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Frühzeitige Beurteilung der Effizienz systemisch-zytotoxischer Therapien bei Patientinnen mit Mammakarzinom durch zirkulierende Nukleinsäuren.

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1 **Einleitung**

1.1 Übersicht zum Mammakarzinom

Das statistische Bundesamt in Deutschland führt das Mammakarzinom mit 17.815 Todesfällen an vierter Stelle der Todesursachen für Frauen in 2011 [1]. Im Jahr 2009 wurde bei insgesamt 73.340 Frauen Brustkrebs diagnostiziert [2]. Innerhalb der letzten Jahre kam es zu einem Anstieg der Brustkrebsdiagnosen. Dies wird zu einem großen Teil auf das seit 2005 eingeführte flächendeckende Früherkennungsprogramm inklusive der Mammographie für Frauen ab dem 50. Lebensjahr zurückgeführt [3]. Seit 2009 sind die Neuerkrankungsraten erstmals wieder leicht rückläufig [2].

Statistisch gesehen erkrankt jede achte Frau im Laufe ihres Lebens an einem Mammakarzinom. 2008 bekamen pro 100.000 Einwohner in Deutschland 171 Frauen und 1 Mann die Diagnose Mammakarzinom. Damit steht das Mammakarzinom an erster Stelle der Krebserkrankungen bei Frauen [2, 4].

Die Therapiemöglichkeiten für Mammakarzinome umfassen verschiedene Säulen: Zum einen die operative Therapie, zum anderen die Radio-, Chemo- und Hormontherapien. Die zunehmende Personalisierung der Therapie ist unter anderem auf die großen Erfolge der Forschung in den letzten Jahren zurückzuführen, zu denen auch die Entdeckung des Her2/neu-Rezeptors und die Entwicklung des spezifischen Antikörpers Trastuzumab gehören.

Die Chemotherapie bei Mammakarzinompatientinnen kann je nach Patienten- und Tumortyp zu verschiedenen Zeitpunkten im Rahmen der Tumortherapie eingesetzt werden.

Bei kurativem Ansatz besteht die Möglichkeit, die Patienten primär mit einer Chemotherapie zu versorgen, um sie anschließend zu operieren. Dieser sogenannte neoadjuvante Ansatz hat mehrere Vorteile: Zum einen wird der Tumor vor der Operation durch die Chemotherapie verkleinert, was die Rate von brusterhaltenden Operationen erhöht [5]. Zum anderen wird das Chemotherapiemedikament quasi in vivo getestet, da die Ansprechrate auf die Therapie nach einigen Wochen radiologisch nachgewiesen werden kann. Nötigenfalls kann man zu diesem Zeitpunkt die Chemotherapie dann umstellen – oder bei Nichtansprechen auch die Operation vorziehen.

Die neoadjuvante Chemotherapie ist das Standardvorgehen, wenn die Patientin unter einem inflammatorischem oder lokal fortgeschrittenem Tumor (T3, UICC 3) leidet. Zusätzlich sollte sie erwogen werden, wenn der Tumor bestimmte Kriterien ausweist, die ein sehr gutes Ansprechen auf die Chemotherapie wahrscheinlich machen. Dazu gehören zum Beispiel ein Alter < 40 Jahre, ein schlecht oder niedrig differenziertes bösartiges Gewebe (Grading 3 nach UICC) und negative Hormonrezeptoren. Außerdem zeigen nicht lobuläre Mammakarzinome ein potentiell gutes Ansprechen auf die Chemotherapie [6].
Zudem wurde die Äquivalenz der neoadjuvanten und der adjuvanten Therapieregime im Bezug auf das tumorfreie Überleben und das Gesamtüberleben in Studien gezeigt [7].
Eine typische neoadjuvante Chemotherapie besteht aus 8 Therapiezyklen im Abstand von jeweils 3 Wochen. Verschiedene Chemotherapeutika werden hierzu kombiniert, ein häufiges Schema ist eine Kombination aus einem Anthrazyklin und Cyclophosphamid für 4 Gaben, gefolgt von eine Taxan-Gabe für weitere 4 Gaben [8]. Im Anschluss erfolgt dann die Operation, sowie gegebenenfalls eine Bestrahlung. Hormontherapie mit Östrogenrezeptormodulatoren wie Tamoxifen oder Aromatasehemmern werden ebenso wie eine Trastuzumab-Therapie in Abhängigkeit des Hormonrezeptorstatus des Tumors hinzugefügt [5, 7].

1.2 Konventionelle Tumormarker bei Mammakarzinoms

Als Tumormarker bezeichnet man im Blut oder sonstigen Körperflüssigkeiten nachweisbare Substanzen, deren Nachweis auf das Vorhandensein von Tumorgewebe oder Tumorzellen hindeutet. Im Fall von Brustkrebs sind die derzeit relevanten Marker carcinoembryonale Antigen (CEA) und das Cancer Antigen 15-3 (CA 15-3). Für beide Tumormarker wurde nachgewiesen, dass sie im Falle eines Tumorwachstums ebenfalls ansteigen [9] und als Prognosefaktor eingesetzt werden können [10].

1.3 Zellfreie DNA und DNA Integrity als potentielle Tumormarker bei Brustkrebs

Zirkulierende zellfreie DNA (cell-free DNA, cfDNA) wurde in mehreren Studien als potentieller diagnostischer Biomarker beschrieben, da erhöhte Werte von zirkulierenden Nukleinsäuren im Blut bei verschiedenen Erkrankungen nachgewiesen werden konnten. Zu diesen Erkrankungen zählen neben Sepsis, rheumatologischen

und traumatologischen Erkrankungen auch Krebserkrankungen [11-13]. In unterschiedlichen Tumorentitäten wie Brust, Kolon, Rektum und Hoden wurden erhöhte Werte für zirkulierende zellfreie DNA gemessen und als Diagnosemarker erprobt [14-16]. Die prädiktive und prognostische Aussagekraft der zellfreien DNA wurde bis jetzt nur in wenigen Studien erprobt, von denen einige einen statistische signifikanten Zusammenhang fanden [17, 18].

Umetani et al. [14, 15] beschrieben im Jahre 2006 eine Rechenformel, die auf gemessenen Werten für zellfreie DNA basiert. Diese Rechenformel beruht auf der Annahme, dass zirkulierende zellfreie DNA mit einer Länge von weniger als 180 Basenpaaren (base pairs, bp) vorwiegend durch apoptotischen Zelltod ins Blut freigesetzt werden, wohingegen längere Basenpaare eher nekrotischen Ursprungs sind [19, 20]. Diese Annahme gründet sich darauf, dass während der Apoptose spezifische Endonukleasen aktiviert werden, die das Chromatin in nukleosomale Fragmente mit einer Größe von 160-180 bp spaltet [21, 22], Ausgehend davon entwickelte diese Forschergruppe Primer zur Messung zweier DNA-Stücke: ALU 115 (115 Basenpaare lang) und ALU 247 (entsprechend 247 bp) und berechnete aus den gemessenen DNA-Mengen im Serum den Quotienten ALU 247/ALU 115. Dieser Quotient wurde DNA Integrity genannt und steht für das Verhältnis von nekrotischen zum apoptotischem Zelltod. Folgt man der Hypothese, dass während des Tumorwachstums alternative Zelltodarten häufiger auftreten als der physiologische apoptotische Zelltod [19, 23], so müsste bei Tumorpatienten die DNA Integrity im Vergleich zu Gesunden erhöht sein und wäre somit ein potentieller Diagnose- wie Prognosemarker für Tumorpatienten.

Wang et al. [24] beschrieben 2003 ausgehend von ähnlichen Überlegungen eine weitere Formel, die sie ebenfalls DNA Integrity nannten. Diese Formel berechnet auch das Verhältnis von längeren zu kürzeren DNA Fragmenten im Plasma, allerdings auf Basis einer komplizierteren Rechenformel $e^{(-\Delta\Delta Cp \times \ln(2))}$. Hierbei wird der im Rahmen der PCR gemessene Cp-Wert jeder einzelnen Probe von einem Standardwert abgezogen. Anschließend werden die so entstandenen ΔCp Werte für ALU 247 von den ΔCp Werten für ALU 115 abgezogen. So entsteht ΔΔCp, welches dann in die o.g. Formel eingesetzt wird.

2 Zielsetzung der Untersuchungen

Die vorliegende Arbeit untersucht die Wertigkeit von zellfreier DNA und DNA Integrity als Diagnosemarker für Brustkrebs. Desweiteren untersucht sie, in wie weit diese noch neuen Tumormarker zur prätherapeutischen Prädiktion des Ansprechens bzw. zum Monitoring einer neoadjuvanten Chemotherapie bei Mammakarzinom-patientinnen verwendet werden können. Hierbei werden die konventionellen Tumormarker CEA und CA 15-3 als Vergleich – wie von den Leitlinien der European Group on Tumor Markers (EGTM) empfohlen – herangezogen [25].

3 Material und Methoden

3.1 Patienten

Die Rekrutierung der Patientinnen für diese Studie erfolgte im Zeitraum von Frühjahr 2007 bis Herbst 2011 in einer hämatoonkologischen Praxis in München.
Zusätzlich zu 65 Brustkrebspatientinnen mit einem lokalisierten Tumor (LBC) wurden prätherapeutische Proben von 47 Patientinnen im metastasierten Stadium (MBC) sowie von 28 gesunden Probandinnen und 12 Patientinnen mit einer benignen Brusterkrankung in der Studie untersucht.
Den 65 Brustkrebspatientinnen wurde zu verschiedenen Zeitpunkten während ihrer neoadjuvanten Chemotherapie Blut abgenommen. Die Erstabnahme erfolgte vor Beginn der Chemotherapie (Zyklus 1, Z1). Die folgenden Abnahmen erfolgten vor Beginn der zweiten Chemotherapiezyklus, ca. 3 Wochen nach der ersten Chemotherapie (Z2), sowie nach ca. 60 Tagen (zwischen Zyklus 5 und 6), kurz vor Ende der neoadjuvanten Chemotherapie.

Alle Brustkrebspatientinnen durchliefen vor Beginn ihrer Therapie ein ausführliches Staging, welches neben radiologischen Untersuchungen (Sonographie Abdomen, Röntgen Thorax, Mammographie und Skelettszintigraphie) auch pathologische Abklärungen (Stanzbiopsien des Tumors mit Bestimmung des Hormonrezeptorstatus) umfasste. Anschließend wurden die Tumore der Patientinnen nach dem TNM-System klassifiziert.
Nach Ende der neoadjuvanten Chemotherapie wurden alle Patientinnen in der LBC Gruppe operiert und in Abhängigkeit des pathologischen Gutachtens des

Operationspräparates wurde das Ansprechen auf die präoperative Chemotherapie eruiert (siehe Abbildung 1).

Alle Patientinnen wurden bezüglich der vorliegenden Studie umfassend informiert und eine schriftliche Einverständniserklärung wurde eingeholt. Das Ethikkommitee der Ludwig-Maximilians-Universität hat die Studie im Jahre 2008 geprüft und gebilligt.

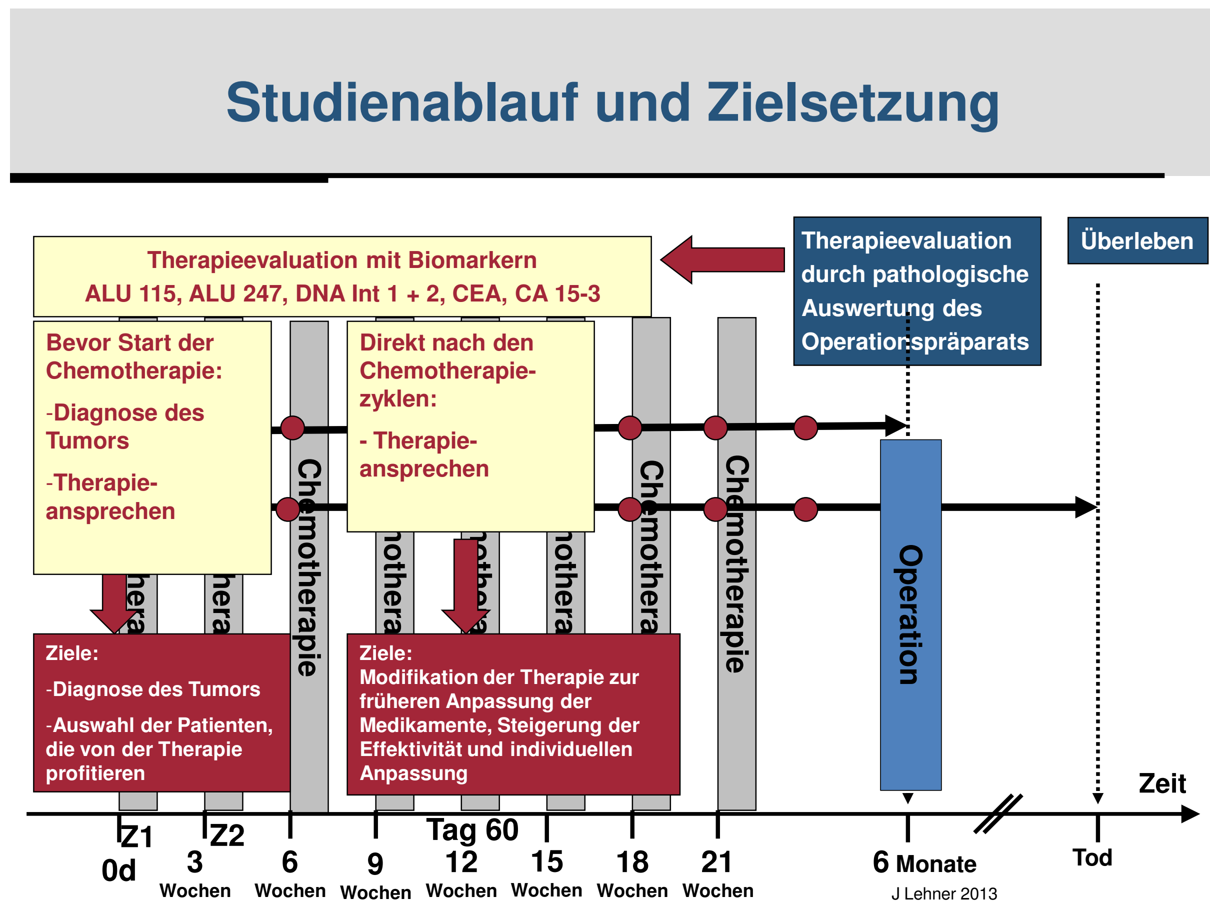

Abbildung 1: Studienablauf und Zielsetzung

3.2 Entnahme der Proben

Allen Patientinnen und allen gesunden Probanden wurde vor Beginn einer etwaigen Therapie Plasmaproben und Serumproben (für die etablierten Tumormarker CEA und CA 15-3) entnommen. Die Proben wurden innerhalb von 2 Stunden zentrifugiert, aliquotiert und bei -80°C eingefroren. Das gleiche Procedere wurde bei Entnahme im weiteren Therapieverlauf der neoadjuvanten Chemotherapie durchgeführt.

