatment in prospective clinical trials. Median TTP was 3.9 months, median OS 7.7 months. Pre treatment CYFRA 21 1 levels were significantly associated with performance status ($P=0.0399$) and stage of disease ($P=0.0001$). Marker values before chemotherapy and at the 2 month staging of all three markers were considered significant predictors for objective treatment response. Pre treatment CYFRA 21 1 levels, as well as CA 19 9 values, could be applied to define subgroups (categorised by tertiles) with a different OS outcome (CYFRA: 14.8 *vs* 7.1 *vs* 4.8 months, CA 19 9: 14.2 *vs* 7.1 *vs* 5.2 months; $P<0.0001$). CYFRA 21 1 and CA 19 9 (both as categorised and as continuous variables) showed a highly significant correlation with TTP and OS at nearly all time points assessed in univariate analysis. In multivariate analysis, only CYFRA 21 1 and performance status were independent predictors for OS.

Conclusions: CYFRA 21 1 may serve as a valuable tool for monitoring treatment response and assessing prognosis in advanced PC.

Pancreatic cancer (PC) is a neoplastic disease known for its unfavourable prognosis and outcome data: the 5 year relative survival rate among patients diagnosed with PC in the United States from 2001 to 2007 was as low as 6% over all stages (Siegel *et al*, 2012). In patients with advanced stages of the disease, palliative gemcitabine based chemotherapy has been the standard of care during the last decade. With the novel FOLFIRINOX regimen, a new treatment option for patients with metastatic PC was recently introduced (Vincent *et al* 2011; Heinemann *et al*, 2012). To date, overall survival (OS) remains the standard clinical end point for clinical trials in PC research, and also for the approval of novel drugs (Heinemann *et al*, 2012). Several efforts have been undertaken to define new (ideally early) surrogate 'biomarker end points' for treatment efficacy and for assessment of

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prognosis. Carbohydrate antigen 19 9 (CA 19 9) is a tumour associated antigen that equals a sialylated hapten of the Lewis blood group antigen and is present in a broad variety of fetal and adult mucosal cells (Boeck *et al*, 2006). It is still the most commonly used tumour marker in PC, as many studies in patients with resectable and advanced disease proved CA 19 9 to be a useful tool for evaluation of treatment response as well as prediction of prognosis (Ferrone *et al*, 2006; Berger *et al*, 2008; Hess *et al*, 2008; Reni *et al*, 2009; Boeck *et al*, 2010; Humphris *et al*, 2012). However, a broad variety of other serum and tissue markers presently investigated in PC have not yet been sufficiently validated for routine clinical use (Duffy *et al*, 2010).

Carcinoembryonic antigen (CEA) is a monomer glycoprotein, which can be found in adult gastrointestinal epithelia, sweat glands, lung epithelia and various epithelial malignancies. In colorectal and lung cancer, pre therapeutic CEA levels are well established as tumour marker for prediction of time to progression (TTP) and OS; furthermore, CEA kinetics are also known for their important role in the diagnosis of tumour recurrence. CYFRA 21 1 is a fragment of cytokeratin 19, a structure protein and part of intermediate filament proteins necessary for stability of epithelial cells. It is thus expressed in a variety of epithelial cells and has already been shown to be a useful biomarker in lung and breast cancer (Nakata *et al*, 2004; Holdenrieder *et al*, 2009; Edelman *et al*, 2012). Moreover, CYFRA 21 1 was recently shown to be a prognostic relevant marker for OS in metastatic colorectal cancer after selective internal radiation therapy (Fahmueller *et al*, 2012). At least to our knowledge, clinical data on CYFRA 21 1 in PC are still very rare (Halm *et al*, 2000; Duffy *et al*, 2010).

The aim of this prospective, single centre biomarker study was to investigate the role of CYFRA 21 1 as serum biomarker in patients with advanced PC undergoing palliative chemotherapy. To this end, a representative group of PC patients underwent an extensive biomarker profiling for CYFRA 21 1, CA 19 9 and CEA levels that were assessed centrally before and weekly after the initiation of chemotherapy.

PATIENTS AND METHODS

Patient population and treatment. Male or female patients with histologically or cytologically confirmed diagnosis of advanced exocrine PC (locally advanced or metastatic stages of disease) were eligible for the current prospective biomarker study. All included patients received palliative chemotherapy. Eighty three consecutive patients meeting the eligibility criteria were recruited from the 'Pancreas Centre' at the Ludwig Maximilians University of Munich between May 2006 and April 2010. Patients treated outside clinical trials received based on the decision of the treating medical oncologist SB and VH standard gemcitabine or gemcitabine based chemotherapy until disease progression, unacceptable toxicity or patient refusal (for detailed treatment regimens see Results section). Routine radiological tumour assessment and response evaluation was performed by CT or MRI according to standard RECIST (response evaluation criteria in solid tumors; version 1.0) every 8 weeks. The study was approved by the local ethics committee of the Ludwig Maximilians University and all patients gave written informed consent before any study specific procedure was performed.

Sample collection and assays. Venous blood samples were collected immediately before initiation of first line chemotherapy (day 0) and thereafter weekly on days 7, 14, 21 and 28, and at the time point of the first radiographic staging after 2 months of chemotherapy (day 56). The samples were centrifuged for 15 min at 3000 g within 2 h of venipuncture. Sera were separated manually, aliquoted into microtubes and without any further treatment frozen at −80 °C for a maximum of 3 years. For measurements, samples were thawed and assessed in batches containing all samples of one single patient. Measurements of CYFRA 21 1, CA 19 9 and CEA were all performed automatically using the Elecsys 2010 (Roche Diagnostics, Penzberg, Germany) in an electro chemi luminescence immuno assay. All assays in this biomarker study were performed blinded to the study end point. In the first step of the assay, the antigens (CYFRA 21 1, CA 19 9 or CEA) are incubated with two types of antigen specific monoclonal anti bodies, one biotinylated, and the other bound to a ruthenium containing complex. The resulting sandwich complex of antigen and the two antigen specific monoclonal antibodies is then bound to streptavidin coated microparticles via biotin streptavidin inter action. Next, the mixture is transferred to the measuring cuvette and the antigen containing complex is magnetically immobilised onto the electrode. After a washing step, chemiluminescence is induced by an electric field and assessed by a photomultiplier. Antigen concentration is determined in the following by use of a calibration curve. CA 19 9*all* was defined as CA 19 9 values for all patients; CA 19 9*syn* was defined as CA 19 9 values for patients, who are supposed to be able to synthesise CA 19 9 (i.e., at least one value $>5\,U\,ml^{-1}$). If not stated otherwise, all analyses were performed with the CA 19 9*syn* population.

Study design and statistical analysis. This prospective single centre biomarker study was designed, conducted and analysed

Table 1. Patient characteristics

	Number	Percentage (%)
	78	100
Gender		
Female	30	38
Male	48	62
Stage of disease at study entry		
Synchronous metastases	51	65
Locally advanced disease	9	12
Relapse	18	23
KPS		
100	16	21
90	42	54
80	13	17
70	6	8
60	1	1
KRAS mutation status (exon 2)		
Wild type	10	13
Mutation	26	33
Not assessed	42	54
Diabetes mellitus		
Negative	30	38
Known	13	17
Newly diagnosed at study entry	12	15
Not assessed	23	30
Best response by imaging		
CR + PR + SD	42	54
PD	26	33
Not assessed	10	13

Abbreviations: CR complete remission; KPS Karnofsky performance status; PD progressive disease; PR partial remission; SD stable disease.

according to the 2005 REMARK guidelines ('REporting recommendations for tumour MARKer prognostic studies') as appropriate (McShane *et al*, 2005). The pre defined end point of this study was to show a correlation of CYFRA 21 1, CA 19 9 and CEA with efficacy outcome parameters of treatment: objective response determined by RECIST, TTP and OS. Time to progression was defined as the interval between initiation of treatment and occurrence of a documented disease progression; OS was defined as the time interval between initiation of treatment and death from any cause. Concentrations of all measured markers before (day 0), and on days 7, 14, 21, 28 and 56 (= staging) after start of chemotherapy as well as their differences compared with pre therapeutic levels were considered for statistical evaluation. Significance of differences was tested using the Wilcoxon test for paired samples. Concerning their response to therapy at staging on day 56, patients with complete remission (CR), partial remission (PR) or stable disease (SD) were combined into a 'non progressive/disease control' group and compared with patients who suffered from progressive disease. For assessment of significance between marker levels in therapy response groups, the Wilcoxon Mann Whitney test was used. Correlations between marker levels were assessed by the Spearman rank correlation coefficient. Overall survival and TTP were estimated by the Kaplan Meier method and survival curves were compared using the log rank test. For this analysis, marker values were separated into tertiles to achieve an equal distribution of the patient number. Marker values were also analysed in univariate Cox regression models, on the one hand as tertiles for the calculation of hazard ratios, and on the other hand as logarithms. In addition, these analyses were done with inclusion of the Karnofsky performance status (KPS) in the models, which was the strongest clinical predictor. All clinical and pre therapeutical biochemical parameters with a P value <0.1 in univariate analysis were included into multivariate Cox regression analysis. To compare the prognostic strength of different models we used the concordance index (C index) proposed by Harrell *et al* (1984). The C index values range from 0.5 to 1, representing the proportion of concordance in all possible pairs of patients, whereby concordance means that the patient with a more favourable value (closer to 1) has the longer survival time. In addition, exploratory subgroup analyses were performed for patients suffering from diabetes mellitus and also for patients with a known KRAS mutation status. A P value of <0.05 was considered statistically significant. All calculations were performed with SAS software (version 9.2, SAS Institute Inc., Cary, NC, USA).

RESULTS

Patient characteristics. Overall, 83 consecutive PC patients from one German Cancer Centre were included in this biomarker study. Patients started treatment between May 2006 and April 2010; 35 patients (45%) in this biomarker study received chemotherapy within a prospective clinical trial. Applied treatment regimens included: gemcitabine plus erlotinib ($n=45$), single agent gemcitabine ($n=12$), gemcitabine plus everolimus ($n=11$), capecitabine plus erlotinib ($n=9$), gemcitabine plus axitinib ($n=2$), single agent capecitabine ($n=2$), gemcitabine plus WX 671 ($n=1$) or nab paclitaxel ($n=1$). Owing to missing baseline marker levels, five cases had to be excluded from statistical analysis. Of the 78 remaining evaluable patients, 68 were assessable for objective response by imaging, which was first performed after a median of

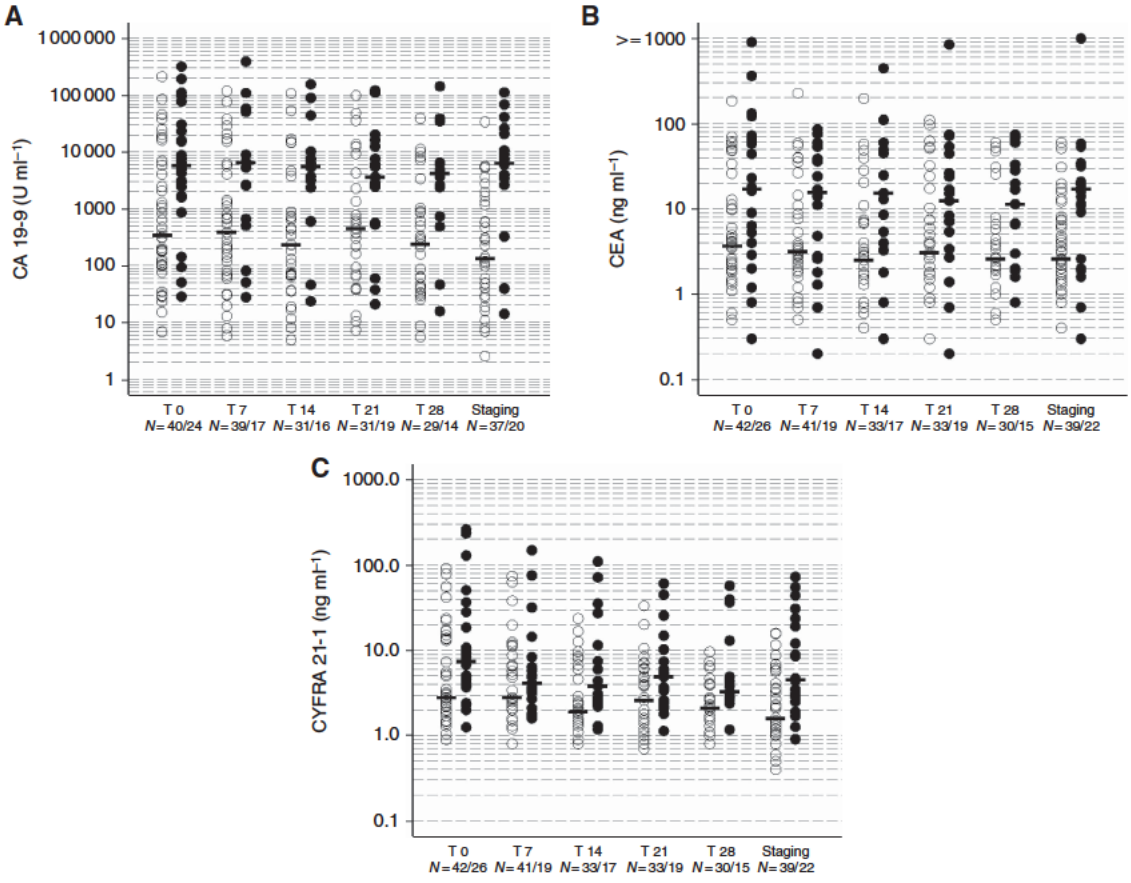

Figure 1. Levels at the time points (T) day 0, 7, 14, 21, 28 and at staging (day 56) in non progressive and progressive patients (dots with medians) for CA 19 9 (**A**), CEA (**B**) and CYFRA 21 1 (**C**) (n 68) (Full dots indicating progressive, empty dots non progressive patients).