Im Rahmen ausführlicher Voruntersuchungen wurde die Stabilität des Materials mittels Präanalytik getestet. Hierbei wurden Untersuchungen bezüglich der

Vergleichbarkeit von Messungen innerhalb eines Ansatzes wie auch zwischen zwei Ansätzen durchgeführt. Das Material zeigte sich hierbei stabil.

3.3 Messmethoden

Die Plasmaproben wurden nach dem Auftauen mithilfe eines DNA-Extraktionskits (Firma Qiagen, Hilden, Deutschland) behandelt. Hierbei wurden jeweils 400µl Plasma eingesetzt und nach mehreren Waschvorgängen 50µl DNA gewonnen. 5µl diesen DNA-Eluats wurden anschließend als Probe in der PCR eingesetzt.
Die quantitative PCR erfolgte an einem Lightcycler 480 der Firma Roche Diagnostics (Mannheim, Deutschland). Hierfür wurden die gleichen Primer wie in Umetani et al. [14, 15] für ALU 115 und ALU 247 verwendet, für die genauen Daten zur Durchführung der PCR, der Primer und der DNA-Extraktion verweist die Autorin auf die mit eingereichten Arbeiten zum Erwerb dieser kumulativen Dissertation. Alle Messungen wurden als Duplikate ausgeführt, Positiv- und Negativkontrollen sowie Standards wurden bei jedem Durchgang zusätzlich gemessen, um die Vergleichbarkeit der einzelnen Messvorgänge zu gewährleisten. Im Anschluss an die Messungen erfolgte die Umrechnung der Messergebnisse in ng/dl mittels der mitgeführten Standardkurve sowie die Berechnung der DNA-Integrity anhand der oben erwähnten Formeln nach Umetani und Wang [14, 15, 24]. Zur besseren Übersicht wurde die DNA-Integrity Formel nach Umetani DNA Int 1 und die Formel nach Wang DNA Int 2 benannt.
Zusätzlich zu den neuen Biomarkern DNA Integrity und zellfreie DNA wurden die konventionellen Tumormarker CEA und CA 15-3 mithilfe eines ECLIA-Essays (Roche Diagnostics) im Serum gemessen.

3.4 Statistik

Diagnose: Um eine übersichtliche statistische Darstellung zu gewährleisten, wurde das Kollektiv in 4 Gruppen geteilt: Gesunde, Benigne, Brustkrebspatienten mit kurativem Ansatz unter neoadjuvanter Chemotherapie und metastasierte Brustkrebspatientinnen.
Prädiktion und Monitoring: Die dritte Gruppe, welche mit neoadjuvanter Chemotherapie versorgt wurde, teilt sich nach Ende der systemischen Therapie anhand des pathologischen Ergebnis nach Operation in 3 Gruppen: Patienten mit exzellentem Ansprechen auf die präoperative Chemotherapie (complete remission,

CR, kein Resttumor im Operationpräparat), Patienten mit gutem bis mäßigem Ansprechen (partial remission, PR, Tumorverkleinerung im Vergleich zu prätherapeutischen Untersuchungen zwischen 30 und 99%) und Patienten mit schlechtem und keinem Ansprechen (no change bzw. progressive disease, NC/PD, Tumorverkleinerung kleiner als 30% oder Tumorwachstum).
Korrelationen mit prätherapeutisch erhobenen Daten wie beispielweise dem TNM-Status oder dem Hormonrezeptorstatus wurden mithilfe des Wilcoxon- oder Kruskal-Wallis-Tests durchgeführt. Spearman-Rank-Korrelationen wurden für die Korrelationen der Biomarker untereinander verwendet.
Zur statistischen Auswertung wurden Mediane, Perzentilen und p-Werte berechnet. Als statistisch signifikant wurde ein p-Wert <0.05 betrachtet.
Alle Berechnungen erfolgten mit der SAS-Software, version 9.2.

4 **Ergebnisse**

Die hier vorgestellten Arbeiten adressieren unterschiedliche Fragestellungen. Die erste Arbeit [26] befasst sich mit der Relevanz der untersuchten Marker im Bezug auf die Diagnose von Brustkrebs. Die zweite Arbeit beleuchtet das Therapiemonitoring während der neoadjuvanten Chemotherapie von Brustkrebspatientinnen [27].

4.1 Diagnostik

Die Diagnostik-Studie umfasste 65 Brustkrebspatientinnen mit kurativem Therapieansatz (Altersmedian 47,0 Jahre), 47 Patientinnen mit metastasiertem Brustkrebs (Median 60,8 Jahre) sowie 28 gesunde Probandinnen (45,5 Jahre) und 12 Patientinnen mit benignen Mammaerkrankungen (41,7 Jahre). In Tabelle 1 sind weitere Patientendaten der neoadjuvant therapierten Patientengruppe aufgeführt.

Prätherapeutische Daten		
Tumorstadium anhand des TNM Systems (T-Stadium)	**N**	**%**
1	8	12.3
2	39	60.0
3	14	21.5
4	3	4.6
X	1	1.6
Lymphknotenbefall anhand des TNM Systems (N-Stadium)	**N**	**%**
0	17	26.1
1	40	61.6
2	1	1.6
3	3	4.6
X	4	6.1
Differenzierung des Tumorgewebes (Grading, G-Stadium)	**N**	**%**
2	22	42.3
3	21	40.4
4	1	1.9
X	8	15.4
Histologie	**N**	**%**
Invasiv duktales Karzinom	57	87,7
Invasiv lobuläres Karzinom	4	6,1
Adenokarzinom	2	3,1
Nicht genauer definiert	2	3,1
Histopathologische Klassifikation	**N**	**%**
Östrogen Rezeptor positiv/negativ	38/27	58.5 / 41.5
Gestagen Rezeptor positiv/negativ	32/33	49.2 / 50.8
Her2neu positiv/negative	21/44	32.3 / 67.7
Triple negativ/Nicht Triple negativ	21/44	32.3 / 67.7

Tabelle 1: Prätherapeutische Daten der Patientinnen mit lokalisiertem Mammakarzinom

Desweiteren wurde die Relevanz der untersuchten Marker ALU 115, ALU 247 (als Repräsentanten für die zirkulierende zellfreie DNA), DNA Int 1 und 2 sowie CEA und CA 15-3 als Diagnosemarker bei Brustkrebs untersucht. Hierbei berechnete sich zuerst je ein p-Wert als Unterscheidungskriterium zwischen den einzelnen Gruppen (siehe Tabelle 2). Alle signifikanten Werte sind fett unterlegt.

Marker	Gruppe	N	Median	Min	Max	Im Vergleich mit		
						Benignen (p-value)	LBC (kurativer Ansatz) (p-value)	MBC (metastasiert) (p-value)
ALU 115	Gesund	28	1.8	0.1	3.2	**< 0.001**	**< 0.001**	**< 0.001**
(ng/mL)	Benigne	12	27.4	1.4	89.2		0.523	0.446
Gesamt	LBC	65	15.9	0.7	871.8			**0.011**
p<0.0001	MBC	47	22.3	3.3	827.1			
ALU 247	Gesund	28	1.9	0.3	4.4	**< 0.001**	**< 0.001**	**< 0.001**
(ng/mL)	Benigne	12	22.3	1.4	63.8		0.950	0.082
Gesamt	LBC	65	16.8	0.8	577.6			**0.001**
p<0.0001	MBC	47	29.8	5.1	835.6			
DNA-Int 1	Gesund	28	1.2	0.5	9.3	**0.006**	0.120	0.738
	Benigne	12	0.9	0.5	1.1		**0.015**	**<0.001**
Gesamt	LBC	65	1.1	0.6	1.7			**0.005**
p=0.0003	MBC	47	1.2	0.6	1.9			
DNA-Int 2	Gesund	28	1.0	0.3	1.9	**0.001**	**0.026**	0.2434
	Benigne	12	0.7	0.4	1.0		0.093	**<0.001**
Gesamt	LBC	65	0.8	0.4	1.5			**<0.001**
p<0.0001	MBC	47	1.1	0.5	2.1			
CEA	Gesund	27	1.0	0.2	4.2	0.402	0.166	**< 0.001**
(ng/mL)	Benigne	12	0.7	0.2	3.4		0.092	**<0.001**
Gesamt	LBC	62	1.3	0.2	14.1			**< 0.001**
p<0.0001	MBC	41	6.0	0.3	2608			
CA 15-3	Gesund	27	17.6	5.6	26.9	0.726	0.303	**< 0.001**
(U/mL)	Benigne	12	17.3	8.2	41.1		0.747	**<0.001**
Gesamt	LBC	62	19.1	6.3	258.0			**< 0.001**
p<0.0001	MBC	41	61.3	10.0	319000			

Tabelle 2 Tumormarkerwerte der verschiedenen Patientengruppen

Die Korrelationen der Marker für alle Patienten untereinander zeigte eine sehr gute, gleichsinnige Korrelation sowohl zwischen ALU 115 und ALU 247 als auch zwischen DNA Int 1 und 2. Die Werte für CA 15-3 korrelierten mit allen untersuchten Biomarkern, wohingegen CEA nur mit den zirkulierenden zellfreien DNA-Stücken, DNA Int 2 und mit CA 15-3 eine gleichsinnige Korrelation zeigte. Eine detaillierte Darstellung findet sich in der Originalarbeit [26].

Die Spezifität und Sensivität der einzelnen Marker wurde mit ROC-Kurven dargestellt. In Abbildung 2 zu sehen ist die ROC-Kurve für den Vergleich LBC gegen gesunde Probanden, der Vergleich, der besonders für die frühe Diagnose relevant ist.

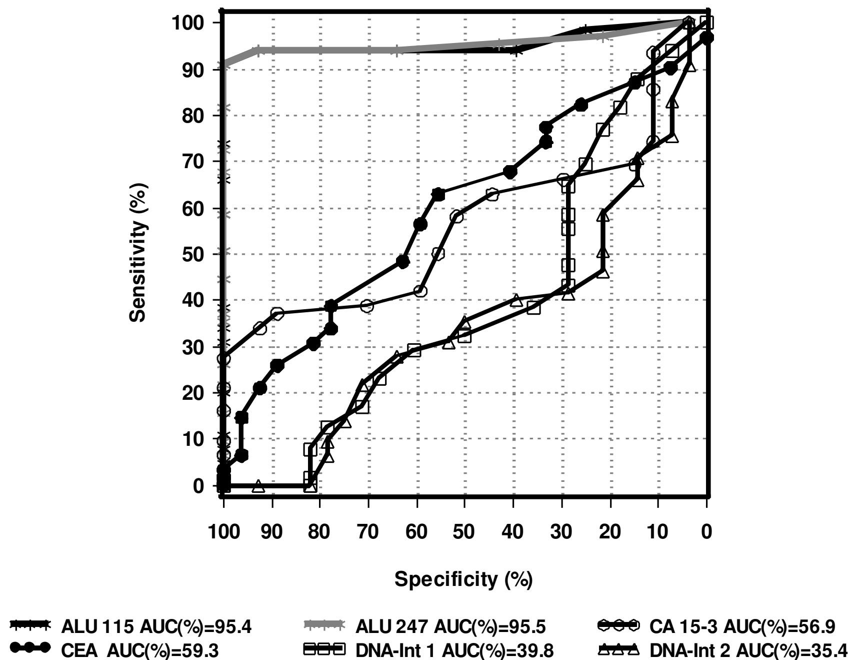

Abbildung 2 ROC Kurven der Plasmawerte von ALU 115, ALU 247, DNA Int 1 und 2 sowie der Serumwerte von CA 15-3 und CEA als Unterscheidung zwischen LBC und gesunden Probanden

Hierbei erwiesen sich ALU 115 und ALU 247 als beste diagnostische Marker mit AUCs von 95,4% respektive 95,5%. CEA und CA 15-3 diskriminierten für LBC deutlich schwächer, allerdings erkannten sie MBCs im Vergleich zu allen anderen Gruppen am besten (siehe Originalarbeit) [26].