Table 2. Correlation of absolute median biomarker levels (Tx) and marker kinetics (Tx T0%) with objective response to chemotherapy ($n=68$)

		PD			Non progressive disease (CR+PR+SD)			*P* value	
Marker	Time (days)	*n*	T_x	T_x T_0%	*n*	T_x	T_x T_0%	T_x	T_x T_0%
CYFRA 21 1 (ng ml^{-1})									
	0	26	7.5	—	42	2.8	—	0.011	—
	7	19	4.1	31.9	41	2.8	8.8	0.262	0.040
	14	17	3.8	32.4	33	1.9	31.6	0.017	0.728
	21	20	5.1	31.6	33	2.6	40.0	0.021	0.267
	28	15	3.3	43.1	30	2.1	28.9	0.005	0.727
	S	23	4.6	20.8	39	1.6	39.1	<0.001	0.311
CEA (ng ml^{-1})									
	0	26	17.1	—	42	3.7	—	0.008	—
	7	19	15.6	12.5	41	3.2	10.2	0.078	0.313
	14	17	15.4	0.0	33	2.5	22.1	0.007	0.037
	21	20	10.4	6.8	33	3.1	27.3	0.130	0.394
	28	15	11.4	3.3	30	2.6	26.9	0.043	0.092
	S	23	18.1	0.0	39	2.6	26.3	0.002	0.078
CA 19 9 (U ml^{-1})									
	0	24	5810.5	—	40	341.5	—	0.006	—
	7	17	6487.0	2.4	39	388.0	10.4	0.023	0.557
	14	16	5590.5	13.8	31	232.0	32.9	0.005	0.119
	21	20	3657.0	20.2	31	446.0	31.2	0.020	0.259
	28	14	4177.0	11.5	29	241.0	42.0	0.013	0.209
	S	21	6428.0	17.4	37	135.0	65.2	<0.001	<0.001

Abbreviations: CR complete remission; PD progressive disease; PR partial remission; S staging (day 56); SD stable disease; Tx marker value at a specific time point (x); Tx – T0% percentage marker decrease or increase based on the difference from baseline (T0), calculated only for patients with data available at the specific time point (*n*).

57 days after initiation of palliative chemotherapy. At the time of final analysis, 74 of the 78 study patients had experienced disease progression and 73 of 78 had died.

Clinical baseline characteristics are summarised in Table 1. Median age was 65 years (range 41 79) and most patients (65%) were diagnosed with synchronous metastatic disease at study entry. KRAS mutation status (exon 2, codon 12 and 13) was known in 36 patients (mainly in patients treated in clinical trials) and 25 patients presented with diabetes mellitus at study registration. Twenty six patients experienced early disease progression based on the 2 month staging interval, whereas 42 patients achieved primary disease control during chemotherapy (1 CR, 4 PR and 37 SD; Table 1). Median TTP for all 78 patients was estimated at 3.9 months (95% CI 2.3 5.3) and median OS at 7.7 months (95% CI 6.3 10.0).

Pre therapeutic biomarker levels. Median baseline CA19 9 levels differed significantly between metastatic and locally advanced disease (3485 *vs* 129 U ml^{-1}, $P=0.0126$) and between primary metastatic and recurrent PC (3485 *vs* 137 U ml^{-1}, $P=0.005$). Pre therapeutic values of CYFRA 21 1 could also be significantly distinguished between the three groups of primary metastatic, locally advanced and recurrent disease (7.5 *vs* 1.5 *vs* 2.6 ng ml^{-1}, $P=0.0001$). At time of study entry, CYFRA 21 1 was the only marker correlating with good or impaired KPS (KPS 90 100%: 3.6 ng ml^{-1} *vs* KPS<90%: 9.8 ng ml^{-1}, $P=0.0399$). CYFRA 21 1 levels did not differ significantly between patients with newly diagnosed diabetes mellitus and patients without impaired glucose tolerance in subgroup analyses (median 3.9 *vs* 7.4 ng ml^{-1}, $P=0.437$). None of the three assessed biomarkers correlated with the KRAS exon 2 mutation status. CYFRA 21 1, CA 19 9 and CEA showed highly significant correlations ($P<0.0001$) with each other on day 0 before onset of systemic chemotherapy (Supplementary Table S1).

Courses of biomarkers during chemotherapy. The course of the median biomarker levels for CYFRA 21 1, CA 19 9 and CEA (grouped with regard to 'progressive' *vs* 'disease control' patients) is illustrated in Figure 1 and Table 2. All three markers showed a significant correlation not only before onset but also at all time points in the course of chemotherapy (Supplementary Table S1). Both CA 19 9 subgroups (CA 19 9*all* and CA 19 9*syn*) correlated significantly with CEA (*P* values ranging from <0.0001 to 0.016 for CA19 9*all* at day 28) and CYFRA21 1 (*P* value ranging from <0.0001 to 0.003 for CA19 9*all* at day 28) at all assessed time points.

Correlation of biomarkers with response. Data from 68 evalu able patients on the correlation of absolute median biomarker levels and marker kinetics with objective response to chemotherapy are summarised in Table 2.

Baseline levels (prediction of response). Pre therapeutic median values of all three markers were significant predictors of treatment response. Baseline CA 19 9 levels in patients suffering from

Table 3a. Correlation of serial biomarker levels with TTP

	Time to progression							
Time (days)	**Median TTP (months) (CI)**	***P* (log rank)**	**Hazard ratio (CI)**	***P* (Cox)**	**C index (CI)**	**Hazard ratio (CI) (adjusted for KPS)**	***P* (Cox)**	**C index (CI)**
d0								
CYFRA 21-1		<0.001			**0.652** (0.592–0.712)			**0.713** (0.658–0.767)
<2.7	9.8 (6.0–19.0)							
2.7–10	2.2 (1.6–3.9)		3.0 (1.7–5.5)	<0.001		3.4 (1.9–6.2)	<0.001	
>10	2.7 (1.6–4.6)		3.3 (1.8–6.0)	<0.001		3.6 (1.9–6.7)	<0.001	
CEA		<0.001			0.633 (0.571–0.694)			0.695 (0.635 0.755)
<2.9	6.5 (3.9–17.9)							
2.9–15	3.8 (2.0–6.2)		1.9 (1.1–3.4)	0.026		2.4 (1.3–4.3)	0.005	
>15	2.1 (1.8–3.6)		3.0 (1.6–5.5)	<0.001		3.3 (1.8–6.2)	<0.001	
CA 19-9		0.016			0.625 (0.555–0.696)			0.675 (0.614–0.736)
<200	6.0 (3.9–17.9)							
200– 6600	3.7 (2.0–6.9)		1.8 (1.0–3.3)	0.05		2.0 (1.1–3.7)	0.022	
>6600	2.1 (1.5–3.9)		2.3 (1.3–4.3)	0.006		2.6 (1.4–4.8)	0.003	
d7								
CYFRA 21-1		0.0046			0.623 (0.553–0.694)			0.651 (0.574–0.727)
<2.5	7.1 (4.1–17.9)							
2.5–6	3.9 (2.0–6.9)		1.8 (1.0–3.3)	0.061		1.8 (0.9–3.3)	0.075	
>6	3.0 (1.6–5.0)		2.8 (1.5–5.3)	0.002		2.8 (1.5–5.4)	0.002	
CEA		0.0183			0.610 (0.535–0.684)			0.657 (0.590–0.724)
<2.6	6.9 (3.9–17.9)							
2.6–14	4.1 (3.0–6.2)		1.3 (0.7–2.5)	0.344		1.7 (0.9–3.1)	0.122	
>14	2.1 (1.8–5.0)		2.4 (1.3–4.4)	0.007		2.7 (1.4–5.1)	0.002	
CA 19-9		0.0021			**0.639** (0.564–0.713)			**0.670** (0.609–0.731)
<200	6.2 (3.9–19.0)							
200–6400	5.2 (2.7–7.6)		1.6 (0.8–3.0)	0.167		1.8 (0.9–3.5)	0.072	
>6400	2.1 (1.6–3.9)		3.1 (1.6–6.1)	0.001		3.4 (1.7–6.7)	0.001	
d14								
CYFRA 21-1		0.0081			**0.646** (0.575–0.718)			0.655 (0.579–0.732)
<1.9	10.3 (4.6–21.4)							
1.9–6	3.9 (1.6–6.2)		1.9 (1.0–3.7)	0.055		2.3 (1.2–4.7)	0.018	
>6	2.8 (1.2–5.3)		2.9 (1.4–5.9)	0.003		3.2 (1.6–6.7)	0.001	
CEA		0.0017			0.626 (0.549–0.703)			**0.658** (0.583–0.733)
<2.1	8.0 (3.4–17.9)							
2.1–11	6.1 (3.7–9.7)		0.9 (0.5–1.8)	0.80		1.1 (0.5–2.2)	0.849	
>11	1.8 (1.3–3.6)		2.7 (1.4–5.4)	0.003		3.0 (1.5–6.1)	0.002	
CA 19-9		0.0232			0.636 (0.556–0.717)			0.650 (0.572–0.728)
<150	6.1 (3.7–19.0)							
150–4000	5.6 (1.9–9.7)		1.4 (0.7–2.8)	0.346		1.4 (0.7–2.7)	0.387	
>4000	1.9 (1.2–3.9)		2.6 (1.3–5.2)	0.009		2.5 (1.2–5.2)	0.011	
d21								
CYFRA 21-1		<0.001			**0.674** (0.605–0.743)			**0.716** (0.652–0.780)
<2.1	9.7 (4.6–19.0)							
2.1–6	3.4 (2.0–4.6)		2.7 (1.3–5.4)	0.005		2.7 (1.3–5.5)	0.005	
>6	2.1 (1.4–4.6)		4.7 (2.2–9.9)	<0.001		3.9 (1.8–8.3)	0.001	
CEA		0.1314			0.578 (0.501–0.654)			0.685 (0.623–0.748)
<2.5	5.3 (2.5–11.9)							
2.5–9	4.0 (1.5–6.9)		1.1 (0.6–2.2)	0.742		1.1 (0.6–2.1)	0.778	
>9	2.2 (1.8–5.0)		1.9 (1.0–3.6)	0.064		1.7 (0.9–3.3)	0.132	

Table 3a. (Continued)