4.2 Prognose und Prädiktion

Dieser Teil der Studie wurde in der 2. Originalarbeit [27] veröffentlicht. Nach Abschluss der Chemotherapie wurde im Zuge der Operation ein histologisches Präparat des Tumors gewonnen. Die Untersuchungsergebnisse sowie weitere Daten bezüglich der Chemotherapie finden sich in Tabelle 3.

Patientendaten nach Abschluss der Chemotherapie (zum Operationszeitpunkt)		
Tumorstadium (T-Stadium)	**N**	**%**
0	16	24.6
1 (1A-1C)	30	46.2
2	12	18.5
3	6	9.2
4	1	1.5
Lymphknotenstadium (N-Stadium)	**N**	**%**
0	40	61.6
1	16	24.6
2	6	9.2
3	3	4.6
Ansprechen auf die Chemotherapie	**N**	**%**
Exzellentes Ansprechen, kein Resttumor (complete remission, CR)	13	20.0
Gutes bis mäßiges Ansprechen, Resttumor 33-99% kleiner (partial remission, PR)	32	49.2
Schlechtes Ansprechen oder Tumorwachstum (no change, NC)	20	30.8
Neoadjuvante Chemotherapeutikakombinationen	**N**	**%**
Cyclophosphamid + Epirubicin + Docetaxel	36	55.3
Cyclophosphamid + Epirubicin	13	20.0
Cyclophosphamid + Epirubicin + Paclitaxel	11	16.8
Cyclophosphamid + Epirubicin + Fluorouracil+ Docetaxel	2	3.1
Cyclophosphamid + Epirubicin + Docetaxel + Paclitaxel	1	1.6
Cyclophosphamid + Epirubicin + Carboplatin	1	1.6
Carboplatin	1	1.6

Tabelle 3 Patientendaten der LBC Gruppe nach Abschluss der Chemotherapie

Die Auswertung aller Biomarker im Bezug auf Prognose und Prädiktion erfolgte sowohl zu den einzelnen Messzeitpunkten (Zyklus 2, Zyklus 6), als auch als kinetisches Auswertung der Marker im Therapieverlauf. Hierbei wurde der prätherapeutische Wert (siehe auch unter Diagnostikmarker bei LBC) mit dem Wert bei Zyklus 2 respektive Zyklus 6 verglichen. Signifikante Ergebnisse zeigte nur die Kinetik der zirkulierenden zellfreien DNA ALU 115 im Vergleich von Zyklus 6 mit dem prätherapeutischen Wert. ALU 115 war in der Lage, Patienten mit einem exzellenten Chemotherapieansprechen (CR) von Patienten ohne Therapieansprechen (NC) zu unterscheiden (p-Wert = 0.033), siehe auch Tabelle 4. Für ALU 247 zeigten sich ähnliche Tendenzen, wenngleich der Unterschied nicht signifikant war. Die

prätherapeutisch erhobenen Klassifikationsfaktoren wie der TNM-Status und der Hormonrezeptorstatus wurden ebenfalls auf ihren prädiktiven Wert untersucht. Hierbei zeigte sich, dass der Her2/neu-Status im Mantel-Haenzsel-Test klar zwischen den einzelnen Gruppen unterscheidet: in der CR Gruppe finden sich 53,9% Her2neu positive Patienten, wohingegen nur 34,4% der PR und 15,0% der NC Gruppe einen positiven Rezeptorstatus aufweisen (p-Wert 0.0193) [27].

	Marker	Gruppe	N	Median	Min	Max	Vergleich mit CR Gruppe (p-Wert)
Zyklus 6 im Vergleich mit prätherapeutischen Werten (in %)	ALU 115	NC	13	109.8	-99.8	5100.6	**0.033**
		PR	26	109.4	-94.8	2744.7	
		CR	9	-39.4	-87.8	175.0	
	ALU 247	NC	13	123.9	-99.9	4223.4	***0.071***
		PR	26	125.6	-98.9	2279.5	
		CR	9	-16.3	-77.8	193.0	
	DNA Int 1	NC	13	-2.0	-89.4	89.1	0.182
		PR	26	-4.5	-78.1	82.7	
		CR	9	26.7	-17.0	85.4	
	DNA Int 2	NC	13	-58.5	-91.8	55.1	0.125
		PR	26	-67.6	-94.9	-22.8	
		CR	9	-30.7	-67.9	89.7	
	CEA	NC	12	-2.9	-66.7	216.7	1.00
		PR	25	40.0	-81.2	900.0	
		CR	8	-1.8	-87.2	166.7	
	CA 15-3	NC	12	19.3	-34.9	158.1	0.787
		PR	25	73.8	-80.1	209.8	
		CR	8	39.9	-48.0	76.6	

Tabelle 4 Biomarkerwerte aus Zyklus 6 im Vergleich mit prätherapeutischen Werten, positive Zahlen zeigen einen Anstieg, negative einen Abfall der Markerwerte an.

4.3 Diskussion des Studiensettings und Perspektiven

Der neoadjuvante Therapieansatz bei Krebserkrankungen ist eine in den letzten Jahren entwickelte und intensivierte Methodik, die zu deutlichen Verbesserungen der Operabilität und der Langzeit-Ergebnisse bei Tumorpatienten geführt hat. Zur Kontrolle der Wirksamkeit von neoadjuvanten Therapien eignen sich im Blut zirkulierende Biomarker im besonderen Maße, da bei Studienbeginn nach Diagnose der Erkrankung zum Teil erhebliche Tumormasse vorhanden ist, die mit einer Ausschwemmung verschiedenster Substanzen ins Blut einher geht. Dies ist bei adjuvanten Therapien nach Entfernung des Primärtumors nicht der Fall. Zudem erfolgt im Anschluss an die neoadjuvante Therapie in der Regel eine Operation

inklusive pathologischer Aufarbeitung des Gewebes, so dass ein äußerst genaues posttherapeutisches Staging vorliegt. Bisherige Ansätze des Therapiemonitorings beim Mammakarzinom zielen auf die Veränderung der Serumkonzentration von Tumor-assoziierten Antigenen wie CEA, CA 15-3 oder HER2-neu, jedoch gibt es bislang für die neoadjuvante Situation noch wenige aussagekräftige Daten.

Weitere potentielle Biomarker sind zirkulierende Nukleinsäuren im Blut. Hierbei kann ähnlich wie bei Tumormarkern die Konzentration von Nukleosomen im Serum oder Plasma quantitativ bestimmt werden. Darüber hinaus ist der qualitative Nachweis von Tumor-spezifischen Veränderungen auf zirkulierender DNA wie genetische und epigenetische Marker möglich. Ferner bietet sich die Bestimmung der DNA Integrity an, welche das Verhältnis von langen zu kurzen DNA-Stücken beschreibt und sowohl für diagnostische Fragestellungen wie auch für die Prädiktion des Therapieansprechens einsetzbar ist.

Die hier durchgeführte Studie verbindet die guten Voraussetzungen des neoadjuvanten Settings mit einem relativ homogenen Kollektiv von Patientinnen in ähnlichen Tumorstadien, die eine vergleichbare diagnostische Untersuchungen und vergleichbare Chemotherapie-Regime erhielten. Für den differentialdiagnostische Bewertung der Marker wurde zudem auf eine homogene Altersverteilung der gesunden Probandinnen im Vergleich zu den erkrankten Probandinnen geachtet. Die Zeitpunkte der Biomarkermessungen richteten sich nach den Therapiezyklen der neoadjuvanten Chemotherapie und orientierten sich an Erfahrungen bereits früher durchgeführter Studien. Hierbei hatte sich eine Abnahme der Blutproben zu Beginn der Chemotherapie sowie Abnahmen im Bereich von 3 Wochen und ca. 60 Tagen nach Start als günstig erwiesen [28]. Eine in früheren Studien ebenfalls durchgeführte Abnahme 8 Tage nach Beginn der Chemotherapie war in diesem Kollektiv aus logistischen Gründen (lange Anfahrtswege der Patientinnen, keine reguläre Vorstellungen der Patientinnen zu diesem Termin) leider nicht durchführbar.

Vor dem Hintergrund, dass präanalytische Faktoren die Messergebnisse prinzipiell beeinflussen können, erfolgte die Blutentnahme und –verarbeitung nach einem standardisierten Protokoll, um die Fehleranfälligkeit zu minimieren. Ein Vorteil hierbei war die unizentrische Anlage der Studie, die gewährleistete, dass alle Proben mit den gleichen Standards aserviert wurden.

Zur Sicherung der Qualität der DNA-Quantifizierung wurden die Proben zunächst bei -80°C gelagert und zu einem späteren Zeitpunkt gesammelt vermessen. Hierbei wurde insbesondere auf eine standardisierte Extraktion sowie quantitative DNA-Messung geachtet. Die hohen Effizienzen der Standardkurve belegen die sehr gute Qualität der Messmethode. Zudem erfolgte die quantitative DNA-Messung im Rahmen der PCR als Doppelbestimmung, wobei nur Proben in die Auswertung eingingen, die einen Variationskoeffizienten kleiner 20% aufwiesen. Zur Kontrolle der Präzision innerhalb sowie zwischen verschiedenen Messläufen wurden mit jeder PCR-Platte verschiedene Kontrollproben mitgeführt. Die Studie erfüllt die MIQE-Richtlinien zur Überprüfbarkeit von Studien, in denen die quantitative PCR als Methode eingesetzt wird (siehe Supplementary Data der Diagnostik-Studie [26]).

Eine weitere Stärke der vorliegenden Studie ist der durchgehende Vergleich der neuen, zu testenden Biomarker mit den oben genannten, beim Mammakarzinom etablierten Tumormarker CEA und CA 15-3. Dies ist ein Qualtitätsmerkmal, welches für Biomarkerstudien von Fachgesellschaften gefordert wird, um eine Einordung im Vergleich zu den Routinemarkern vornehmen zu können [25].

Außerdem ist hervorzuheben, dass die Erhebung und Dokumentation der Patientendaten, die Messung der Biomarker sowie die statistische Auswertung jeweils unabhängig voneinander an verschiedenen Orten durch unterschiedliche Personen erfolgte und somit ein „Erwartungs-Bias“ vermieden wurde.

Einige Studien befassten sich bereits mit dem potentiellen diagnostischen und prognostischen Nutzen von zellfreier DNA und DNA Integrität bei verschiedenen Tumorentitäten [29, 30]. Allerdings gibt es bislang keine Daten zur DNA Integrity hinsichtlich der Prädiktion des Therapieansprechens bei Tumorpatienten während einer neoadjuvanten Therapie. Somit erlaubten die Ergebnisse unserer Studie erstmals eine Einschätzung dieses neuen Markers bei neoadjuvant therapierten Patientinnen mit einem Mammakarzinom sowohl für die differentialdiagnostische Abgrenzung zu Kontrollgruppen wie auch für die Prädiktion des Therapieansprechens.

Wir wählten hierfür einen mehrfach genutzte Methode zur DNA-Integrität, bei der so genannte ALU Sequenzen verwendet werden. Die ausgewählten DNA-Fragmente mit charakteristischer Länge kleiner und größer der nukleosomalen Fragmentgröße

(115 bp und 247 bp) weisen jeweils eine große Menge an repetitiven DNA Sequenzen auf und sind somit methodisch leichter zu detektieren. Zudem erhöht sich die Aussagekraft durch die DNA Menge, da kleine Messabweichungen keine starken Auswirkungen auf die Gesamtmenge der DNA zeigen. Andere Arbeiten bedienen sich anderer Primer, längerer oder kürzerer Amplicons oder Einzel-Gen-Sequenzen zur Berechnung der DNA-Integrität. Nachdem wir die Brauchbarkeit bereits bekannter Marker für den diagnostischen Einsatz testen wollten, haben wir uns für die bekannten ALUs (wie von Umetani beschrieben [15]) entschieden. Allerdings haben wir zusätzlich zwei Rechenmethoden zur Bestimmung der DNA Integrität herangezogen, um zu sehen, welcher Algorithmus besser geeignet ist. Auch dieser Vergleich wurde bislang noch nicht durchgeführt.

Wenngleich im Rahmen dieser Studie eine repräsentative Anzahl an Patientinnen untersucht werden konnte, ist der hierbei gewählte, für eine Hypothesenbildung gedachte, explorative Ansatz der Arbeit als gewisse Limitation zu werten. Aufgrund der Vielzahl an Markern, Algorithmen und Messzeitpunkten können einzelne zufällige Zusammenhänge nicht sicher ausgeschlossen werden. Deshalb sind diese Ergebnisse an einem unabhängigen Kollektiv noch einmal gezielt zu validieren. Ein interessanter Aspekt wäre zudem die Bestimmung der Kinetik von ALU 115, ALU 247 und DNA Integrity innerhalb der ersten Tage nach Gabe einer Chemotherapie, wie sie für andere Biomarker durchgeführt wurde [28]. Möglicherweise erlauben bereits Veränderungen dieser Marker unmittelbar nach Therapiegabe Rückschlüsse auf das weitere Ansprechen der Behandlung.

5 **Zusammenfassung**

5.1 DNA Integrity und zirkulierende zellfreie DNA als Biomarker für die Diagnostik, Prognose und Prädiktion bei Mammakarzinompatientinnen unter neoadjuvanter Chemotherapie

Brustkrebs ist mit 23% aller jährlichen weltweiten Krebsdiagnosen der am häufigsten diagnostizierte Tumor weltweit [3].

Zirkulierende zellfreie DNA ist ein neuer Biomarker, der im Blut bei Patienten mit vielen verschiedenen Krebsentitäten als erhöht nachgewiesen werden konnte. Bei DNA Integrity handelt es sich um eine Rechenformel, die das Verhältnis von langen zu kurzen DNA Stücken im Blut berechnet. Hypothesen besagen, dass bei Tumorpatienten die Art des Zelltodes zu einer vermehrten Freisetzung von langen DNA Stücken im Vergleich zu Gesunden führt. Daher stiege das errechnete Verhältnis dieser beiden DNA Stücke bei Tumorpatienten im Vergleich zu Gesunden.

Die vorliegende Studie befasst sich zum einen mit der diagnostischen Aussagekraft der zirkulierende zellfreie DNA und DNA Integrity. Hierzu wurden 65 Mammakarzinompatientinnen mit lokalisiertem Tumor, 47 Patientinnen mit metastasiertem Mammakarzinom sowie 28 gesunde Probandinnen und 12 Patientinnen mit benignen Brusterkrankungen vor Start einer etwaigen Therapie Plasmaproben entnommen. Hierbei zeigte sich eine gute Diskriminationsfähigkeit von ALU 115 (ein 115 Basenpaare langes zirkulierendes DNA Stück) und ALU 247 (247 Basenpaare lang). Beide Marker unterschieden gesunde Probanden von malignen wie benignen Erkrankungen (p-Werte alle <0.001). Die DNA Integrity, bei der es zwei verschiedene Berechnungsformeln gibt, grenzte zudem die benignen von den malignen Erkrankungen (p-Werte 0.015 bzw. <0.001) ab. Die konventionellen Tumormarker CEA und CA 15-3 unterschieden klar zwischen metastasierten Patientinnen und anderen Gruppen (p-Werte alle < 0.001).

Der zweite Teil der Studie befasste sich mit den 65 Patientinnen mit lokalisiertem Brustkrebs, die eine neoadjuvante Chemotherapie erhielten, während derer ihnen zu bestimmten Zeitpunkten Plasmaproben entnommen wurden. Zielsetzung war, bereits im Laufe der Chemotherapie das Ansprechen der Patientinnen auf die systemische Therapie zu evaluieren (Prädiktion und Prognose). Hierbei zeigte sich, dass ALU 115 in der Lage war, Patientinnen mit exzellentem Ansprechen auf die Chemotherapie von Patientinnen ohne Ansprechen auf die Chemotherapie zu trennen. Patientinnen

mit exzellentem Ansprechen verringerten ihre ALU 115 Werte 60 Tage nach Beginn der Therapie um durchschnittlich 40% im Vergleich zu den prätherapeutischen Werten, wohingegen Patientinnen ohne Ansprechen ihre Werte um durchschnittlich 100% steigerten (p-Wert 0.033). In der Gruppe mit exzellentem Ansprechen zeigte sich zudem ein höherer Anteil an Her2/neu positiven Patienten.

Die hier vorliegende Studie zeigt den Wert zirkulierender zellfreier DNA im Rahmen der Diagnostik von Brustkrebserkrankungen. Soweit den Autoren bekannt ist, behandelt sie als erste Studie überhaupt den prädiktiven und prognostischen Wert von zirkulierender zellfreier DNA und DNA Integrity im Rahmen einer neoadjuvante Chemotherapie bei Brustkrebserkrankungen. Weitere Studien werden zeigen, in wie weit zellfreie DNA und DNA Integrity in Zukunft als Tumormarker bei Brustkrebs von Nutzen für den klinischen Alltag sein können.

5.2 DNA Integrity and circulating cell-free DNA as biomarker for diagnosis, prediction and prognosis in breast cancer patients undergoing neoadjuvant chemotherapy

With 23% of all diagnosed cancer yearly, breast cancer is the most frequently diagnosed cancer in women worldwide [3].
Circulating cell-free DNA is a new biomarker, that was shown to be elevated in plasma and serum of patients with different sorts of cancer. DNA Integrity on the other hand is a formula, that calculates a quotient of longer to shorter DNA fragments. Longer DNA fragments are supposed to be elevated in blood of cancer patients when compared to healthy individuals, due to the fact that different cell death occurs in tumor patients. Following this hypothesis, DNA Integrity should be elevated in tumor patients when compared to healthy individuals.
The following study was conducted to find out about the diagnostic capability of DNA Integrity and circulating cell-free DNA (cfDNA) in breast cancer. Plasma samples of 65 patients with localized breast cancer, 47 patients with metastasized breast cancer, 28 healthy individuals and 12 patients with benign breast diseases were taken before the start of any therapy. The results showed a high discriminative power of both ALU 115 and ALU 247 (two circulating cell-free DNA fragments of 115 and 247 basepair length) when comparing healthy individuals to both benign and malign diseases ($p<0.001$). DNA Integrity, which was calculated with two different formula, was able to differ between benign and malign diseases ($p=0.015$ and $p<0.001$). CEA and CA 15-3, which were measured in order to compare the new biomarkers to established ones, were higher in metastasized breast cancer patients when compared to all other groups ($p<0.001$).
In the second part of the study, plasma samples 65 patients with localized breast cancer were taken at several points while undergoing a neoadjuvant chemotherapy. The aim of the study was to find out whether cfDNA, DNA Integrity, CEA and CA 15-3 were able to find out about the response to chemotherapy while undergoing neoadjuvant treatment (prediction and prognosis). Results showed ALU 115 to be the only measured marker to distinguish patients with excellent chemotherapy response from patients without chemotherapy response. Values of ALU 115 at day 60 after start of neoadjuvant chemotherapy were compared with pretherapeutic values. Patients with excellent response had a median decrease of 40% in values, where as patients without response had a median increase of 110% (p-value 0.033).

Furthermore, the complete remission group contained a significantly higher percentage of Her2/neu positive patients.

The present study shows the value of circulating cell-free DNA in breast cancer diagnosis. As far as to our best knowledge, this study is the first to investigate the prognostic and predictive values of circulating cell-free DNA and DNA Integrity in breast cancer patients undergoing neoadjuvant chemotherapy. Further research will have to be done in order to find out about the clinical use of these tumor markers in breast cancer patients.

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7 Eigenanteil an den vorgelegten Arbeiten

Die Konzeption der vorliegende Studie erfolgte durch Herrn PD Dr. Stefan Holdenrieder (SH) sowie Herrn Dr. Oliver J. Stötzer (OJS). Im Rahmen der Studienkonzeption wurde eine Einverständniserklärung der Ethikkommision eingeholt.

Die Patientenrekrutierung, klinische Dokumentation und Validierung des Ansprechens auf die Chemotherapie mittels Auswertung der Pathologiebefunde erfolgte durch OJS, Frau Dr. Debora Fersching-Gierlich (DFG) und Frau Doktorandin Julia Lehner (JL) in der Praxis Dr. Stötzer/Prof. Salat in der Franz-Schrank-Str. 2 in München, in der auch die Patientinnen diagnostiziert und behandelt wurden.

Ebenfalls in obengenannter Praxis durchgeführt wurde die Probenentnahme und die sofortige Weiterverarbeitung des Blutmaterials. Anschließend wurden die Proben nach Bonn transportiert. Für diesen Teil der Studie zeichnen sich die Doktorandin JL, DFG, SH sowie OJS verantwortlich.

Die Methodenetablierung, die eigentlichen Messungen sowie das Qualitätsmanagement wurde durch die Doktorandin JL unter Supervision von SH am Institut für Klinische Chemie und Klinische Pharmakologie der Rheinischen Friedrich-Wilhelms-Universität in Bonn durchgeführt. Diese Kooperation entstand durch den Wechsel von Herrn PD Dr. med. S. Holdenrieder an die Universität Bonn im Sommer 2010.

Anschließend erfolgte die statistische Auswertung der gemessenen Werte durch Frau Dr. rer. nat. D. Nagel (DN). Die weitere Diskussion sowie Interpretation der Ergebnisse wurde durch JL und SH durchgeführt. DN stand zudem bei graphischen Darstellungen der Ergebnisse unterstützend zur Seite.

Beide Publikationen wurden von der Doktorandin JL verfasst, anschließend von SH revidiert und in Kooperation mit OJS in ihre hier vorgelegte Fassung gebracht. Die Präsentation auf mehreren wissenschaftlichen Kongressen erfolgte durch JL, SH und OJS.

Zusammenfassend war die Doktorandin JL an der Patientenrekrutierung, der klinischen Dokumentation, der Probenverarbeitung und Logistik beteiligt. Die laborchemischen Messungen führte sie an der Universität Bonn selbstständig durch.

Im Anschluss an die statischen Auswertungen durch Frau Dr. D. Nagel erfolgte die Diskussion und Interpretation der Daten durch die Doktorandin in Zusammenarbeit mit SH. Anschließend fertigte die Doktorandin selbstständig beide Publikationen an.

8 **Originalarbeiten**

8.1 Publikationen für die kumulative Dissertation

Die in dieser Arbeit erläuterten Ergebnisse wurden in mehreren Originalarbeiten veröffentlich. Als Grundlage zum Erwerb der kumulativen Dissertation werden eine Arbeit zur Diagnostischen Relevanz und eine Arbeit zum Verlauf im Rahmen der neoadjuvanten Chemotherapie eingereicht.

- Diagnostic relevance of plasma DNA and DNA integrity for breast cancer.
  Stötzer OJ, Lehner J, Fersching-Gierlich D, Nagel D, Holdenrieder S.
  Tumour Biol. 2013 Sep 10. [Epub ahead of print]

Dieser Artikel befasst sich mit der Stellung der Biomarker ALU 115, ALU 247, DNA Integrity 1 und 2 sowie CEA und CA 15-3 im Rahmen der Diagnose von Mammakarzinomen im Vergleich zu benignen Erkrankungen und gesunden Probanden.
Tumor Biology ist die Zeitschrift der International Society of Oncology and Biomarkers (ISOBM) und gehört zum Springer Verlag. Herausgeber ist Prof. Torgny Stigbrand von der Umea Universität in Schweden. Der Impact Faktor im Jahre 2012 liegt bei 2,518 (2012 Journal Citation Reports® Science Edition; Thomson Reuters).

- Circulating plasma DNA and DNA integrity in breast cancer patients undergoing neoadjuvant chemotherapy.
  Lehner J, Stötzer OJ, Fersching D, Nagel D, Holdenrieder S.
  Clin Chim Acta. 2013 Aug 2;425C:206-211. doi: 10.1016/j.cca.2013.07.027

Diese Arbeit beleuchtet den Nutzen von zellfreier DNA und DNA Integrity als Prädiktions- und Prognosemarker im Rahmen der neoadjuvanten Chemotherapie.
Die Zeitschrift Clinica Chimica Acta wird durch den Verlag Elsevier aufgelegt. Sie vertritt die International Federation of Clinical Chemistry and Labaratory Medicine (IFCC). Im Jahre 2012 lag der Impact Faktor bei 2,850 (2012 Journal Citation Reports® Science Edition; Thomson Reuters). Damit belegt Clinica Chimica Acta im Fachbereich Medizinische Labor Technologie bei ISI Web of Knowledge Platz 6 (von 31).

8.2 Weitere Publikationen

Elevated DNA Integrity In Colorectal Cancer
*Leszinski G, **Lehner J**, Gezer U, Holdenrieder S*
Anticancer Research 2014, accepted

Plasma DNA Integrity indicates response to neoadjuvant chemotherapy in patients with locally confined breast cancer
***Lehner J**, Stötzer OJ, Fersching DM, Nagel D, Holdenrieder S.*
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8.3 Posterbeiträge

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Diagnostic and predictive relevance of plasma DNA and DNA integrity in patients with localized breast cancer undergoing neoadjuvant chemotherapy.
CESAR Annual Meeting Abstract Book S. 51
Jahrestagung der Central European Society on Anticancer Drug Research (CESAR) 2012 in Essen, *Posterpräsentation*

Stötzer OJ, **Lehner J**, Fersching D, Salat C, Nagel D, Holdenrieder S.
Circulating immunogenic cell death markers HMGB1 and RAGE in breast cancer patients during neoadjuvant chemotherapy.
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Wittwer C, **Lehner J**, Fersching D, Siegele B, Stötzer OJ, Holdenrieder S.
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Eidesstattliche Versicherung

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Name, Vorname

Ich erkläre hiermit an Eides statt,

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Frühzeitige Beurteilung der Effizienz systemisch-zytotoxischer Therapien bei Patientinnen mit Mammakarzinom durch zirkulierende Nukleinsäuren.