Time to progression								
Time (days)	Median TTP (months) (CI)	*P* (log rank)	Hazard ratio (CI)	*P* (Cox)	C index (CI)	Hazard ratio (CI) (adjusted for KPS)	*P* (Cox)	C index (CI)
CA 19-9		0.0492			0.615 (0.529–0.70)			0.687 (0.626–0.748)
<310	5.9 (3.4–11.9)							
310–4000	3.9 (1.8–5.3)		1.9 (0.9–3.7)	0.071		1.6 (0.8–3.2)	0.200	
>4000	2.1 (1.6–4.6)		2.3 (1.1–4.5)	0.022		1.8 (0.9–3.7)	0.120	
d28								
CYFRA 21-1		0.0674			**0.623** (0.544–0.703)			0.637 (0.554–0.719)
<2.1	6.2 (3.7–19.0)							
2.1–3.8	3.9 (1.6–5.8)		1.6 (0.8–3.3)	0.169		1.9 (0.9–4.0)	0.077	
>3.8	2.3 (1.6–5.3)		2.3 (1.1–4.6)	0.025		2.3 (1.1–4.7)	0.024	
CEA		0.1012			0.574 (0.485–0.662)			0.639 (0.552–0.726)
<2.0	5.3 (1.6–9.7)							
2.0–6	5.0 (2.3–11.5)		0.7 (0.3–1.5)	0.378		0.6 (0.3–1.4)	0.235	
>6	2.3 (1.8–5.1)		1.6 (0.8–3.2)	0.216		1.5 (0.7–3.1)	0.265	
CA 19-9		0.0961			0.621 (0.532–0.710)			**0.661** (0.582–0.741)
<100	5.9 (3.4–19.0)							
100–2500	5.0 (2.3–7.1)		1.6 (0.8–3.3)	0.217		1.7 (0.8–3.6)	0.150	
>2500	2.1 (1.6–3.9)		2.2 (1.1–4.6)	0.035		2.4 (1.1–5.1)	0.024	
d56								
CYFRA 21-1		<0.001			0.672 (0.613–0.731)			0.697 (0.636–0.759)
<1.7	7.1 (6.0–12.0)							
1.7–5	2.6 (1.8–5.8)		2.0 (1.1–3.7)	0.024		2.4 (1.3–4.4)	0.007	
>5	2.1 (1.4–3.6)		3.2 (1.7–6.1)	<0.001		3.0 (1.6–5.7)	0.001	
CEA		<0.001			0.641 (0.572–0.710)			**0.725** (0.664–0.786)
<2.5	7.0 (4.6–17.9)							
2.5–13	5.5 (2.7–7.6)		1.5 (0.8–2.8)	0.173		1.3 (0.7–2.4)	0.394	
>13	2.0 (1.6–3.6)		3.3 (1.7–6.3)	<0.001		2.9 (1.5–5.7)	0.002	
CA 19-9		<0.001			**0.688** (0.625–0.751)			0.719 (0.663–0.776)
<120	8.4 (3.7–19.0)							
120–3960	5.8 (2.7–7.7)		1.5 (0.8–2.9)	0.205		1.6 (0.8–3.1)	0.144	
>3960	1.9 (1.4–2.1)		4.6 (2.3–9.3)	<0.001		4.4 (2.1–8.9)	<0.001	

Abbreviations: CA 19-9 Carbohydrate antigen 19-9; CEA carcinoembryonic antigen; CI 95% confidence interval; d day; KPS Karnofsky performance status; TTP time to progression.
The bold entries indicate the highest C index (assessed at each specific time point for all 3 markers.

progressive disease (median 5811 U ml^{-1}, range 29 320 000) were elevated compared with patients with non progressive disease (median 342 U ml^{-1}, range 7 214 000, $P = 0.006$). Median CEA baseline levels were higher in progressive (17.1 ng ml^{-1}, range 0.3 908.0) than in non progressive cases (3.7 ng ml^{-1}, range 0.5 185, $P = 0.008$) and CYFRA 21 1 levels were also significantly higher in progressive patients (median 7.5 ng ml^{-1}, range 1.3 263) than in patients with disease control (median 2.8 ng ml^{-1}, range 0.9 91.5, $P = 0.01$).

Marker levels during the first 2 months of chemotherapy (early estimation of response). Absolute levels of CYFRA 21 1, CA 19 9 and CEA showed an excellent correlation with treatment response at almost all assessed time points (see Table 2). On day 14, CEA kinetics could best distinguish between the two response groups (0% decrease in the progressive *vs* 22% decrease in the non progressive group, $P = 0.037$). At the time of staging (day 56), absolute levels of each analysed marker again showed a significant correlation with objective response. Median CA 19 9 levels in patients suffering from progressive disease (6428 U ml^{-1}, range 14 113 000) were exceedingly higher than those in patients with disease control (135 U ml^{-1}, range 2.6 34 047, $P < 0.0001$). A similar discriminatory power was detected for CYFRA 21 1 on day 56 (4.6 *vs* 1.6 ng ml^{-1}, $P < 0.001$).

Correlation of biomarkers with prognosis. The impact of clinical parameters on efficacy end points is shown in Supplementary Table S2. As expected, KPS was significantly associated with both TTP and OS ($P < 0.001$), and stage of disease was correlated with OS ($P = 0.03$). KPS had the highest C index in this model, with 0.611 (95% CI 0.555 0.668) for TTP and 0.619 (95% 0.562 0.676) for OS, indicating KPS as the clinical variable with the strongest impact on outcome.

Correlation of baseline biomarker values and of levels during therapy with outcome in univariate analysis. For this complex analysis, all biomarker levels on day 0, 7, 14, 21, 28 and 56 were first categorised into tertiles to ensure equal distribution of patient numbers (see Tables 3a and 3b). Concerning baseline levels, all three markers had a significant impact on TTP and OS. Tables 3a and 3b show data analysed by the log rank test, by an (unadjusted) univariate Cox model and by a Cox model that was adjusted for

Table 3b. Correlation of serial biomarker levels with OS

Overall survival								
Time (days)	**Median OS (months) (CI)**	***P* (log rank)**	**Hazard ratio (CI)**	***P* (Cox)**	**C index (CI)**	**Hazard ratio (CI) (adjusted for KPS)**	***P* (Cox)**	**C index (CI)**
d0								
CYFRA 21-1		<0.001			**0.693** (0.637–0.748)			**0.746** (0.694–0.799)
<2.7 2.7–10 >10	14.8 (8.4–23.7) 7.1 (5.4–9.5) 4.8 (3.2–6.3)		2.9 (1.6–5.4) 5.0 (2.6–9.5)	0.001 <0.001		3.1 (1.6–5.8) 5.5 (2.9–10.8)	0.001 <0.001	
CEA		<0.001			0.618 (0.553–0.684)			0.693 (0.624–0.761)
<2.9 2.9–15 >15	12.9 (8.2–23.7) 7.1 (4.4–8.4) 6.3 (4.6–8.2)		2.6 (1.4–4.7) 3.0 (1.6–5.7)	0.002 0.001		3.1 (1.7–5.8) 3.1 (1.6–5.9)	<0.001 0.001	
CA 19-9		<0.001			0.660 (0.589–0.731)			0.709 (0.647–0.772)
<200 200–6600 >6600	14.2 (9.4–24.3) 7.1 (5.8–8.3) 5.2 (2.9–7.7)		3.0 (1.6–5.7) 3.6 (1.9–6.9)	0.001 <0.001		3.1 (1.6–6.0) 4.3 (2.2–8.5)	0.001 <0.001	
d7								
CYFRA 21-1		<0.001			**0.683** (0.623–0.744)			**0.713** (0.647–0.780)
<2.5 2.5–6 >6	14.2 (8.4–23.3) 9.4 (6.6–10.3) 5.0 (3.2–7.6)		2.2 (1.1–4.1) 4.6 (2.3–9.0)	0.021 <0.001		2.1 (1.1–4.1) 4.8 (2.4–9.6)	0.028 <0.001	
CEA		0.021			0.607 (0.535–0.680)			0.679 (0.607–0.750)
<2.6 2.6–14 >14	11.9 (8.2–19.0) 8.3 (7.1–10.8) 6.4 (4.7–8.2)		1.4 (0.8–2.5) 2.4 (1.3–4.6)	0.296 0.007		1.8 (0.9–3.3) 3.0 (1.5–5.9)	0.084 0.001	
CA 19-9		<0.001			0.658 (0.585–0.731)			0.705 (0.641–0.769)
<200 200–6400 >6400	14.8 (9.4–24.3) 7.7 (6.4–11.9) 6.0 (3.0–9.5)		2.1 (1.1–4.0) 3.9 (1.9–8.0)	0.028 <0.001		2.6 (1.3–5.1) 4.2 (2.1–8.7)	0.006 <0.001	
d14								
CYFRA 21-1		<0.001			**0.677** (0.610–0.745)			**0.687** (0.615–0.759)
<1.9 1.9–6 >6	16.1 (8.4–23.3) 10.1 (6.2–14.8) 5.9 (3.5–7.6)		1.6 (0.8–3.1) 4.4 (2.1–9.2)	0.197 <0.001		1.9 (0.9–3.8) 4.8 (2.3–10.4)	0.084 <0.001	
CEA		0.0223			0.609 (0.531–0.686)			0.650 (0.575–0.725)
<2.1 2.1–11 >11	12.9 (7.0–19.0) 9.4 (7.1–17.9) 6.4 (4.7–8.3)		1.1 (0.6–2.2) 2.4 (1.2–4.9)	0.787 0.013		1.3 (0.6–2.7) 2.6 (1.3–5.4)	0.455 0.008	
CA 19-9		0.0032			0.648 (0.572–0.724)			0.671 (0.593–0.749)
<150 150–4000 >4000	15.5(8.2–25.0) 10.3 (6.8–13.9) 6.4 (3.6–8.3)		1.9 (0.9–3.8) 3.5 (1.6–7.4)	0.090 0.001		1.9 (0.9–3.9) 3.5 (1.6–7.4)	0.078 0.002	
d21								
CYFRA 21-1		<0.001			**0.684** (0.621–0.748)			**0.733** (0.671–0.795)
<2.1 2.1–6 >6	11.9 (7.1–19.0) 7.1 (5.4–10.0) 4.6 (2.0–5.8)		1.5 (0.8–2.9) 4.2 (2.1–8.6)	0.251 <0.001		1.4 (0.7–2.8) 3.5 (1.7–7.3)	0.291 0.001	
CEA		0.1853			0.581 (0.510–0.653)			0.701 (0.637–0.766)
<2.5 2.5–9 >9	10.2 (5.6–16.1) 7.1 (3.0–10.8) 5.9 (3.6–8.2)		1.3 (0.7–2.5) 1.9 (0.9–3.7)	0.464 0.073		1.2 (0.6–2.4) 2.0 (1.0–3.9)	0.514 0.050	

Table 3b. (Continued)

	Overall survival							
Time (days)	Median OS (months) (CI)	*P* (log rank)	Hazard ratio (CI)	*P* (Cox)	C index (CI)	Hazard ratio (CI) (adjusted for KPS)	*P* (Cox)	C index (CI)
CA 19-9		0.0104			0.644 (0.569–0.720)			0.722 (0.656–0.787)
<310	11.4(7.1–19.0)							
310–4000	7.0 (4.6–10.2)		2.0 (1.0–4.1)	0.046		1.9 (1.0–3.9)	0.066	
>4000	5.8 (3.0–6.6)		2.9 (1.4–5.9)	0.004		2.4 (1.2–5.0)	0.020	
d28								
CYFRA 21-1		0.0096			**0.637** (0.552–0.722)			0.651 (0.564–0.739)
<2.1	11.9 (6.8–19.0)							
2.1–3.8	8.3 (6.2–11.3)		1.2 (0.6–2.4)	0.663		1.3 (0.6–2.6)	0.499	
>3.8	5.8 (3.0–7.7)		2.8 (1.3–5.8)	0.006		2.9 (1.4–6.1)	0.005	
CEA		0.2343			0.545 (0.446–0.644)			0.621 (0.571–0.725)
<2.0	9.4 (5.4–11.9)							
2.0–6	9.25 (4.6–23.3)		0.7 (0.3–1.4)	0.264		0.6 (0.3–1.2)	0.161	
>6	6.8 (5.8–8.4)		1.3 (0.6–2.6)	0.537		1.2 (0.6–2.4)	0.665	
CA 19-9		0.0487			0.616 (0.526–0.705)			**0.662** (0.585–0.738)
<100	13.3 (5.6–24.3)							
100–2500	7.4 (4.7–10.8)		1.7 (0.8–3.6)	0.143		1.9 (0.9–4.0)	0.091	
>2500	6.4 (4.6–7.7)		2.5 (1.2–5.5)	0.017		2.5 (1.1–5.4)	0.021	
d56								
CYFRA 21-1		<0.001			0.681 (0.620–0.743)			0.714 (0.648–0.779)
<1.7	10.8 (8.2–17.9)							
1.7–5	9.8 (6.3–11.9)		1.2 (0.7–2.3)	0.492		1.4 (0.8–2.7)	0.259	
>5	4.7 (2.0–6.0)		3.7 (1.9–6.9)	<0.001		3.3 (1.7–6.3)	<0.001	
CEA		0.0075			0.630 (0.562–0.699)			0.731 (0.666–0.795)
<2.5	12.9 (8.2–19.0)							
2.5–13	7.7 (4.7–10.3)		1.9 (1.0–3.5)	0.037		1.6 (0.8–3.0)	0.156	
>13	6.3 (3.6–7.7)		2.7 (1.4–5.2)	0.003		2.3 (1.2–4.5)	0.012	
CA 19-9		<0.001			**0.699** (0.638–0.760)			**0.742** (0.679–0.805)
<120	14.5 (8.2–24.3)							
120–3960	7.6 (5.8–10.8)		2.5 (1.3–5.0)	0.009		2.8 (1.4–5.6)	0.004	
>3960	5.8 (2.0–6.8)		5.3 (2.5–11.0)	<0.001		5.0 (2.4–10.6)	<0.001	

Abbreviations: CA 19-9 Carbohydrate antigen 19-9; CEA carcinoembryonic antigen; CI 95% confidence interval; d day; KPS Karnofsky performance status; OS overall survival. The bold entries indicate the highest C index (assessed at each specific time point for all 3 markers).