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RESEARCH ARTICLE

Diagnostic relevance of plasma DNA and DNA integrity for breast cancer

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Abstract Levels of ALU 115, ALU 247, DNA integrity ([1, 2]) and of the tumour markers CA 15–3 and CEA were analysed in the blood of 152 patients. Plasma levels of ALU 115 and ALU 247 were significantly higher in patients with locally confined (LBC; $N=65$), metastatic breast cancer (MBC; $N=47$), and benign diseases ($N=12$) than in healthy controls ($p<0.001$ for all comparisons). DNA integrity, CEA, and CA 15–3 were significantly higher in MBC than in benign controls and LBC but could not identify LBCs. The best discrimination of LBC from healthy controls was achieved by ALU 115 and ALU 247 (AUC 95.4 and 95.5 %) and of MBC from all control groups by CA 15–3 and CEA (AUC 83.2 and 79.1 %). Plasma DNA is valuable for the detection of LBC, while established tumour markers are most informative in MBC.

Keywords Plasma DNA · DNA integrity · Breast cancer · Neoadjuvant chemotherapy

Introduction

With 1.38 million new cases in 2008, breast cancer still represents the most frequently diagnosed cancer in women worldwide [3]. About 458,000 women die due to this disease each year [3]. An increased incidence of breast cancer (60 % of all cases) is known for developed countries like Western/Northern Europe, North America, and Australia, which is also due to early stage detection as a result of screening programs [3]. Whereas radiological screening programs (mammography) have demonstrated to be useful in detecting breast cancer in earlier stages, no valuable blood biomarkers have been identified for that purpose up to now. [4].

Several studies analysing the benefit of using the established tumour markers cancer antigen 15–3 (CA 15–3) and carcinoembryonic antigen (CEA) in breast cancer have been published. Whereas multiple investigations demonstrated the efficacy of CEA and CA 15–3 in monitoring the course of metastatic breast cancer, this has not yet been addressed in the neoadjuvant setting [5]. While a few studies support an effect of these markers on earlier relapse detection in breast cancer after curable surgery [6], there is still no evidence that CEA and CA 15–3 are valuable tools for early breast cancer detection and screening [7]. Therefore, there is yet a need for reliable biomarkers as an aid in breast cancer screening, early detection of local or distant relapse, and monitoring and predicting response to primary systemic chemotherapy.

Elevated levels of circulating cell-free DNA (cfDNA) have been detected in diseases of different origins, such as trauma, stroke, burns, sepsis, autoimmune diseases, and also cancer [8–12]. This broad prevalence of diseases with potentially elevated cfDNA levels limits to a certain extent the diagnostic specificity [13]. However, cfDNA has been identified to offer high sensitivity for cancer detection [1, 14, 15] and to indicate a high prognostic and predictive value in various solid tumour diseases [16]. Several approaches have been used to measure cfDNA in plasma and serum, including non-coding DNA (like repetitive ALU sequences [1, 2] or LINE1 (long interspersed nucleotide elements) [14]). These repetitive DNA sequences are known to be distributed everywhere in the genome, with approximately 1.4 million copies per genome for the ALUs [17, 18].

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Umetani et al. described primers and a quantitative PCR method to measure ALU 115 and ALU 247 in which the smaller ALU 115 fragments were an integral part of the larger ALU 247 fragments [1, 2]. During apoptotic cell death, DNA is cleaved by specific endonucleases to nucleosomal or to subnucleosomal fragments smaller than 180 bp, while during necrotic cell death longer fragments are produced by a non-specific cleavage [19, 20]. Following this hypothesis, ALU 247 is then supposedly a marker of necrotic cell death, while ALU 115 is associated with either form of cell death. As elevated cellular proliferation and, in parallel, elevated rates of diverse forms of cell death are characteristic biological features of tumour growth [21], elevated levels of cfDNA and a higher portion of longer DNA fragments (DNA integrity) are supposedly useful blood markers for cancer detection [20].

Concerning the so-called DNA integrity that potentially mirrors the relation between the necrotic and overall cell death rate, different calculations have been used. Umetani et al. simply calculate the ratio of the concentrations of longer DNA fragments (ALU 247) to shorter DNA fragments (ALU 115) [1, 2], while Wang et al. [22] use a more sophisticated formula based on Cp value differences. Both groups demonstrate significantly higher portions of long fragments in the plasma and the serum of cancer patients than in healthy controls. However, they do not compare their results with each other or with established protein tumour markers.

The present study was conducted to find out whether quantitative levels of ALU 115 and ALU 247 and the two DNA integrity formulas are powerful biomarkers for the diagnosis of breast cancer as well as for tumour characterization and staging purposes. Furthermore, we compared these biomarkers with the already established and routinely used cancer biomarkers CEA and CA 15–3 to identify their specific relevance in the clinical setting.

Patients and methods

Patients

Between 2007 and 2011, plasma samples of 112 breast cancer patients were collected at the time of diagnosis and before the therapy started. Forty-seven of the patients suffered from metastatic breast cancer (MBC); 65 had a locally confined breast cancer (LBC; UICC stages II and III). Additionally, we collected plasma samples of 40 controls, including 28 healthy female controls and 12 patients with benign breast diseases.

In all breast cancer patients, complete relevant histopathological staging (subtype, grading, oestrogen receptor status, progesterone receptor status, Her2/neu-status) was pretherapeutically assessed. Further clinical and radiological staging—including mammography, ultrasound, chest X-ray, abdomen ultrasound, and bone scintigraphy—were performed. In the neoadjuvant setting, the histology was done by fine needle biopsy or vacuum biopsy and underwent a clinical classification according to the TNM system. In all other cases, a complete pathological and clinical TNM status was available. In breast cancer patients, venipunctures were regularly performed before starting a neoadjuvant or palliative systemic chemotherapy in controls before any therapy was started.

The study was approved by the local ethics committee. Patients were intensively informed of the study; prospective and written informed consent was obtained from all patients before study entry.

Plasma preparation for qPCR

Plasma samples (4.4 ml) and serum samples (10 ml) were collected in K2-EDTA and gel separation tubes, respectively (Sarstedt, Nürnbrecht, Germany). All samples were centrifuged within 1 to 2 h after venipuncture. Plasma and sera were separated, aliquoted, and cryopreserved at −80 °C.

DNA isolation was done with a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Initially, 400 μl of plasma sample and 400 μl of lysis buffer were added to a vial containing 20 μl of Qiagen protease. After the mixture of the reagents and 30 min of incubation at 56 °C, 400 μl of 100 % ethanol was pipetted into the vials and mixed. Subsequently, a vacuum pump was used to wash the two washing buffers (750 μl each) through spin columns. Afterwards, the spin columns were centrifuged, 50 μl of lysis buffer was added, and one more centrifugation followed to elute the DNA from the column filter. Five microliters of this eluate was used as a template for the qPCR.

Quantitative PCR of ALU repeats

For the qPCR of the ALU repeats, we used the same primers as described by Umetani et al. [1] (“Electronic supplementary material 1”). The reaction mixture for the qPCR contained 5 μl of template, 0.25 μl of uracile DNA glycosylase (UNG, Roche Diagnostics, Mannheim, Germany) to prevent carryover contamination, 2 μl of each primer (forward and reverse), 6.75 μl of PCR grade H_2O, and 4 μl of Mastermix SYBR Green (Roche Diagnostics), resulting in 20 μl of reaction volume.

Real-time PCR amplification was performed using the LightCycler® 480 Instrument II (Roche Diagnostics, Mannheim Germany). It started with 10 min of incubation time for the uracil–DNA–glycosylase at 40 °C, followed by 10 min of UNG inactivation time at 95 °C. The real-time PCR amplification was conducted with 45 cycles of denaturation (at 95 °C for 10 s), annealing (at 62 °C for 15 s), and extension (at 72 °C for 15 s). To determine the absolute quantitative amount of DNA in the samples, a standard curve was calculated. We used serial dilutions of 20 to 0.076 ng/ml of DNA (Roche Diagnostics) in ten dilution steps. The standard curve for ALU

115 had an efficiency of 1.95; for ALU 247 the efficiency was 1.84 (“Electronic supplementary material 2”). Additionally to the samples, a negative and a positive control, two patient plasma pools with high and low DNA levels as well as three dilution step samples of the standard curve were performed with every plate for quality control. All measurements were done in duplicates (description according to MIQE standards; see “Electronic supplementary material 3”).

Calculation of the DNA integrity index

DNA integrity was calculated according to two different algorithms according to Wang et al. [22] and Umetani et al. [1, 2]. For the calculation of the DNA integrity index according to Umetani et al. (DNA Int 1), the ratio of the concentration of ALU 247 sequences to the concentration of ALU 115 sequences was calculated. This ratio can theoretically vary between 0 and 1 as the ALU 115 sequences are represented within the annealing sites of ALU 247 [1]. Assuming that DNA fragments originating from apoptosis are mainly sized below 180 bp, a high index would indicate a considerable contribution of non-apoptotic cell death, such as necrosis.

For the calculation of the DNA integrity index according to Wang et al. (DNA Int 2), the difference between the Cp value of a standard pool of human genomic DNA (which was measured with every PCR plate) and the Cp value of each sample for ALU 115 and for ALU 247 to obtain ΔCp 115 and ΔCp 247 was used. These two ΔCp values were subtracted (ΔCp115−ΔCp247) to obtain ΔΔCp. Subsequently, DNA integrity was calculated using the formula: $e^{(-\Delta\Delta Cp \times \ln(2))}$.

Determination of established tumour markers

CA 15–3 and CEA were measured by enzymatic chemiluminescent immunoassay (ECLIA) on the ElecSys 2010 immunoassay analyser of Roche Diagnostics, Germany, in sera of breast cancer patients.

Statistics

The concentrations of all measured markers before the start of a therapy in the breast cancer groups as well as the measurements of the healthy and benign group were considered for statistical evaluation.

Medians, percentiles, and ranges are presented in tables for biomarker concentrations within the different groups. Dot plots show the individual marker distribution. Discriminative power between the groups was tested by overall analysis of variance on ranks of data followed by the Ryan–Einot–Gabriel–Welsch multiple-range test to assess the significance of differences between single groups. Additionally, results are illustrated in receiver operating characteristic (ROC) curves.

The correlation of biomarkers with disease characteristics, such as TNM stage and receptor status (oestrogen receptor, progesterone receptor, and Her2/neu receptor), was done by Wilcoxon test or Kruskal–Wallis test. The correlation of biomarkers with each other was done by Spearman rank–correlation test.

A p-value of <0.05 was considered to be statistically significant. All calculations were performed with SAS software (version 9.2, SAS Institute Inc., Cary, NC, USA).

Results

Clinical data of patients with primary breast cancers

Clinical and histopathological data of patients suffering from breast cancer and controls, including age, tumour subtype, grading, receptor-status, Her2/neu status, clinical and/or pathological TNM, and UICC stage, and radiological results are given in Table 1.

Table 1 Patient characteristics

Patients and controls, *N* (age, median)		
Locally confined breast cancer	65	47.0
Metastatic breast cancer	47	60.8
Benign breast diseases	12	45.5
Healthy controls	28	41.7
Characteristics of patients with locally confined breast cancer, *N* (%)		
T stage		
1	8	12.3
2	39	60.0
3	14	21.5
4	3	4.6
X	1	1.6
N stage		
0	17	26.1
1	40	61.6
2	1	1.6
3	3	4.6
X	4	6.1
Histology		
Invasive ductal carcinoma	57	87.7
Invasive lobular carcinoma	4	6.1
Adenocarcinoma	2	3.1
Unknown	2	3.1
Histopathological classification, *N* (%)		
Oestrogen receptor positive/negative	38/27	58.5/41.5
Progesterone receptor positive/negative	32/33	49.2/50.8
Her2/neu receptor positive/negative	21/44	32.3/67.7
Triple negatives/non-negatives	21/44	32.3/67.7

Biomarker values in different patient groups; diagnostic value

Plasma levels of ALU 115 were discriminated significantly between the single groups by Ryan–Einot–Gabriel–Welsch multiple-range test ($p<0.0001$). Median values in healthy females (1.8 ng/mL) were significantly lower than in patients with benign diseases (27.4 ng/mL) and in patients with LBC (15.9 ng/mL) and with MBC (22.3 ng/mL).

Similar results were obtained for ALU 247. Overall significance for the discrimination of the single groups was $p<0.0001$. There was a significant difference between median ALU 247 levels in healthy controls (1.9 ng/mL) and benign diseases (22.3 ng/mL), LBC (16.8 ng/mL), and MBC (29.8 ng/mL). In addition, ALU 247 levels significantly discriminated between benign diseases and MBC as well as between LBC and MBC (Table 2; Fig. 1a, b).

For both DNA integrities, overall significances for the discrimination of the single groups were $p=0.0003$ and $p<0.0001$, respectively. DNA integrity index 1 (DNA Int 1), representing the ratio of ALU 247 to ALU 115, was able to distinguish between healthy controls (median 1.2) and benign diseases (0.9) and between benign diseases and both LBC (1.1) and MBC (1.2). DNA integrity index 2 (DNA Int 2) showed significant differences between healthy controls (1.0) and benign diseases (0.7) as well as between benign diseases and LBC (0.8) and MBC (1.2), respectively (Table 2; Fig. 1c, d).