KPS. With HR ranging from 3.0 (unadjusted) to 3.6 (adjusted), pre treatment CYFRA 21 1 levels had the strongest impact on the end point TTP. This is also reflected by the C index of 0.652 (unadjusted) and 0.713 (adjusted), respectively (see Table 3a). Similar data were observed in the OS analysis, where baseline CYFRA 21 1 had the strongest influence in patient survival (Table 3b). The C index for CYFRA 21 1 was most favourable on days 0, 7, 14, 21 and 28 (unadjusted only). On day 56 (staging), CA 19 9 had the strongest impact on OS with a C index of 0.699 and 0.742, respectively. The Kaplan Meier plots for the correlation of baseline CA 19 9, CEA and CYFRA 21 1 levels with TTP and OS are shown in Figure 2 (TTP) and Figure 3 (OS).

Biomarker levels of CYFRA 21 1, CA 19 9 and CEA were also analysed as continuous variables (after logarithmic transforma tion), as this may potentially represent a more adequate method for the evaluation of a quantitative variable with a broad range (Boeck *et al*, 2010). Results of these investigations (*P* values only) are summarised in Table 4. For univariate analysis on TTP and OS, the absolute marker values (Tx) as well as the marker kinetics in relation to the pre treatment baseline level (Tx − T0) are presented. CYFRA 21 1 showed a strong correlation with TTP and OS at all assessed time points in the univariate evaluation ($P<0.0001$, for details see Table 4). Absolute levels of CA 19 9 had an equal predictive power for TTP as well as OS, whereas CEA showed a lack of statistical significance at only a few time points (day 21 for TTP and day 28 for TTP and OS, see Table 4). CA 19 9 kinetics were significantly correlated with TTP and OS only at time of staging (day 56). However, marker kinetics generally proved to be not as powerful as the absolute marker levels in determining prognosis.

Multivariate analysis. The significant association of CYFRA 21 1 as continuous variable with OS was maintained in the multivariate analysis, confirming the independent prognostic role of CYFRA 21 1 (Table 4). Of note, again only the absolute marker values of CYFRA 21 1 but not marker kinetics had a significant impact on OS. Within a second multivariate Cox model including all clinical and biomarker variables (categorised as tertiles) with a *P* value <0.1 in univariate analysis ($n=78$), only KPS (HR 3.3, 95% CI 1.7 6.5, $P=0.0003$) and pre treatment CYFRA 21 1 (tertile 2: HR 2.4, 95% CI 1.1 5.3, $P=0.027$; tertile 3: HR 4.0, 95% CI 1.7

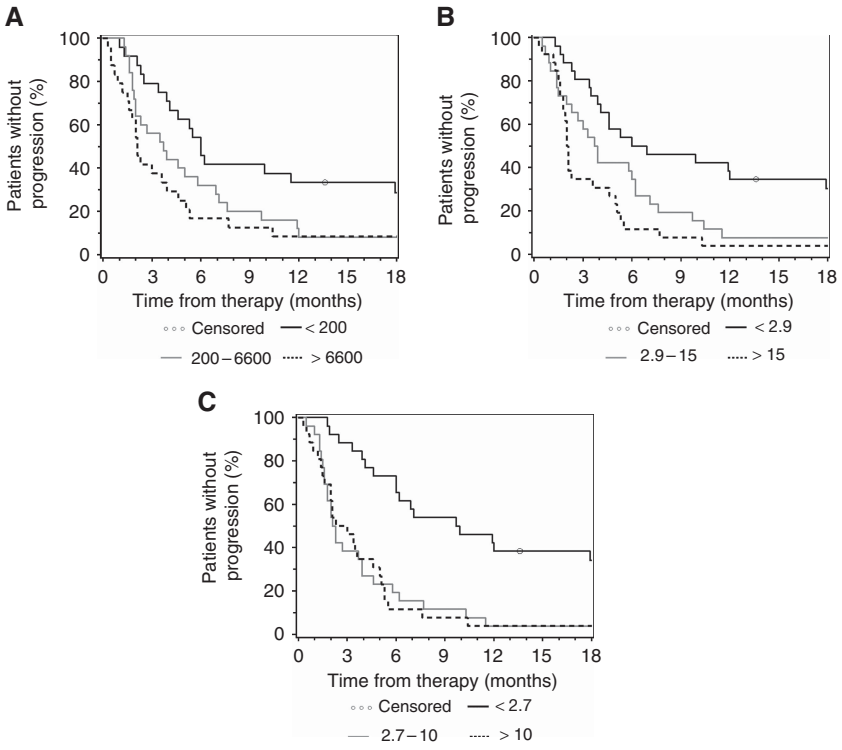

Figure 2. Kaplan Meier curves for TTP based on pre therapeutic (**A**) CA 19 9 (U ml ^{1}), (**B**) CEA (ng ml ^{1}) and (**C**) CYFRA 21 1 levels (ng ml ^{1}) (categorised by tertiles).

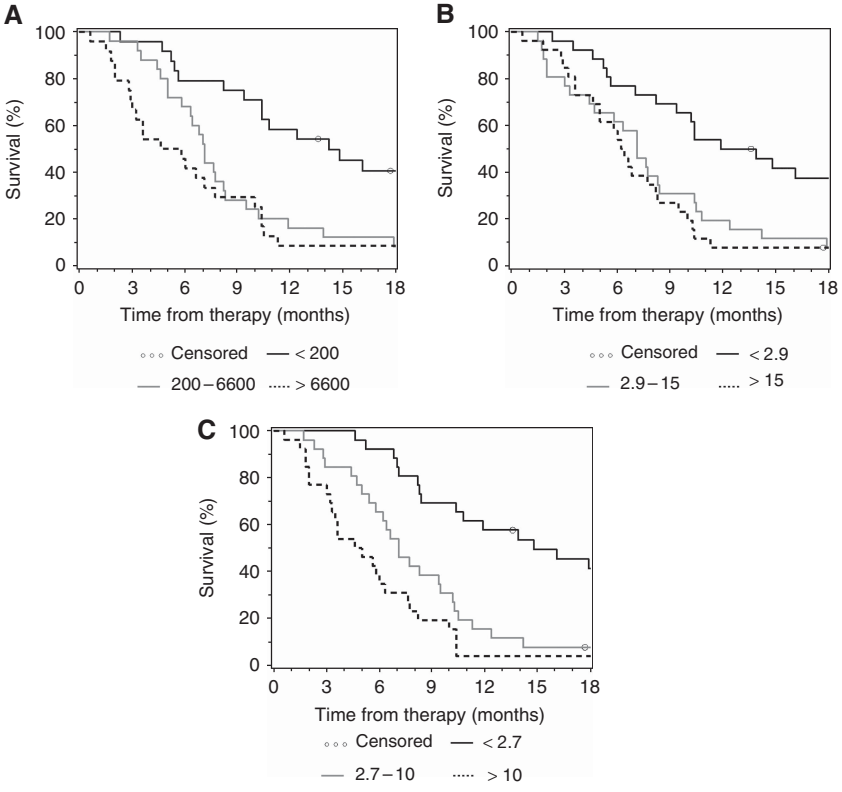

Figure 3. Kaplan Meier curves for OS based on pre therapeutic (**A**) CA 19 9 (U ml ^{1}), (**B**) CEA (ng ml ^{1}) and (**C**) CYFRA 21 1 levels (ng ml ^{1}) (categorised by tertiles).

9.7, $P = 0.002$) but not CA 19 9 or CEA retained their independent prognostic significance for OS. The C index for that model was 0.769 (95% CI 0.716 0.822). When this model was re analysed with only CA 19 9*syn* patients ($n = 73$), the corresponding C index was 0.776 (95% CI 0.724 0.828), and apart from KPS (HR 3.4, 95% CI 1.7 6.8, $P = 0.0005$) and CYFRA 21 1 (tertile 2: HR 3.1, 95% CI 1 3 7.2, $P = 0.0091$; tertile 3: HR 5.1, 95% CI 1.9 13.7, $P = 0.0013$), also CA 19 9*syn* (tertile 2: HR 2.6, 95% CI 1.2 5.6, $P = 0.0144$, tertile 3: NS) became statistically significant for the end point OS.

Table 4. Uni and multivariate analyses for correlation of biomarkers (each analysed as continuous variable; (Tx) indicates the absolute value, (Tx T_0) the marker kinetics in relation to the pre treatment baseline level) with outcome

		Univariate time to progression		Univariate overall survival		Multivariate overall survival	
Marker	**Time (days)**	***P* (Tx)**	***P* (Tx T_0)**	***P* (Tx)**	***P* (Tx T_0)**	***P* (Tx)**	***P* (Tx T_0)**
CYFRA 21 1							
	0	<0.001	—	<0.001	—	<0.001	—
	7	<0.001	0.131	<0.001	0.130	<0.001	0.542
	14	<0.001	0.262	<0.001	0.123	<0.001	0.641
	21	<0.001	0.995	<0.001	0.261	<0.001	0.761
	28	<0.001	0.018	<0.001	0.006	0.014	0.171
	Staging	<0.001	0.907	<0.001	0.413	<0.001	0.673
CEA							
	0	<0.001	—	<0.001	—	0.013	—
	7	0.002	0.043	0.002	0.037	0.051	0.450
	14	0.002	0.763	0.003	0.476	0.064	0.612
	21	0.062	0.260	0.043	0.207	0.273	0.954
	28	0.198	0.104	0.542	0.045	0.978	0.206
	Staging	<0.001	0.321	<0.001	0.236	0.036	0.006
CA 19 9							
	0	<0.001	—	<0.001	—	0.005	—
	7	0.001	0.919	<0.001	0.367	0.013	0.891
	14	0.002	0.229	<0.001	0.939	0.023	0.456
	21	0.002	0.154	<0.001	0.880	0.027	0.760
	28	0.012	0.173	0.008	0.473	0.145	0.590
	Staging	<0.001	<0.001	<0.001	0.003	<0.001	0.001

Abbreviations: CA 19-9 Carbohydrate antigen 19-9; CEA carcinoembryonic antigen. *P*-values by uni- and multivariate Cox model; *n* 78.

DISCUSSION

To date, CA 19 9 is the only established and validated serum biomarker in PC that also is applied routinely in daily clinical practice. A broad variety of other potential biomarkers is currently under investigation, for example, genetic (tissue) biomarkers, epigenetic markers and blood markers including circulating tumour cells (Bhat *et al*, 2012). Unfortunately, there is still no biomarker available predicting the benefits of a specific treatment (e.g., chemotherapy or targeted therapy) in advanced PC. Early evidence suggests that the tumour KRAS mutational status (for erlotinib treatment) or VEGF pathway genetic variants (for bevacizumab treatment) may serve as such predictive markers. However, these translational data still require prospective validation (Costello *et al*, 2012; Lambrechts *et al*, 2012; Boeck *et al*, 2013).