With respect to the established marker CEA, locally confined tumours could not be distinguished from the control groups of healthy women and from those with benign breast diseases. However, women with MBC (6.0 ng/mL) had significantly higher median CEA levels than healthy women (1.0 ng/mL), women with benign breast diseases (0.7 ng/mL), and patients with LBC (1.3 ng/mL). Comparable results were obtained for CA 15–3 that also revealed highly significant differences of median values in patients with MBC (61.3 U/mL) and all other groups, such as healthy women (17.6 U/mL), patients with benign diseases (17.3 U/mL), and patients with LBC (19.1 U/mL). Similar to CEA, CA 15–3 was not able to discriminate between locally confined tumours and either control group. Overall significances for the

Table 2 Biomarker values in different patient groups

Marker	Group	Number of cases	Median	Minimum	Maximum	Comparison with		
						Benign (p-value)	Localized breast cancer (p-value)	Metastatic breast cancer (p-value)
ALU 115 (ng/mL); overall $p<0.0001$	Healthy	28	1.8	0.1	3.2	Sig.	Sig.	Sig.
	Benign	12	27.4	1.4	89.2		Not sig.	Not sig.
	Localized breast cancer	65	15.9	0.7	871.8			Not sig.
	Metastatic breast cancer	47	22.3	3.3	827.1			
ALU 247 (ng/mL); overall $p<0.0001$	Healthy	28	1.9	0.3	4.4	Sig.	Sig.	Sig.
	Benigns	12	22.3	1.4	63.8		Not sig.	Sig.
	Localized breast cancer	65	16.8	0.8	577.6			Sig.
	Metastatic breast cancer	47	29.8	5.1	835.6			
DNA-Int 1; overall $p=0.0003$	Healthy	28	1.2	0.5	9.3	Sig.	Not sig.	Not sig.
	Benign	12	0.9	0.5	1.1		Sig.	Sig.
	Localized breast cancer	65	1.1	0.6	1.7			Not sig.
	Metastatic breast cancer	47	1.2	0.6	1.9			
DNA-Int 2; overall $p<0.0001$	Healthy	28	1.0	0.3	1.9	Sig.	Not sig.	Not sig.
	Benign	12	0.7	0.4	1.0		Not sig.	Sig.
	Localized breast cancer	65	0.8	0.4	1.5			Sig.
	Metastatic breast cancer	47	1.1	0.5	2.1			
CEA (ng/mL); overall $p<0.0001$	Healthy	27	1.0	0.2	4.2	Not sig.	Not sig.	Sig.
	Benign	12	0.7	0.2	3.4		Not sig.	Sig.
	Localized breast cancer	62	1.3	0.2	14.1			Sig.
	Metastatic breast cancer	41	6.0	0.3	2,608			
CA 15–3 (U/mL); overall $p<0.0001$	Healthy	27	17.6	5.6	26.9	Not sig.	Not sig.	Sig.
	Benign	12	17.3	8.2	41.1		Not sig.	Sig.
	Localized breast cancer	62	19.1	6.3	258.0			Sig.
	Metastatic breast cancer	41	61.3	10.0	319,000			

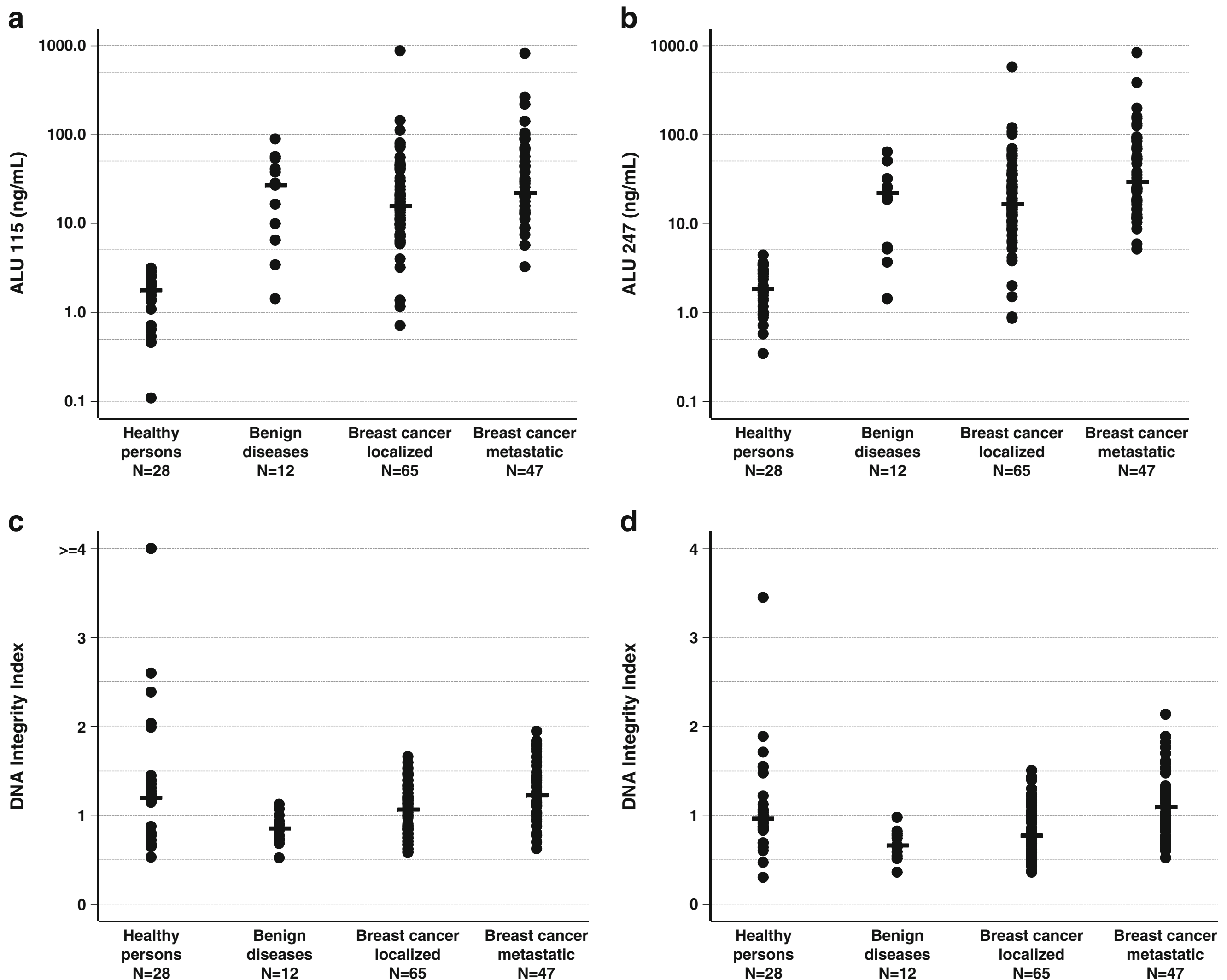

Fig. 1 Value distribution and medians of ALU 115 (**a**), ALU 247 (**b**), DNA integrity index 1 (**c**), and DNA integrity index 2 (**d**) in the plasma of healthy persons, patients with benign breast diseases, patients with locally confined and metastatic breast cancer

discrimination of the single groups were $p<0.0001$ for CEA and CA 15–3, respectively (Table 2; "Electronic supplementary materials 4 and 5").

For a comparison of LBC with healthy persons, the diagnostic efficiency was highest for ALU 115 and ALU 247, reaching an area under the curve (AUC) of ROC curves of 95.4 and 95.5 %, respectively. AUCs of CA 15–3 and CEA were 56.9 and 59.3 % only, of DNA Int 1 39.8 %, and of DNA Int 2 35.4 %. Sensitivities for cancer detection at 95 % specificities were 93.8 % (ALU 115), 92.3 % (ALU 247), 0 % (DNA Int 1 and 2), 30.6 % (CA 15–3), and 8.1 % (CEA) (Fig. 2a).

For a comparison of MBC from either control group, the diagnostic efficiency was highest for CA 15–3 and CEA, reaching AUCs in ROC curves of 83.2 and 79.1 %, respectively. AUCs for ALU 115, ALU 247, and DNA Int 1 and 2 were slightly lower with 73.0, 76.4, 64.5, and 71.1 %, respectively. Sensitivities for MBC detection at 95 % specificities were 19.1 % (ALU 115), 29.8 % (ALU 247), 2.1 % (DNA Int 1), 17.0 % (DNA Int 2), 48.8 % (CA 15–3), and 56.1 % (CEA) (Fig. 2b).

Correlation with disease characteristics (TNM and UICC stage, receptor status)

Information on the clinical TNM and receptor status (oestrogen, progesterone, and Her2/neu receptor) was gained by pretherapeutic biopsy. When locally confined tumours (T stage 1 and 2) were compared with locally advanced tumours (T stage 3 and 4), none of the markers were able to differentiate between these groups. Concerning the nodal (N) stage, CEA was higher in lymph node positive (N1–3) as compared with lymph node negative (N0) patients ($p=0.045$), while the other markers showed no differences. In addition, ALU 247 correlated with the progesterone receptor status, with higher levels for the receptor-positive group ($p=0.041$). No differences of biomarker levels were observed between patients

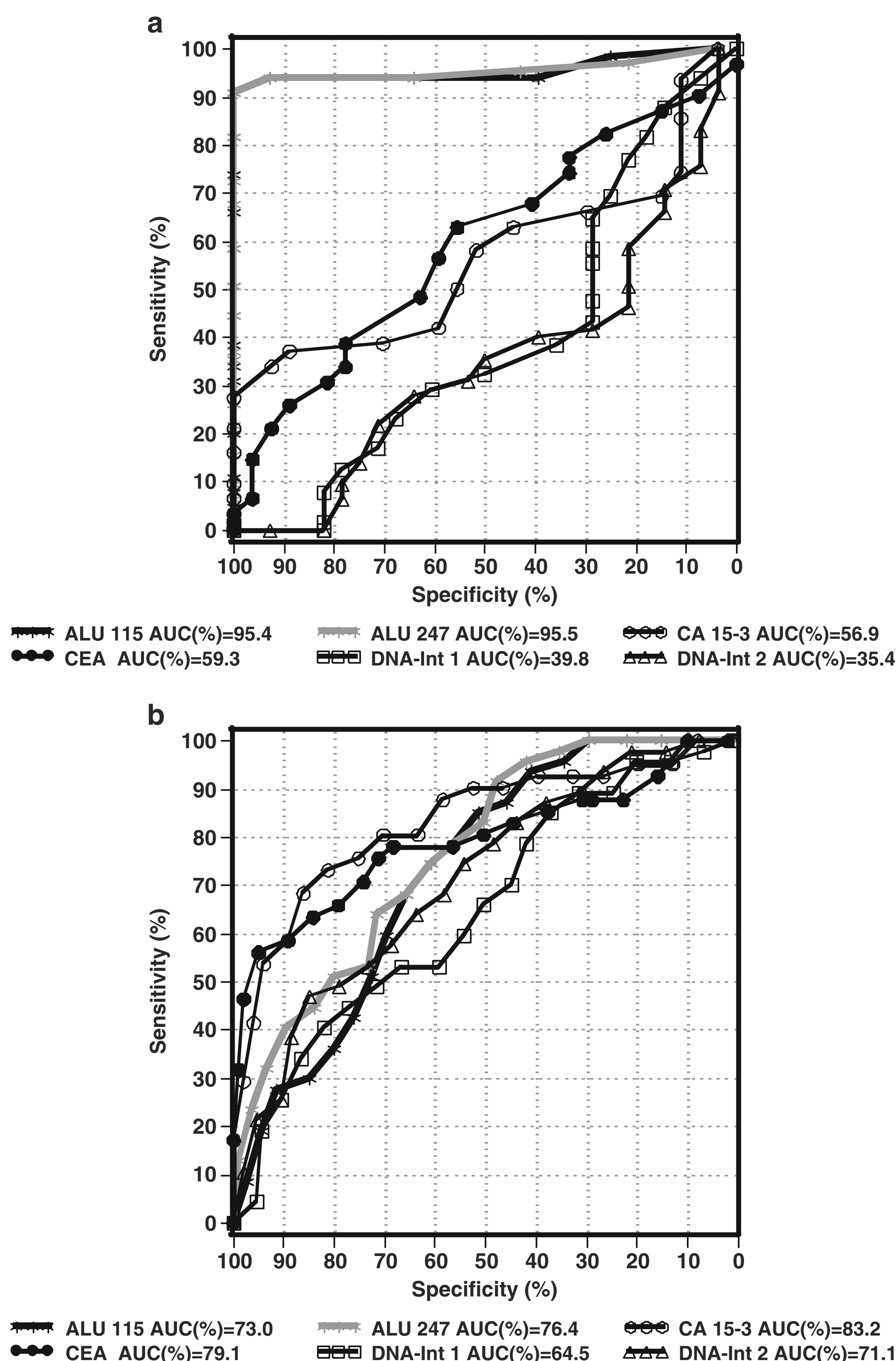

Fig. 2 ROC curves of plasma ALU 115, ALU 247, DNA integrity index 1 and 2 as well as of serum CA 15–3 and CEA for the discrimination between **a** locally confined breast cancer and healthy control groups as well as between **b** metastatic breast cancer and all control groups (healthy, benign, locally confined breast cancer)

with Her2/neu receptor-positive or -negative tumours, and neither between oestrogen receptor-positive and -negative tumour patients. When comparing the patients with a triple-negative receptor status (oestrogen receptor, progesterone receptor, and Her2/neu receptor negative) with all other patients, no significant difference of marker values was found.

Correlation of the markers with each other

Biomarker levels showed highly significant positive correlations with each other for the conventional tumour markers, ALUs, and DNA integrity indices. A highly significant correlation was found between both DNA integrity indices for LBC

and MBC as well as for both ALUs when compared with each other. The conventional tumour markers showed a very good correlation with ALU 115 and ALU 247, particularly in metastatic patients (see Table 3).

Discussion

In order to establish new serum biomarkers for breast cancer, we investigated the diagnostic value of cell-free DNA in breast cancer patients.

Multiple studies have indicated elevated absolute levels of cell-free DNA (cfDNA) in breast, colorectal, lung, testicular, prostate, and ovarian cancer among others [1, 2, 17, 23–27]. Results from these studies suggest that cfDNA levels might be valuable in order to determine the tumour cell turnover.

In addition, DNA integrity—as the relation of longer to smaller DNA fragments—has been elevated in the plasma [2] and the serum of cancer patients [2]. As other studies reported inconsistent data not supporting these results [13, 28, 29], we tried to verify the clinical validity of DNA integrity in plasma as a diagnostic tool for breast cancer. As diverse algorithms are used for the calculation of the DNA integrity index, we used the two most popular methods to compare their clinical validity and to determine whether the combination of the indices, the absolute cfDNA levels, and the conventional markers CEA and CA 15–3 increases the diagnostic sensitivity.

In line with most studies that have investigated absolute levels of cfDNA in plasma, we found significantly higher levels of plasma cfDNA in cancer patients as compared to healthy controls. Unfortunately, no significant difference was obtained from this comparison of benign and malignant diseases. Indeed this is not really surprising as benign breast diseases often occur together with inflammation, and inflammation is known to increase cfDNA levels in the blood as well [9]. On the other hand, cfDNA levels can be low in cancer patients due to low cell death rates and a low half-life time of cfDNA in the plasma as a result of high DNA clearance [30].

With DNA Int 1 [1, 2], it was possible to differentiate between healthy controls and benign diseases and between benign diseases and both LBC and MBC. Interestingly, the DNA integrity of healthy individuals and of patients with malignant diseases did not differ, which is in contrast to the findings of Umetani et al., who report a clear discriminative difference [1].

Similar results were obtained for DNA Int 2 [22], presenting significant differences between healthy controls with benign diseases as well as between MBC with

Table 3 Correlation between the biomarkers

	CA153	CEA	ALU 115	ALU 247	DNA Int 1	DNA Int 2
All patients						
CA 15–3		0.399	0.361	0.412	0.234	0.273
		<.001	<.001	<.001	0.005	0.001
		142	142	142	142	142
CEA	0.399		0.282	0.323	0.158	0.199
	<.001		0.001	<.001	0.061	0.017
	142		142	142	142	142
ALU 115	0.361	0.282		0.977	−0.168	−0.003
	<.001	0.001		<.001	0.039	0.965
	142	142		152	152	152
ALU 247	0.412	0.323	0.977		0.021	0.148
	<.001	<.001	<.001		0.798	0.069
	142	142	152		152	152
DNA Int 1	0.234	0.158	−0.168	0.021		0.752
	0.005	0.061	0.039	0.798		<.001
	142	142	152	152		152
DNA Int 2	0.273	0.199	−0.003	0.148	0.752	
	0.001	0.017	0.965	0.069	<.001	
	142	142	152	152	152	
Locally confined breast cancer						
CA 15–3		0.047	0.024	0.045	0.221	0.134
		0.715	0.855	0.729	0.084	0.298
		62	62	62	62	62
CEA	0.0473		−0.087	−0.052	0.119	0.161
	0.715		0.503	0.686	0.355	0.212
	62		62	62	62	62
ALU 115	0.024	−0.087		0.961	−0.377	−0.146
	0.855	0.503		<.001	0.002	0.246
	62	62		65	65	65
ALU 247	0.045	−0.052	0.961		−0.141	0.629
	0.729	0.686	<.001		0.263	0.619
	62	62	65		65	65
DNA Int 1	0.221	0.119	−0.377	−0.141		0.832
	0.084	0.355	0.002	0.263		<.001
	62	62	65	65		65
DNA Int 2	0.134	0.161	−0.146	0.629	0.832	
	0.298	0.212	0.246	0.619	<.001	
	62	62	65	65	65	
Metastatic breast cancer						
CA 15–3		0.713	0.536	0.554	0.104	0.123
		<.001	0.001	0.001	0.519	0.443
		41	41	41	41	41
CEA	0.713		0.430	0.464	0.045	0.018
	<.001		0.005	0.002	0.781	0.910
	41		41	41	41	41
ALU 115	0.536	0.430		0.962	−0.060	0.207
	0.001	0.005		<.001	0.688	0.163
	41	41		47	47	47

Table 3 (continued)

	CA153	CEA	ALU 115	ALU 247	DNA Int 1	DNA Int 2
ALU 247	0.554	0.464	0.962		0.167	0.355
	0.001	0.002	<.001		0.262	0.014
	41	41	47		47	47
DNA Int 1	0.104	0.045	−0.060	0.167		0.734
	0.519	0.781	0.688	0.262		<.001
	41	41	47	47		47
DNA Int 2	0.123	0.018	0.207	0.355	0.734	
	0.443	0.910	0.163	0.014	<.001	
	41	41	47	47	47	

benign breast diseases and LBC. Notably, both formulas showed a high correlation with each other in all patients ($R=0.75$), LBC ($R=0.83$), and MBC ($R=0.73$) despite the level of absolute values being different. However, there was no or only a weak correlation with either ALU 115 and ALU 247 on the one hand and DNA integrity on the other hand. Interestingly, it was possible to differentiate between benign and malignant diseases by use of both types of DNA integrities, a feature which is an important tool for diagnostic markers. This information was not obtained with cfDNA or the conventional tumour markers. However, due to elevated DNA integrity levels in some healthy controls, its use as diagnostic markers is limited in the individual patient case.

It has to be pointed out that the levels of DNA integrity were often above the value of 1.0. This is theoretically implausible as, according to Umetani et al. [1], the annealing sites of ALU 115 are represented within the annealing sites of ALU 247, implying that ALU 115 is always present when ALU 247 can be measured. Several reasons may explain this phenomenon: Lower absolute cfDNA levels could be caused by the shorter denaturation, annealing, and extension times in the qPCR. However, this argument cannot clarify why the longer ALU fragments were measured more often, as a shorter extension time during qPCR would preferably affect the amplification of longer DNA fragments. Alternatively, primer binding to DNA could have been impaired. To improve primer binding, we additionally included different add-ons (DMSO and BSA) to the PCR setting; however, results were unchanged.

To assure the quality of pPCR measurements, plasma pools with high and low DNA levels were included in every run, resulting in quite constant levels in the inter-run comparison. Interestingly, the level of ALU 247 was higher even in both pools compared to the level of ALU 115 in the same pools. This finding confirms that the ALU 247 levels were elevated compared to the ALU 115 levels not only in cancer patients. In a recently published paper about cfDNA in patients with testicular germ cell cancer using another primer pair (where the annealing sites of the short DNA fragment were also within the longer DNA fragment), calculated DNA integrity levels often were above 1, too [23]. Nevertheless, it should be mentioned that both levels of ALU sequences were highly elevated in the plasma of cancer patients compared to healthy controls.

The direct comparison of our results with other studies remains difficult due to the use of different types of blood samples (serum or plasma). Furthermore, DNA isolation in the different studies [31] is not always comparable as the amount of isolated DNA varies highly between the different extraction kits [27]. Additionally, many different primers are used to determine the DNA integrity, impairing further the direct comparison of different studies [27]. In fact, studies using single-copy sequences have also been successfully applied for the quantification of DNA integrity [29]. Thus, further clinical validation of these assays is crucial to determine the relevance of both cfDNA and DNA integrity as a diagnostic tool under routine conditions [17].

As it is generally requested that new promising biomarkers are compared with already established ones [17], we included the breast tumour markers CA 15–3 and CEA in our evaluations. As expected, they had significantly higher values in MBC than in all other control groups. However, these markers could not distinguish between LBC and healthy controls. There was a highly significant correlation of CA 15–3 and CEA with each other in MBC but not in LBC patients. The same applies to comparisons of these tumour markers with ALU 115 and ALU 247, while there was only a slight correlation with DNA integrity in the all patients group. As a consequence, tumour markers performed best for the detection of MBC. However, for the detection of LBC, ALU 115 and ALU 247 were considerably better indicators, showing the potential diagnostic impact of these new markers for the early detection of breast cancer patients.

Conclusion

Although DNA integrity could not improve the diagnostic performance of the established markers, ALU concentrations were highly promising for the detection of locally confined breast cancers and surpassed the conventional biomarkers CEA and CA 15–3 by far for this indication. For the detection of MBC, CA 15–3 and CEA showed the overall best diagnostic profile.

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Conflicts of interest None

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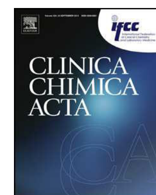

Circulating plasma DNA and DNA integrity in breast cancer patients undergoing neoadjuvant chemotherapy

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ABSTRACT

Background: In breast cancer patients undergoing neoadjuvant chemotherapy before surgery, biomarkers for predicting response to therapy are urgently required.

Patients and methods: In 65 patients with locally confined breast cancer who had completed the course of chemotherapy until surgery, plasma DNA biomarkers obtained before and during therapy were evaluated concerning (early) estimation of therapy response. Levels of repetitive ALU 115 and ALU 247 elements as well as DNA integrity calculated according the formulas of Umetani (1) and Wang (2) were correlated with changes in histopathological staging at surgery and compared with conventional tumor markers CEA and CA 15-3.

Results: At surgery, 13 patients presented complete remission (CR), 32 partial remission (PR) and 20 no change of disease (NC). Pretherapeutic Her2/neu status was positively correlated with therapy response ($p = 0.019$). DNA biomarkers before onset of therapy cycles 1, 2 and 6 did not indicate outcome after therapy. However, kinetics of ALU 115 from cycle 1 to 6 showed decreases in CR patients, while in NC patients, an increase was observed ($p = 0.033$). Similar tendencies were found for ALU 247 fragments. DNA integrity index as well as CEA and CA 15-3 were not informative for therapy outcome.

Conclusion: Kinetics of plasma DNA (ALU 115) is associated with response to neoadjuvant chemotherapy in patients with locally confined breast cancer.

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1. Introduction

Breast cancer is the most frequently diagnosed cancer in women worldwide with 23% of all global cancer diagnoses and 14% of cancer deaths [1]. While in early stage, localized breast cancer, patients undergo surgical tumor resection eventually followed by adjuvant radiation and chemotherapy [2], patients with locally advanced (>T3 stage) and inflammatory breast cancer can be treated by neoadjuvant chemotherapy to downstage the tumor before surgery [3]. Furthermore, patients <40 years, grade 3 tumors, negative hormone receptors and non-lobular histology are considered for neoadjuvant chemotherapy [4,5] typically consisting of six to eight cycles of chemotherapy combinations of docetaxel, adriamycin and cyclophosphamide (TAC) or epirubicin and cyclophosphamide (EC), followed by docetaxel or paclitaxel [3,6]. Trastuzumab is added to the regimen depending on the Her2/neu expression status [3].

As only a small portion of patients achieve the prognostically relevant complete remission [7], predictive markers for pretherapeutic stratification and for the early estimation of response to neoadjuvant therapy are urgently required. Currently, response is only assessed after several therapy cycles and before surgery by radiology. Serial measurement of blood-based markers is an attractive approach because this can be performed easily, rapidly and with minimal invasiveness. Depending on the results, therapy could be adapted to the individual patient's needs thereby optimizing efficiency and reducing toxicity of the treatment.

To date, prognostic and predictive markers are mainly on tissue basis. Apart from tumor size, lymph node and metastasis status and tumor cell grading, diverse immunohistological markers particularly for estrogen receptor (ER), progesterone receptor (PR), Her2/neu and Ki67 are used to stratify the relapse risk of the patient [8–10]. The conventional serum tumor markers CA 15-3 and CEA frequently used for therapy monitoring [11] or early recurrence detection [12], show lack in diagnostic sensitivity especially for the detection of small tumor nodules [13]. At time of surgery, they provide important prognostic information [14]. However, their usefulness in pretherapeutic stratification and early estimation of therapy response in patients receiving neoadjuvant chemotherapy is still unclear.

A promising approach is the quantification of tumor related cell-free DNA (cfDNA) in plasma or serum of cancer patients [15] that has shown potential in cancer detection [15–17] and prognosis [18]. However, cfDNA was also found to be elevated in various benign diseases, such

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as stroke, burns, sepsis and autoimmune diseases [19–23]. Frequently, single gene copies such as ß-actin, GAPDH or ERV-DNA are quantified [24]. Due to the limited sensitivity, highly repetitive sequences such as ALUs [15,25], which are distributed throughout the genome [26], were suggested as alternatives.

Two ALU fragments with 115 and 247 base pairs (bp) were used to differentiate between apoptotic and necrotic cell death. As DNA fragmentation during apoptosis is known to result in nucleosomal pieces of around 150–180 bp, the shorter ALU 115 should represent the total amount of DNA while the longer ALU 247 fragments are considered a necrotic products [27,28]. Since necrotic cell death is particularly relevant in progressive tumor disease [29], a higher portion of longer DNA fragments was proposed as biomarker for cancer detection [28].