Thus, the scientific rationale still exists to study other serum tumour markers besides CA 19 9 in order to obtain an easy determinable biomarker that provides prognostic information and also allows patient stratification, for example, within the setting of a clinical trial. Based on data from this prospective single centre study, CYFRA 21 1 could possibly serve as such a biomarker in advanced PC. We could show that pre treatment CYFRA 21 1 levels are significantly correlated with TTP and OS, and that CYFRA 21 1 may also predict treatment response to chemother apy. As patients with high CYFRA 21 1 values before the initiation of palliative chemotherapy are less likely to achieve objective disease control, a more intensive treatment (e.g., with the FOLFIRINOX regimen) might be considered in such a poor prognosis patient population (Heinemann *et al*, 2012). Interest ingly, the main determinant for response was the absolute CYFRA 21 1 level (at any of the assessed time points) rather than the kinetics during chemotherapy (see Table 2); an observation that also holds true for CA 19 9 and CEA. When CYFRA 21 1 was analysed as continuous variable the strongest prognostic informa tion was again based on the absolute CYFRA 21 1 values at each assessed time point and not on the marker kinetics during treatment (see Table 4).

Most importantly, CYFRA 21 1 was shown to be independent of CA 19 9 in multivariate analysis, and was apart from KPS the only significant prognostic factor for OS in a multivariate Cox model. Based on the determined C indices from our study, it may be postulated that for end point OS CYFRA 21 1 is a potentially more powerful prognostic factor than CA 19 9 (Table 3b). Of note, the C index for our multivariate Cox model that included all biomarker data as categorised variable was 0.769, whereas when only CA 19 9*syn* patients were included in this model, the C index slightly increased up to 0.776 and CA 19 9*syn* remained significant for OS. Thus, it could be concluded that an additional prognostic information from CA 19 9 is potentially only obtained when patients able the synthesise CA 19 9 are considered. Furthermore, CYFRA 21 1 offers the opportunity to serve as novel serum tumour marker in PC patients who are Lewis antigen negative (about 5 10%) or who present significant cholestasis conditions that are well known limitations for the application of CA 19 9 (Boeck *et al*, 2006).

Also, only limited data exist on the role of CEA (a widely used tumour marker in colorectal cancer) in PC (Duffy *et al*, 2010). Based on the data from this study, CEA may also have a role as tumour marker in pancreatic malignancies because CEA levels at baseline and at staging (day 56) were significantly correlated with objective response and also yielded prognostic information regarding TTP and OS in univariate analysis (Tables 2 4).

The strength of the current biomarker study is based on its prospective design (according to the REMARK guidelines) with the serial (weekly) assessment of marker values throughout the course of the first 2 months of first line palliative chemotherapy and the use of a unique assay for a centralised marker determination. The investigated patient cohort represents a population comparable to other study populations from PC trials with a median OS of 7.7 months (Table 1). Notably, 45% of the included patients were treated within a prospective clinical study. The main limitation arises from the fact that this study was conducted at a single high volume German Cancer Centre and not within a multicentre setting. Thus, an external validation of these provocative novel data on CYFRA 21 1 within a prospective multicentre investigation is recommended, ideally conducted as translational biomarker study accompanying a clinical trial.

In conclusion, CYFRA 21 1 serves as a novel, potent serum biomarker in PC providing independent prognostic information. If other prospective multicentre trials confirm these data, CYFRA 21 1 may have a relevant role in pre therapeutic prognostic models of clinical factors and laboratory parameters that support patient stratification and may be used for the application of different treatment strategies.

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CONFLICT OF INTEREST

PS and SH receive honoraria for conference lectures from Roche Diagnostics. The remaining authors declare no conflict of interest.

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Circulating nucleosomes and immunogenic cell death markers HMGB1, sRAGE and DNAse in patients with advanced pancreatic cancer undergoing chemotherapy

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Serum biomarkers are urgently needed for patient stratification and efficient treatment monitoring in pancreatic cancer (PC). Within a prospective diagnostic observation study, blood samples were obtained from 78 patients with advanced PC before and weekly during the course of palliative chemotherapy. Circulating nucleosomes and immunogenic cell death markers, high-mobility group box 1 (HMGB1), soluble receptors of advanced glycation end products (sRAGE) and DNAse activity, were measured by enzyme-linked immunosorbent assay and correlated with results of radiological staging after 2 months of treatment, with time to progression (TTP) and overall survival (OS). Median TTP and OS of PC patients were 3.9 and 7.7 months, respectively. Pretherapeutic baseline biomarker levels did not correlate with objective response; however, nucleosome levels on day (d) 28 were higher ($p = 0.048$) and sRAGE levels at time of staging (d56) were lower in progressive patients ($p = 0.046$). Concerning estimation of prognosis, high nucleosome levels (d7, d14, d21 and d56), low sRAGE levels (d56) and DNAse activity courses (d0–d7) correlated with TTP, whereas high nucleosomes (d7, d14 and d56), high HMGB1 (d21 and d56) and DNAse (d0–d7) were associated with OS. After adjustment to Karnofsky performance score, nucleosomes and HMGB1 (both d56) and DNAse (d0–d7) remained independent prognostic factors. Thus, courses of circulating nucleosomes and immunogenic cell death markers HMGB1 and sRAGE show prognostic relevance in PC patients undergoing chemotherapy.

Tumor Immunology

Pancreatic cancer (PC) is known for its unfavorable prognosis with a 5 year survival rate of only 6% making it to the fourth leading cause of cancer death in the United States and 11th leading cause of cancer death worldwide (WHO database 2008). At time of diagnosis more than 80% of PC patients are already in an advanced stage of disease that impairs the potentially curative surgical resection.[1,2] In those palliative situations, systemic chemotherapy with single agent gemcitabine or gemcitabine based combinations (by adding the oral epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor erlotinib, the oral fluoropyrimidine capecitabine or platinum analogs like oxaliplatin and cisplatin to gemcitabine) is regarded as an international standard of care.[3–5]

Standard response evaluation criteria in solid tumors (RECIST) are defined according the change of tumor size in radiological staging techniques such as computed tomography (CT). However, there have been debates on its relevance because of well known interinvestigator differences, misinterpretations of tumor shrinkage in only one dimension, tumor activity changes irrespective of tumor size and, particularly in PC, nonspecific stroma reactions and inflammation that may complicate an accurate interpretation.[6,7] Therefore, estimation

Key words: nucleosomes, HMBG1, sRAGE, DNAse activity, pancreatic cancer, prognosis

Abbreviations: CA 19 9: cancer antigen 19 9; CEA: carcinoembryonic antigen; CT: computed tomography; CYFRA 21 1: cytokeratin 19 fragments; DAMP: damage associated molecular pattern; DNAse: desoxyribonuclease; EGFR, epidermal growth factor receptor ELISA: enzyme linked immunosorbent assay; HMGB1: high mobility group box 1; KPS: Karnofsky performance status; MRT: magnetic resonance tomography; OS: overall survival; RECIST: response evaluation criteria in solid tumors; sRAGE: receptor of advanced glycation end products; TTP: time to progression; SGA: small for gestational age

Additional Supporting Information may be found in the online version of this article.

Conflict of interest: PS and SH received honoraria for conference lectures from Roche Diagnostics.

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What's new?

Serum biomarkers are urgently needed for prognosis and treatment monitoring in pancreatic cancer (PC). In this study, the authors assessed biomarkers of cell death that are known to affect the immune response against tumor cells. They found that high serum levels of nucleosomes and the protein HMGB1 (high mobility group box-), as well as low serum levels of sRAGE (soluble receptor of advanced glycation end products), were associated with a poorer response to therapy and poorer prognosis. These indicators may therefore provide useful tools for early estimation of therapeutic response and prognosis in advanced PC.

of prognosis in terms of time to progression (TTP) and overall survival (OS) remains important endpoints for clinicians for therapeutic decision making. In addition, an early and specific estimation of therapy response already during the first weeks of chemotherapy would be desirable to early identify nonresponders, to move to a potentially more efficient therapy and to reduce unnecessary side effects. Those approaches would be ideally addressed by blood related biomarkers that are easy to obtain, reliable and cost effective to assess and that give objective information on the biological activity of the tumor.

Recent studies have shown the high potential of carbohydrate antigen 19 9 (CA 19 9) for therapy monitoring, recurrence detection and estimation of prognosis in PC.[8–10] Early changes of nucleosomes during the first days of chemoradiotherapy have further been shown to serve as relevant markers for prognosis in locally advanced PC.[11] In addition, nucleosomes were valuable predictors of treatment response and prognostic biomarkers in colorectal, lung, breast and liver cancers undergoing systemic or local chemotherapy or chemoradiotherapy.[12–17] In most cases of advanced cancer disease, high nucleosome levels before or during therapy were associated with poor outcome. This might arise either from higher levels of spontaneous or induced cell death or from impaired degradation in serum that, among others, is influenced by the activity of serum DNAse.[18]

Although there are plenty reports on the release of cell death biomarkers in cancer disease, little is known about their immunogenic role and their clinical implications. Stimulation of the immune system as a side effect of chemotherapeutic treatment has been demonstrated to be relevant for a sustained response to the treatment.[19,20] On the other hand, a chronic stimulation of the tumor surrounding microenvironment is known to lead to immune paralysis that facilitates tumor growth and invasiveness.[21,22] One essential mechanism is the release of damage associated molecular pattern (DAMP) molecules such as calreticulin, adenosine triphosphate or high mobility group box 1 (HMGB1) that bind to diverse surface receptors on immune cells and trigger inflammatory responses.[23]

HMGB1 is a highly conserved member of the HMG box protein family, abundantly expressed and present in virtually all human cell types. It is released passively from degrading, necrotic cells and is secreted actively by cells undergoing apoptosis or facing hypoxic conditions, as well as activated cells of the innate immune system. Once in the extracellular space, HMGB1 can bind to diverse receptors such as the receptor of advanced glycation end products (RAGE) and also to different toll like receptors (TLRs). HMGB1 induces expression and secretion of proinflammatory cytokines and improves phagocytation and cross presentation of pathogenic cell death products for T cell activation. These immune stimulatory effects are augmented if HMGB1 is complexed with nucleosomes for activation of TLR2 or with single stranded DNA for activation of TLR 9. Via RAGE and TLR 4, HMGB1 can induce maturation and homing of dendritic cells, which is important for efficient antigen presentation and for therapeutic success.[21,24] Selected knockdown or impairment of either HMGB1 or TLR 4 leads to restricted anticancer immune response in vivo and in vitro and was shown to correlate with poor therapy response in human breast cancer.[25]

The complex functions of HMGB1 in cancer development and growth show dual and opposite effects, probably depending on the microenvironmental context, state and rate of released HMGB1 as well as on the extracellular "combinatorial cocktail" of cytokines and their "spatiotemporal sequence."[26] Thus, HMGB1 is not only essential for efficient anticancer immune responses but, on the other hand, can also promote tumor growth, angiogenesis and metastasis.[27] Indeed, overexpression of HMGB1 and RAGE was shown to correlate with malignant potential such as invasion and metastasis in colorectal and gastric cancers.[28] Consistently, targeted knockdown of RAGE or HMGB1 leads to increased apoptosis and decreased viability of pancreatic tumor cells and enhances their sensitivity to anticancer chemotherapy. This is achieved via inhibition of a HMGB1/RAGE dependent pathway that sustains autophagy as an important tumor survival mechanism.[29] However, the role of soluble RAGE needs to be examined further, as sRAGE may either originate from alternative splicing of pre m RNA or from proteolytic cleavage of the membrane bound receptor and thus simply reflect high cellular RAGE concentrations. In its soluble state RAGE may even act as a decoy receptor with neutralizing effects on HMGB1 and its extracellular functions.[30]

Therefore, our prospective study was designed to assess these new promising biomarkers with multiple functions in the field of immunogenic tumor cell death to reveal their potential roles as tools for the assessment of prognosis and early monitoring of therapy response in patients with advanced PC undergoing palliative chemotherapy.

Table 1. Baseline patient characteristics

	Number	Percentage (%)
Patients	78	100
Gender		
Female	30	38
Male	48	62
Karnofsky performance status (KPS)		
100	16	21
90	42	54
80	13	17
70	6	8
60	1	1
Stage of disease at study entry		
Synchronous metastasis	51	65
Locally advanced disease	9	12
Relapse	18	23
KRAS mutation status (exon 2)		
Wild type	10	13
Mutation	26	33
Not assessed	42	54
Diabetes mellitus		
Negative	30	39
Known	13	17
Newly diagnosed at study entry	12	15
Not assessed	23	30
Best response by imaging		
CR + PR + SD	42	54
PD	26	33
Not assessed	10	13
	Median	**Range/95% CI**
Age	65 years	41 79
Time to first staging	57 days	9 176
Time to progression (TTP)	3.9 months	2.3 5.3
Overall survival (OS)	7.7 months	6.3 10.0

Abbreviations: CR: complete response; PD: progressive disease; PR: partial response; SD: stable disease.

Patient and Methods

Patient population

Within a prospective, single center observation study, 83 consec utive patients with (histologically or cytologically) confirmed advanced PC treated between May 2006 and April 2010 at the Department of Internal Medicine III of the University Hospital Munich Grosshadern were recruited. Five of these 83 patients were excluded from the evaluation because of missing baseline marker values resulting in 78 evaluated patients. At study entry, nine patients suffered from locally advanced disease, 51 from syn chronously metastasized and 18 from recurrent PC (Table 1). All patients received standard first line palliative chemotherapy (based on the decision of the treating oncologists SB and VH) with either gemcitabine plus erlotinib (n 43), single agent gem citabine (n 11), gemcitabine plus everolimus (n 11), capeci tabine plus erlotinib (n 8), gemcitabine plus axitinib (n 2), single agent capecitabine (n 2) or nab paclitaxel (n 1). Thirty five of the 78 patients (45%) were treated within prospec tive clinical trials. In all patients, blood drawings were performed directly before the initiation of chemotherapy and weekly until the first radiographic staging investigation that was generally scheduled 2 months after start of chemotherapy. Additionally, clinical characteristics, among others, Karnofsky performance status (KPS), presence of diabetes mellitus and KRAS exon 2 mutation status (codon 12 and 13) were assessed (see Table 1). The study was approved by the local Ethics Committee of the University Munich and all patients gave written informed con sent before any study specific procedure.

Classification of response to therapy and survival

Radiological staging was performed in 68 patients after a median of 57 days after start of chemotherapy treatment. Response was classified according to RECIST, version 1.0. Only in single cases, time to first imaging varied considerably (9 176 days) because of multimorbidity and/or newly developed complications. Generally, staging was performed by CT or magnetic resonance tomography (MRT). Ten patients were not evaluable for objective response by imaging; therefore, these patients did not enter into the statistical evaluation of therapy response. Applying these criteria to our study population of 68 patients, complete remission could be observed in one patient (2%) and partial remission in four patients (6%). Dis ease remained stable in 37 cases (54%) and showed signs of pro gression in 26 patients (38%). Of the remaining ten patients without radiological staging, two died before the first staging exam.

TTP was defined as the interval between the initiation of chemotherapy and the occurrence of (objective or clinical) disease progression under first line treatment; OS was defined as the time interval between the initiation of chemotherapy and death from any cause. For the evaluation of TTP and OS, all 78 patients were included of which a majority of 74 patients suffered from progression of disease and 73 have died during the follow up period.

Sample collection and assays

Blood samples were collected prospectively directly before the start of first line systemic chemotherapy and then weekly in the course of therapy until the first radiologic staging after 2 months. They were centrifuged for 15 min at 3,000g within 2 hr of collec tion. The resulting sera were aliquoted into microtubes and either immediately frozen at 80°C or previously stabilized with 10 mM EDTA (pH 8) for nucleosome measurement. After a maximum of 3 years of storage, the samples were thawed and parameters were measured in batches containing all samples of one single patient. Nucleosome concentrations were measured

by use of the Cell Death Detection ELISAplus (CDDE) of Roche Diagnostics (Mannheim, Germany) as reported previously.[31] The CDDE assay is based on the quantitative "sandwich enzyme immunoassay" principle. The serum samples are pipetted onto a streptavidin coated microplate and covered with a mixture of anti histone biotin and anti DNA peroxidase (HRP, horseradish peroxidase). Mouse monoclonal antibodies directed against DNA and histones allow for specific determination of mononu cleosomes and oligonucleosomes. Color reaction with ABTS substrate solution is then photometrically quantified in ng/mL by use of a calibration curve.[31,32]

HMGB1 levels were also assessed in a manually performed sandwich enzyme linked immunosorbent assay (ELISA) assay of IBL International GmbH (Hamburg, Germany). Soluble HMGB1 binds to these immobilized anti HMGB1 specific anti bodies and enzyme triggered substrate reaction with enzyme marked detection antibodies lead to color change, which is assessed photometrically at 450 nm (reference wavelength at 540 570 nm). In general, preparation steps and assay perform ance were done following the test's procedure instructions; dilu tions of the standard were adjusted to the "high sensitive" mode of the assay (0.31, 0.63, 1.25, 2.5, 5, 10 and 20 ng/mL). sRAGE concentrations were measured by the Quantikine Human sRAGE ELISA test kits of R&D Systems (Abingdon, UK). Fol lowing the same procedure of quantitative sandwich ELISA tech nique, the test also involves an incubation step with immobilized RAGE specific antibodies and enzyme linked polyclonal anti bodies (directed against the extracellular domain of RAGE), as well as a final colorimetric determination of sRAGE levels at 450 nm, with the reference wavelength again set at 540 570 nm. The amount of DNase present in the serum samples was measured using the solid phase ELISA of Orgentec Diagnostika GmbH (Mainz, Germany), which determines the activity reduction (%AR) of human serum DNase. Thawed serum samples are given onto microtiter plates with immobile specific DNase sub strate. During incubation time this substrate is enzymatically cleaved by any DNase contained in the sample. Next to the sub sequent washing step anti DNase substrate conjugated with HRP detects the remaining membrane bound substrate. Again the plate is washed and then TMB substrate solution (3,3′,5,5′ tetramethylbenzidine) added. The following color reaction is stopped after 15 min and the color intensity determined photo metrically at 450 nm and a reference wavelength at 600 690 nm. The assessed color intensity is inversely proportional to the DNase activity. The serum levels of tumor associated antigens CA19 9, CEA and CYFRA 21 1 were measured automatically by use of Elecsys 2010 of Roche Diagnostics.

Statistics

Levels of the four biomarkers measured in sera of PC during our study were compared with sera levels of healthy subjects who were investigated in earlier studies[33,34] by means of the Wilcoxon Mann Whitney test.

In PC patients, concentrations of all markers assessed before (day 0), and on days 7, 14, 21, 28 and 56 (staging) after start of chemotherapy as well as their differences com pared to pretherapeutic levels were considered for statistical evaluation. Significance of differences was tested using the Wilcoxon test for paired samples. Patients were divided into two groups according to their response to therapy: patients with complete remission, partial remission and stable disease were combined into the "no progression" group (n 42) in contrast to patients who suffered from progressive disease (n 26). Significance between marker levels in therapy response groups was assessed by use of the Wilcoxon Mann Whitney test. Correlations between marker levels were eval uated by means of the Spearman rank correlation coefficient.

For analysis of TTP, defined as the interval between the initiation of treatment and the occurrence of a documented disease progression, and OS, defined as the time interval between the initiation of treatment and death from any cause, all marker values were logarithmized and used as con tinuous variables in univariate Cox regression models. In addition, the KPS which was found to be the strongest clin ical predictor for outcome in our patient population was included into these models, leading to KPS adjusted p values. To illustrate results and make them more applicable, signifi cant variables of this first approach were split into tertiles and analyzed by the Kaplan Meier method and log rank tests. The Cox model was used to calculate raw and KPS adjusted hazard ratios for the tertiles. The prognostic strength of the different models was also compared by the concord ance index (C index) introduced by Harrell et al.[35] This C index mirrors the proportion of concordance in all possible pairs of patients, with concordance implying that the patient with more favorable value has the longer survival. C indices can range from 0.5 to 1.0 with higher values indicating a higher proportion of concordance. Because of the exploratory nature of the analysis adjustment for multiplicity of testing was not applied. A p value of <0.05 was considered statisti cally significant. All calculations were performed with SAS software (version 9.2, SAS Institute, Cary, NC).

Results

Pretherapeutic biomarker values

Initial sRAGE levels were significantly lower in PC patients (median 0.68 ng/mL, range 0.25 2.36 ng/mL) than in 28 healthy individuals that additionally were examined (sRAGE median 1.12 ng/mL, range 0.52 1.56 ng/mL, p 0.0001). DNAse activity was highly significantly lower [DNAse activ ity reduction (AR%) was higher] in PC patients (median 23.2 %AR, range 4.4 50.2 %AR) before initiation of therapy when compared to the healthy individuals (median 12.6 %AR, range 7.3 26.6 %AR, $p < 0.0001$). Pretherapeutic values of circulating nucleosomes (median 80.6 ng/mL, range 18.1 2288.0 ng/mL) and HMGB1 (median 2.0 ng/mL, range 0.1 39.5 ng/mL) were slightly higher in advanced PC patients when compared to the healthy controls (nucleosomes median 70.2 ng/mL, range 19.2 283.0 ng/mL; HMGB1 median 1.3 ng/mL, range 0.2 4.9 ng/mL); however, this

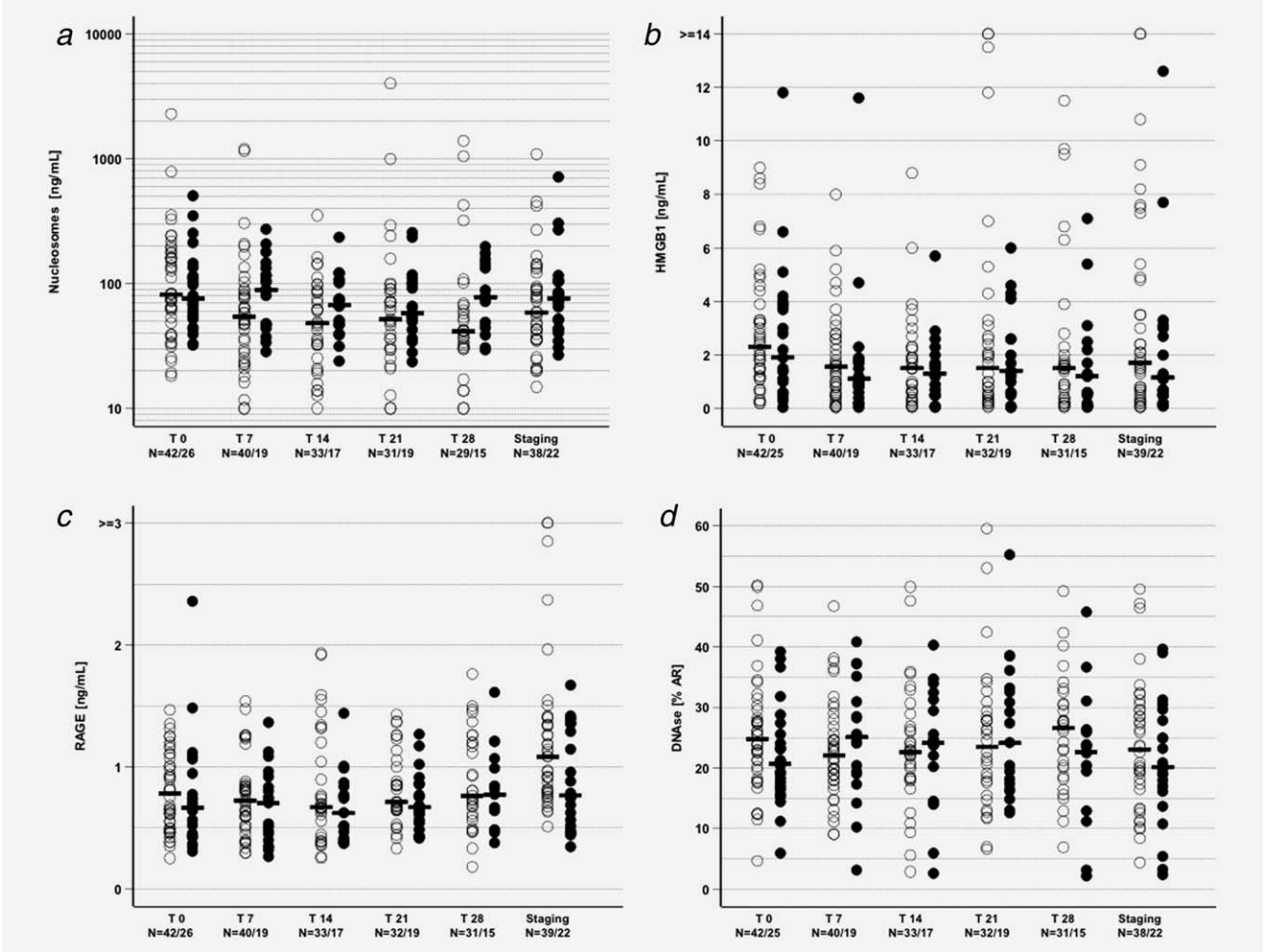

Figure 1. Marker levels at time point (T) day 0, 7, 14, 21, 28 and at staging in non progressive (empty circles with medians) and progres sive patients (filled circles with medians) for nucleosomes (A), HMGB1 (B), sRAGE (C), and DNAse activity reduction (D).

difference was not statistically significant (p 0.114 and p 0.053, respectively).

Baseline levels of all biomarkers were not significantly dif ferent in patients with metastatic or locally advanced PC. Baseline levels of all investigated markers did not correlate with the KPS at treatment initiation. In an exploratory subgroup analysis, there were neither differences of marker levels in patients with (n 25) or without (n 30) diabetes nor in patients with (n 26) and without (n 10) a KRAS exon 2 mutation (data not shown).

Courses of biomarkers during chemotherapy

As illustrated in Figure 1, weekly determined nucleosome lev els during chemotherapy decreased in nonprogressive patients, whereas they remained stable with considerable vari ation in progressive patients. DNAse activity was stable in both patient groups, whereas HMGB1 levels decreased in both patient groups. sRAGE levels were stable in progres sive patients, whereas they increased in nonprogressive patients (see Fig. 1 and Table 2). As shown in Supporting Information Table S1, HMGB1 showed a highly statistically significant correlation with circulating nucleosomes at all time points assessed (R between 0.52 and 0.73; all p values <0.0001). In addition, some minor correlations between markers were observed (Supporting Information Table S1).

Correlation of biomarkers with therapy response

In 68 patients available for evaluation of objective radio graphic response, 42 had stable disease or a remission and 26 suffered from progressive disease. With respect to immuno genic biomarkers, no significant difference was found for pre therapeutic baseline values between the two response groups (as summarized in Table 2). During most time points of the following weeks, differences of biomarker levels in both response groups were not statistically significant as well. However, nucleosome levels on d28 were significantly higher in progressive patients (median 77.6 vs. 41.6 ng/mL; p 0.048) and sRAGE levels at time of staging (d56) were lower in progressive patients (median 0.79 vs. 1.09 ng/mL; p 0.046), respectively. Of note, percentual changes in kinetic

Table 2. Correlation of median absolute biomarker levels (T_x) and marker kinetics ($T_x - T_0$, %) with objective response to chemotherapy (n 68)

		Progressive disease (PD)			Stable disease and remission (CR + PR + SD)			p value	
Marker	Time (days)	n	T_x	$T_x - T_0$, %	n	T_x	$T_x - T_0$, %	T_x	$T_x - T_0$, %
HMGB1 (ng/mL)	0	25	1.9		42	2.3		0.508	
	7	19	1.1	−33.8	40	1.6	−28.1	0.381	0.853
	14	17	1.3	−49.0	33	1.5	−53.3	0.806	0.862
	21	20	1.4	0.0	32	1.5	−38.2	0.895	0.714
	28	15	1.2	−44.5	31	1.5	−37.5	0.598	0.864
	S	23	1.1	−51.2	39	1.7	−16.7	0.155	0.553
sRAGE (ng/mL)	0	26	0.668		42	0.784		0.264	
	7	19	0.705	−2.0	40	0.725	−4.9	0.413	0.968
	14	17	0.626	−3.3	33	0.674	−5.4	0.486	0.854
	21	20	0.678	13.3	32	0.714	2.7	0.367	0.167
	28	15	0.775	25.5	31	0.764	1.8	0.606	0.232
	S	23	0.788	16.8	39	1.085	42.7	**0.046**	0.088
Nucleosomes (ng/mL)	0	26	76.1		42	82.0		0.677	
	7	19	89.5	−14.3	40	54.8	−37.9	0.069	0.063
	14	17	66.9	−2.8	33	48.0	−38.4	0.155	0.183
	21	20	61.0	−11.8	31	52.2	−33.9	0.297	0.493
	28	15	77.6	5.8	29	41.6	−34.2	**0.048**	0.225
	S	23	74.8	−17.5	38	58.9	−49.0	0.587	0.188
DNAse (%AR)	0	25	20.7		42	24.8		0.100	
	7	19	25.1	10.8	40	22.2	−6.1	0.343	0.068
	14	17	24.2	6.9	33	22.7	−6.2	0.532	0.287
	21	20	23.7	3.3	32	23.6	8.7	0.632	0.771
	28	15	22.7	9.5	31	26.6	9.7	0.223	0.951
	S	23	20.5	3.5	38	23.1	−8.7	0.484	0.421

Abbreviations: CR: complete response; PD: progressive disease; PR: partial response; SD: stable disease; T_x: absolute biomarker values; $T_x - T_0$: relative changes of marker values to day 0. Significant results are marked in bold.

investigations were not meaningful and did not show a difference for any marker (see Table 2).

Correlation of biomarkers with prognosis

Median TTP of all 78 patients was estimated with 3.9 months (95% CI 2.3 5.3 months) and median OS with 7.7 months (95% CI 6.3 10.0 months). Concerning baseline patient characteristics, patients with KPS of 90 100% had significantly improved TTP and OS (each $p < 0.0001$, Supporting Information Table S2); moreover, KPS proved to have the highest C index for TTP (0.611) and OS (0.619) of all clinical variables included into statistical analysis. Patients with locally advanced disease had a longer median OS (14.2 months) than patients with recurrent disease (8.3 months) or with synchronous metastases (6.6 months; p 0.02). As expected, patients without progression of disease at the 2 month staging had a prolonged OS compared to progressive patients (10.4 vs. 6 3 months; $p < 0.0001$).

The univariate and KPS adjusted analyses for all biomarker data that were logarithmized and handled as continuous variables and evaluated for the endpoints TTP and OS are shown in Table 3. Thereby, absolute median marker values (T_x) and marker kinetics (T_x T_0; change in value when compared to baseline levels) were examined. In the univariate model for TTP and OS, circulating nucleosomes specifically the absolute marker values showed best correlation with prognosis of all markers assessed: absolute levels of nucleosomes on days 7, 14, 21 and at staging as well as percentual changes from day 0 to 14 and days 0 to 28 were significantly associated with TTP. Consistent with this observation, nucleosome levels on days 7, 14 and at staging also significantly affected OS. Thereby, high nucleosome values were associated with poor outcome. Early kinetics of DNAse activity and HMGB1 from day 0 to 7 as well as the absolute sRAGE level at staging (day 56) all were prognostically relevant for TTP. When adjusted to KPS, the absolute nucleosome and

Table 3. Univariate and KPS adjusted analyses for correlation of biomarkers (continuous variables) with outcome*

		Univariate time to progression		Univariate overall survival		Overall survival adjusted to KPS	
Marker	Time (days)	$p\ (T_x)$	$p\ (T_x - T_0)$	$p\ (T_x)$	$p\ (T_x - T_0)$	$p\ (T_x)$	$p\ (T_x - T_0)$
Nucleosomes	0	0.183		0.086		0.090	
	7	**0.006**	0.303	**0.027**	0.679	0.262	0.834
	14	**0.002**	**0.046**	**0.036**	**0.025**	0.126	0.158
	21	**0.024**	0.284	0.113	0.604	0.086	0.454
	28	0.076	0.058	0.104	0.123	0.207	0.779
	56 (Staging)	**0.007**	0.998	**0.006**	0.867	**0.007**	0.324
DNAse	0	0.591		0.245		0.107	
	7	0.103	**0.012**	0.833	**0.026**	0.725	**0.014**
	14	0.327	0.266	0.686	0.356	0.910	0.465
	21	0.414	0.857	0.548	0.678	0.780	0.783
	28	0.652	0.777	0.904	0.773	0.868	0.524
	56 (Staging)	0.493	0.250	0.822	0.374	0.609	0.856
HMGB1	0	0.500		0.168		0.166	
	7	0.269	0.050	0.051	0.781	0.554	0.788
	14	0.203	0.055	0.269	0.784	0.691	0.834
	21	0.071	0.075	**0.039**	**0.031**	0.165	0.300
	28	0.337	0.083	0.118	0.096	0.150	0.184
	56 (Staging)	0.121	0.674	**0.023**	0.852	**0.006**	0.551
sRAGE	0	0.209		0.507		0.766	
	7	0.059	0.243	0.482	0.462	0.350	0.802
	14	0.117	0.590	0.561	0.834	0.800	0.607
	21	0.457	0.645	0.959	0.503	0.449	0.706
	28	0.402	0.573	0.749	0.371	0.297	0.381
	56 (Staging)	**0.034**	0.364	0.351	0.842	0.947	0.537

Abbreviations: KPS: Kamofsky performance status; T_x: absolute biomarker values; $T_x - T_0$: relative changes of marker values to day 0.
*Significant results are marked in bold.

HMGB1 level at staging and early kinetics of DNAse activity (from day 0 to 7) remained independent prognostic factors for OS (Table 3). Thereby, high HMGB1 and low sRAGE levels were associated with poor outcome.

In the analysis of tertiles, results on nucleosomes were essentially confirmed showing higher levels in univariate and KPS adjusted analyses being related to poor prognosis, particularly with regard to TTP (Tables 4 and 5). This is also highlighted by the corresponding C indices that revealed that nucleosomes on days 7 56 were the best predictor for TTP of all four markers assessed. The corresponding Kaplan Meier plot for nucleosomes on day 7 is illustrated in Figure 2*a*. In addition, high HMGB1 levels at days 21 and 56 were confirmed to indicate short OS in univariate and KPS adjusted analyses (Fig. 2*b*).

Discussion

In clinical practice, CA19 9 is currently the only established tumor marker for PC patients; no other prognostic or even predictive biomarker for the efficacy of a specific treatment or drug has yet been defined.[8,36] New diagnostic and prognostic tools, however, may be found in the field of novel serum biomarkers, especially those involved in biological processes relevant for development and sustain of tumors, such as cell proliferation, apoptosis, tissue homeostasis and inflammation. As some of these new biomarkers have already proven their prognostic ability in other tumor entities,[10–15] we examined circulating nucleosomes, sRAGE, HMGB1 and DNAse activity on their potential role as prognostic tools also in PC. This prospective biomarker study is, at least to our knowledge, the first one to examine such a marker panel in advanced PC patients.

Our study found that pretherapeutic levels of sRAGE were significantly lower in PC patients when compared to healthy controls (p 0.0001), whereas DNAse activity reduction was significantly elevated in cancer patients ($p < 0.0001$). In contrast to previous findings in lung, liver, colorectal and squamous cell cancers,[37–41] baseline values of nucleosomes or

Table 4. Correlation of (dichotomous) biomarker levels with time to progression (TTP) at different time points (T, day) assessed

	Median TTP (months) (95% CI)	*p* Value (log rank)	Hazard ratio (95% CI)	*p* Value (Cox)	C index (95% CI)	Hazard ratio (adjusted for KPS) (95% CI)	*p* Value (adjusted for KPS) (Cox)	C index (adjusted for KPS) (95% CI)
Nucleosomes (ng/mL) day 7		**0.0153**			**0.614 (0.539 0.689)**			**0.647 (0.571 0.724)**
<47	6.7 (3.3 19.0)							
47 89	5.0 (2.1 6.0)		1.9 (1.0 3.6)	**0.043**		1.9 (1.0 3.7)	**0.040**	
>89	2.5 (1.6 4.6)		2.5 (1.3 4.7)	**0.006**		2.2 (1.1 4.2)	**0.024**	
Nucleosomes (ng/mL) day 14		**0.0112**			**0.590 (0.503 0.676)**			**0.632 (0.542 0.721)**
<40	7.6 (2.1 23.7)							
40 80	3.9 (1.6 6.0)		2.7 (1.3 5.7)	**0.010**		3.0 (1.4 6.3)	**0.005**	
>80	3.7 (1.6 6.2)		2.6 (1.3 5.5)	**0.009**		2.4 (1.1 5.0)	**0.022**	
Nucleosomes (ng/mL) day 21		0.0864			0.609 (0.527 0.691)			0.694 (0.618 0.771)
<45	5.7 (2.1 19.0)							
45 91	3.6 (1.6 5.3)		2.4 (1.2 5.0)	**0.02**		1.9 (0.9 4.1)	0.089	
>91	2.5 (1.8 4.6)		3.3 (1.5 7.0)	**0.002**		3.3 (1.5 7.2)	**0.002**	
Nucleosomes (ng/mL) day 56		0.0942			0.584 (0.510 0.659)			0.661 (0.589 0.733)
<45	7.0 (2.1 9.9)							
45 90	3.1 (1.8 6.2)		1.6 (0.9 3.0)	0.135		1.1 (0.5 2.2)	0.790	
>90	3.8 (1.5 5.0)		1.9 (1.0 3.6)	**0.038**		1.6 (0.9 3.1)	0.141	
sRAGE (ng/mL) day 56		0.2351			0.577 (0.496 0.658)			0.653 (0.576 0.729)
<0.77	2.1 (1.5 5.3)		1.7 (0.9 3.1)	0.107		1.2 (0.6 2.4)	0.514	
0.77 1.20	5.3 (2.7 9.7)		1.1 (0.6 2.1)	0.685		1.1 (0.6 2.1)	0.671	
>1.20	5.3 (2.5 9.9)							

Significant results are marked in bold.

Table 5. Correlation between (dichotomous) biomarker levels and overall survival (OS) at different time points (T, day) assessed

	Median OS (months) (95% CI)	p Value (log rank)	Hazard ratio (95% CI)	p Value (Cox)	C index (95% CI)	Hazard ratio (adjusted for KPS) (95% CI)	p Value (adjusted for KPS) (Cox)	C index (adjusted for KPS) (95% CI)
Nucleosomes (ng/mL) day 7		0.0751			0.600 (0.526 0.675)			0.651 (0.575 0.727)
<47	11.2 (7.1 19.0)							
47 89	8.2 (6.2 10.4)		1.5 (0.8 2.8)	0.199		1.6 (0.8 2.9)	0.165	
>89	6.0 (4.6 10.2)		2.0 (1.1 3.8)	**0.026**		1.6 (0.8 3.1)	0.172	
Nucleosomes (ng/mL) day 14		0.1878			0.584 (0.491 0.678)			0.643 (0.556 0.730)
<40	14.2 (7.0 23.7)							
40 80	8.3 (6.2 10.5)		1.6 (0.8 3.3)	0.185		1.7 (0.8 3.4)	0.144	
>80	7.7 (3.6 10.4)		1.8 (0.9 3.6)	0.081		1.7 (0.8 3.2)	0.142	
HMGB1 (ng/mL) day 21		**0.0359**			**0.610** (0.535 0.684)			**0.690** (0.622 0.759)
<0.8	10.6 (6.6 16.1)							
0.8 2.4	6.4 (4.6 8.4)		1.4 (0.7 2.8)	0.335		1.4 (0.7 2.9)	0.314	
>2.4	5.6 (3.2 8.2)		2.4 (1.2 4.8)	**0.014**		1.8 (0.9 3.7)	0.121	
Nucleosomes (ng/mL) day 56		0.1493			0.580 (0.507 0.653)			0.640 (0.556 0.725)
<45	10.5 (7.1 14.8)							
45 90	6.6 (4.6 10.3)		1.5 (0.8 2.8)	0.234		1.0 (0.5 2.0)	0.994	
>90	7.4 (4.7 10.4)		1.8 (1.0 3.4)	0.058		1.5 (0.8 2.8)	0.227	
HMGB1 (ng/mL) day 56		0.0933			0.584 (0.508 0.660)			0.678 (0.609 0.748)
<0.8	10.8 (7.7 16.1)							
0.8 2.7	7.1 (5.0 8.4)		1.7 (0.9 3.2)	0.105		1.9 (1.0 3.7)	**0.046**	
>2.7	7.1 (4.7 10.4)		1.9 (1.0 3.5)	**0.043**		2.0 (1.1 3.7)	**0.034**	

Significant results are marked in bold.

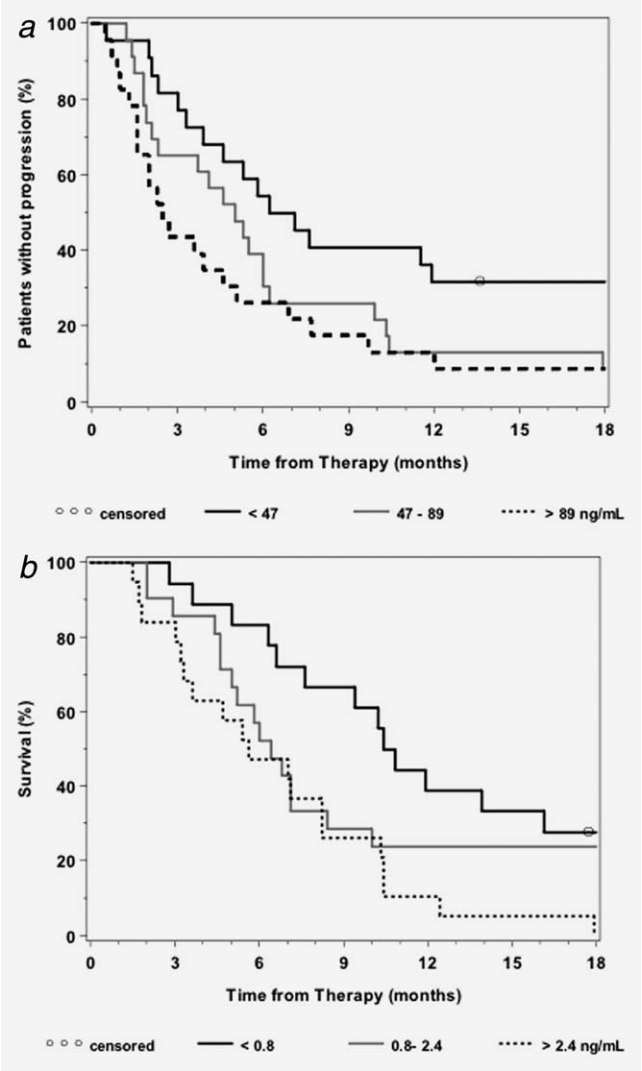

Figure 2. Kaplan Meier curves for Time to Progression based on nucleosomes on day 7 (A) and for Overall Survival based on HMGB1 levels on day 21 (B).

HMGB1 were not elevated to the level of statistical significance in PC; however, patients with locally advanced tumors had a significantly higher pretherapeutic level of nucleosomes than those with recurrent disease. The exceptionally good correlation of HMGB1 and nucleosomes throughout all time points measured ($p < 0.0001$) could be very well explained by a combined release from the degrading tumor cells. In vitro HMGB1 was already shown to stay tightly bound to nucleosomes when released from apoptotic cells and HMGB1 nucleosome complexes were found to bind to TLR 2 with subsequent proinflammatory activation.[42] During the first weeks after initiation of chemotherapy no statistically relevant differences in marker kinetics could be observed between the two response groups. A reason for this may lie in the different abilities of certain chemotherapeutics to induce release of cell death markers, as previously shown, e.g., for oxaliplatin and HMGB1.[43] After 4 weeks of therapy, at d28, nucleosome levels were significantly higher in progressive than in nonprogressive patients and sRAGE levels at time of staging were significantly lower in progressive than in nonprogressive patients (see Table 2). Earlier studies of our group support these results observed in this PC study that high HMGB1 and low sRAGE levels show a correlation with poor outcome.[17,44] These previous studies in colorectal and liver cancers also showed an additional prognostic information provided by marker values assessed 24 and 48 hr after treatment application; however, as we analyzed PC patients who all received mainly weekly chemotherapies in an outpatient setting, we unfortunately were not able to capture these early changes in marker levels.

For the evaluation of a correlation with prognosis (TTP and OS), the biomarker data were logarithmized and handled as continuous variables to see whether there is an prognostic effect over the whole value range. In addition to univariate analyses, biomarkers were adjusted to KPS that was found to be the most relevant clinical factor. Remarkably, absolute levels of circulating nucleosomes were found to be related to TTP and OS at most days during chemotherapy investigated. Although percentual changes of nucleosome levels during therapy were prognostically relevant as well, it turned out that absolute values assessed at different time points provided superior prognostic information.

Further single observations indicated some prognostic relevance for early kinetics of DNAse activity from day 0 to 7 as well as the absolute sRAGE level at staging (day 56) for TTP and of DNAse activity kinetics and absolute HMGB1 levels (days 21 and 56) for OS. Once again high nucleosome and HMGB1 levels and low sRAGE levels indicated poor prognosis. Notably, the absolute nucleosome and HMGB1 level at staging and early kinetics of DNAse activity (from day 0 to 7) remained independent prognostic factors for OS (Table 3) when adjusted to KPS and may potentially add to existing multivariate prognostic models.

When including biomarkers into cutoff related prognostic models for confirmation and better visualization in Kaplan Meier survival curves, cohorts were split into tertiles to ensure an equal patient distribution. This approach avoids arbitrary cutoffs and provides insight into the nature of the relationship between biomarker and prognosis. Remarkably, results of the general evaluation particularly on nucleosomes measured at different time points during chemotherapy were confirmed by this approach especially for estimating TTP. These results match with findings of former studies on a rather late release of nucleosomes after tumor cell death. Results remained statistically significant when adjusted to KPS and yielded high C indices in univariate and KPS adjusted evaluations. Thus, circulating nucleosomes proved to be a relevant additive to KPS, a clinical parameter with a known strong predictive power for TTP and OS. As further informative biomarker, immunogenic cell death marker HMGB1 on days 21 and 56 was identified as valuable prognostic parameter for OS.

Recently, we have evaluated tumor markers CA 19 9, CYFRA 21 1 and CEA in the same patient setting and have found them to be highly relevant predictive and prognostic variables.[45] Although immunogenic biomarkers were not as strong as these markers, they should be considered as promising, additional, biologically functional markers in further prospective investigations.

The strength of our study is based on extensive, standardized methodical assay evaluation, including intra and inter assay imprecision, linearity and analyte stability under different storage conditions before and after centrifugation.[33,34] Patient samples were acquired weekly following a clearly defined study protocol to get homogeneous and complete sample sets to match with complete sets of clinical data. Studies on those close biomarker kinetics during chemotherapy and their relevance for therapy monitoring and prognosis are not available so far. ELISA tests were performed in batches including all samples of one patient. Final statistical evaluation was independently performed by our institute's biostatistician. However, a drawback may be found in missing marker values during the first week after therapy, especially as 24 and 48 hr values have been shown to be valuable for evaluation of tumor metabolism and cell death.'[17,41,46]

It has to be pointed out that the evaluations presented in our article address several clinical questions that are not completely independent from each other but that will have different clinical consequences:

a. The prediction of response to therapy before start of chemotherapy is relevant for the decision whether and which therapy will be applied.
b. The monitoring of response to therapy during chemotherapy is relevant as an additional tool to radiology for the decision whether the therapy should be continued or not.
c. The estimation of prognosis in terms of TTP and OS (irrespective of the radiological response to therapy) is most relevant for patient counselling and the decision whether additional therapies should be offered.

Despite there are, of course, some interrelations of these events it was not self evident that the same markers are relevant for all indications in the same manner. As we could show, they were more meaningful for prognostic than for predictive or monitoring purposes. Further, the time point of the biomarker exams remains a matter of debate. Of course, the marker values during the therapy will be influenced by the efficacy of the treatment. However, it is not known so far which time points would be ideal for marker determination and whether absolute values or relative changes are more meaningful. To respect these uncertainities, a considerable number of statistical comparisons and analyses have been carried out. As a consequence, the character of our study is strongly exploratory, forming the basis for future validation studies.

In conclusion, circulating nucleosomes and biomarkers of immunogenic cell death (specifically sRAGE and HMGB1) are novel and valuable tools for assessing response to chemotherapy and for estimating the prognosis in patients with advanced PC. An external validation of these innovative data is recommended, ideally with the setting of a multicenter biomarker study.

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dass ich die vorliegende Dissertation mit dem Thema

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