In order to objectify the "DNA integrity index" as ratio of longer and smaller fragments, different formulas were suggested: Umetani et al. calculated the pure ratio of ALU 247 and ALU 115 concentrations in the serum of patients [15,25], while Wang et al. used a formula based on delta-Cp values for the quantification of DNA integrity in patient plasma [30]. Both authors reported higher DNA integrities in serum and plasma of patients with ovarian, breast, and colorectal cancer as compared with controls [15,25]. However, other reports could not find a difference of DNA integrity values in the same tumor types [31–33]. Unfortunately, no study has compared the diagnostic utility of DNA integrity in comparison with established cancer biomarkers. Concerning prediction and monitoring of therapy response in cancer patients undergoing systemic therapies, little data has become available to date [15,24,34].

In the present study, we measured ALU 115 and ALU 247 in breast cancer patients undergoing neoadjuvant systemic chemotherapy, calculated the DNA integrity according to both formulas and compared them with the established breast cancer biomarkers CA 15-3 and CEA concerning their relevance in the pretherapeutic prediction and the intratherapeutic monitoring of tumor response to treatment.

2. Patients and methods

2.1. Plasma samples, clinical and pathology information

Plasma samples of 65 breast cancer patients undergoing neoadjuvant chemotherapy were collected between 2007 and 2011. The 65 patients with localized breast cancer received pretherapeutic staging (mammography, mamma-sonography, chest x-ray, abdomen sonography and bone scintigraphy) before onset of neoadjuvant chemotherapy. During the study, the TNM-stage of all patients before onset of chemotherapy, and after surgery, as well as the receptor status (estrogen, progesterone and Her2/neu status) were documented. Furthermore, histopathological findings such as tumor subtype and patient's age were reported.

When neoadjuvant chemotherapy was completed after six to eight chemotherapy cycles, patients underwent surgery. The histopathological findings at surgery were compared with pretherapeutic staging and were used to determine the outcome of the neoadjuvant chemotherapy. The patients were classified into three groups according to RECIST criteria [35]: no change (NC, i.e. less than 30% tumor regression), partial remission (PR, 30 to 99% tumor regression) and complete remission (CR).

During the chemotherapy cycles, plasma sample were taken before onset of the neoadjuvant chemotherapy (cycle 1), before cycle 2 (approximately 3 weeks after cycle 1) and between cycles 5 and 6 (C6, approximately 60 to 70 days after therapy start) when therapy was almost finished. Detailed patient data are provided in Table 1. The study was approved by the local ethics committee. Patients were informed in detail on the study prospective and written informed consent was obtained from all patients before study entry.

2.2. Plasma preparation for qPCR

From each patient, three plasma samples (4.4 ml) were collected in an EDTA collection tube (Sarstedt, Nürnbrecht, Germany). The samples were centrifuged within a maximum of two hours after venipuncture, separated, aliquoted and cryopreserved at −80 °C.

For DNA isolation, the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was used: 400 μl of plasma sample and 400 μl of lysis buffer was mixed with 20 μl of Qiagen protease. After 30 minutes of incubation at 56 °C, 400 μl of 100% ethanol was added to the vial. Two washing steps were performed using buffers (750 μl each), a vacuum pump and spin columns as recommended. Spin columns were centrifuged, before 50 μl of lysis buffer was added. After an additional centrifugation, DNA was eluated from the column. 5 μl of this eluate was used as a template for each qPCR performed.

2.3. Quantitative PCR for ALU repeats

Two primers for the detection of ALU 115 and ALU 247 were used as described by Umetani et al. [15] (see Supplemental Data). The reaction mixture for the qPCR contained the following ingredients: 5 μl template, 0.25 μl of UNG DNA Glycosylase (UNG, Roche Diagnostics, Mannheim, Germany), 2 μl of each primer (forward and reverse, only one primer combination (ALU 115 or 247) per PCR plate), 6.75 μl PCR grade H_2O and 4 μl of Mastermix SYBR Green (Roche Diagnostics). A reaction volume of 20 μl was achieved.

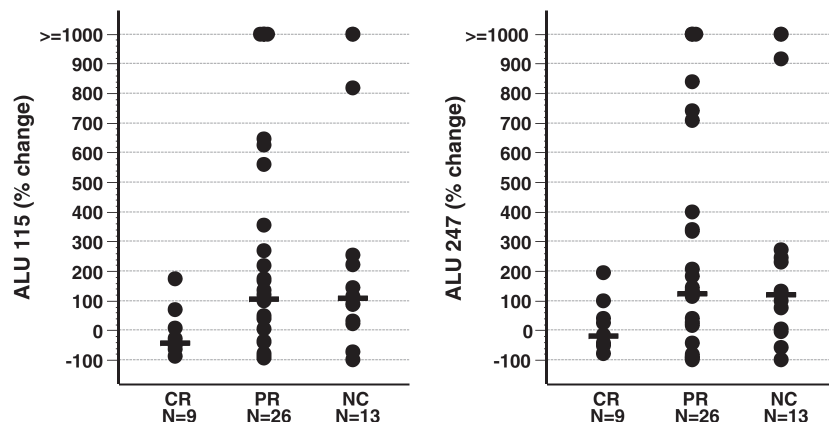

Fig. 1. Dot-plots showing the distribution of individual relative changes of ALU 115 and ALU 247 from courses 1 to 6 in patients with complete (CR), partial remission (PR) and no change of disease (NC).

The Light Cycler® 480 Instrument II (Roche Diagnostics) was used to perform the RT qPCR amplification. UNG DNA glycosylase was incubated for 10 min at 40 °C followed by 10 min UNG-inactivation time at 95 °C. Each PCR amplification was performed with 45 cycles of denaturation (95 °C for 10 s), annealing (62 °C for 15 s and extension (72 °C for 15 s). A standard curve was calculated after measuring serial dilutions of a DNA (Roche Diagnostics). Efficiencies were calculated at 1.95 for ALU 115 and 1.84 for ALU 247 (See Supplemental data). All measurements were performed in duplicates. For quality controls, positive and negative controls, two plasma patient pools with high and low DNA levels as well as three dilution steps of the standard curve were measured with each PCR plate.

2.4. Calculation of the DNA Integrity Index

DNA Integrity Index as the relation of ALU 247 to ALU 115 was calculated according to the formulas of Umetani et al. (DNA Int 1) [15,25] and of Wang et al. (DNA Int 2) [30]: DNA Int 1 represents the ratio of ALU 247 to ALU 115 concentration. As the annealing sites of ALU 247 are represented within those of ALU 115, this ratio can theoretically vary between 0 and 1.

DNA Int 2 was calculated using the following formula: DNA Int $2 = e^{(-\Delta\Delta Cp \times \ln(2))}$. Therein, ΔΔCp is the difference between ΔCp 115 and ΔCp 247, and ΔCp 115 and ΔCp 247 was calculated as the difference between the Cp value of a standard pool of human genomic DNA and the Cp value of both ALU 115 and ALU 247.

2.5. Determination of established tumor markers

Carcinoembryonic antigen (CEA) and cancer antigen (CA) 15-3 were measured in patient sera by enzymatic chemiluminescent immunoassay (ECLIA) on the ElecSys 2010 immunoassay analyzer of Roche Diagnostics, Germany.

2.6. Statistics

The concentration of all measured markers (both ALUs, both DNA Integrity Indices, CEA and CA 15-3) was measured before therapy onset, before cycle 2 and before cycles 5 or 6. For the latter, the percentage differences to pre-therapeutic values were also considered for statistical evaluation. The patients were separated into three groups according to their surgery results as described above.

Concentrations of biomarker values are given as medians and ranges for the three groups. Significance of differences between the three groups was tested by Wilcoxon–Mann–Whitney test. First, all marker values were compared between the complete remission (CR) group and the no change (NC) group. If this test was significant, patients with partial remission (PR) were additionally compared with the CR and NC patients. For significant markers, receiver operating characteristic (ROC) curves are given. Here, the PR and NC groups were combined and compared to the CR group resulting in sensitivities and specificities for complete versus non-complete remission.

Pre-therapeutic clinical factors in the three therapy response groups were tested for significance by means of the Mantel–Haenszel χ^2 test for overall trend.

A p-value < 0.05 was considered statistically significant. All calculations were performed with SAS software (version 9.2, SAS Institute Inc., Cary, N.C., USA).

3. Results

3.1. Clinical characteristics and treatment response

During neoadjuvant chemotherapy, 65 patients were followed: 47 breast tumors were classified as T1 or T2 tumors while 17 were classified as T3 or T4. Seventeen patients presented lymph-node negative stage (N0), while 44 were classified as lymph node positive (N1–3). Histological subtypes included 57 invasive ductal, four invasive lobular carcinoma, two adenocarcinoma and two without classification. Thirty-eight patients were estrogen receptor positive (27 negative), 32 progesterone receptor positive (33 negative) and 21 Her2/neu receptor positive (44 negative). Twenty-one patients were found to have a "triple negative" receptor status with negative results for all three receptor classes.

In histopathological staging at time of surgery, 13 patients achieved a pathological complete remission (CR), and 32 a partial remission (PR), while no change of disease (NC) was found in 20 patients (for details see Table 1).

Concerning pretherapeutic clinical factors (TNM-status, grading, hormone receptors), only the Her2Neu receptor was found to be predictive of therapy response in the Mantel–Haenszel test (p-value = 0.019). A

Table 1
Patient characteristics.

Patient characteristics	Median	Min/Max
Age	47.0	26.5/72.7
Characteristics of patients before neoadjuvant chemotherapy		
T stage	*N*	%
1	8	12.3
2	39	60.0
3	14	21.5
4	3	4.6
X	1	1.6
N stage	*N*	%
0	17	26.1
1	40	61.6
2	1	1.6
3	3	4.6
X	4	6.1
G stage	*N*	%
2	22	42.3
3	21	40.4
4	1	1.9
X	8	15.4
Histology	*N*	%
Invasive ductal carcinoma	57	87.7
Invasive lobular carcinoma	4	6.1
Adenocarcinoma	2	3,1
Unknown	2	3.1
Histopathological classification	*N*	%
Estrogen receptor positive/negative	38/27	58.5/41.5
Progesterone receptor positive/negative	32/33	49.2/50.8
Her2/neu positive/negative	21/44	32.3/67.7
Characteristics of patients at time of surgery after neoadjuvant chemotherapy		
T stage (ypT)	*N*	%
0	16	24.6
1 (1A-1C)	30	46.2
2	12	18.5
3	6	9.2
4	1	1.5
N stage (ypN)	*N*	%
0	40	61.6
1	16	24.6
2	6	9.2
3	3	4.6
Response to therapy	*N*	%
Complete remission (CR)	13	20.0
Partial remission (PR)	32	49.2
No change (NC)	20	30.8
Neoadjuvant chemotherapies (trastuzumab was added depending on Her2Neu receptor status)	*N*	%
Cyclophosphamide + Epirubicine + Docetaxel	36	55.3
Cyclophosphamide + Epirubicine	13	20.0
Cyclophosphamide + Epirubicine + Paclitaxel	11	16.8
Cyclophosphamide + Epirubicine + Fluorouracil + Docetaxel	2	3.1
Cyclophosphamide + Epirubicine + Carboplatin	1	1.6
Cyclophosphamide + Epirubicine + Docetaxel + Paclitaxel	1	1.6
Carboplatin	1	1.6

Table 2
Values and *p*-values in groups (NC compared with CR).

	Marker	Group	*N*	Median	Min	Max	Comparison with CR group (*p*-value)
Cycle 1	ALU 115	NC	20	14.3	4.0	61.3	0.126
		PR	32	15.2	0.7	112.1	
		CR	13	16.6	8.9	143.9	
	ALU 247	NC	20	14.4	4.1	48.7	0.294
		PR	32	15.9	0.9	119.8	
		CR	13	19.2	8.4	100.9	
	DNA Int 1	NC	20	1.2	0.6	1.5	0.117
		PR	32	1.1	0.6	1.7	
		CR	13	0.8	0.6	1.4	
	DNA Int 2	NC	20	0.8	0.4	1.5	0.179
		PR	32	0.8	0.4	1.2	
		CR	13	0.7	0.5	1.3	
	CEA	NC	19	1.5	0.5	5.9	0.105
		PR	31	1.4	0.2	6.9	
		CR	12	0.7	0.2	14.1	
	CA 15-3	NC	19	19.0	8.9	102.0	0.824
		PR	31	19.3	6.3	164.0	
		CR	12	18.1	9.9	46.5	
Cycle 2	ALU 115	NC	13	28.1	0.1	51.4	0.369
		PR	23	23.5	0.2	559.6	
		CR	10	20.0	6.6	33.9	
	ALU 247	NC	13	30.8	0.3	67.3	0.515
		PR	23	24.7	0.05	370.5	
		CR	10	25.0	10.5	57.2	
	DNA Int 1	NC	13	1.2	1.0	2.2	0.403
		PR	23	1.3	0.2	1.8	
		CR	10	1.3	0.7	1.7	
	DNA Int 2	NC	13	0.4	0.2	1.0	0.336
		PR	23	0.3	0.08	0.6	
		CR	10	0.6	0.2	1.3	
	CEA	NC	13	1.1	0.4	6.2	0.291
		PR	21	1.5	0.2	5.8	
		CR	10	0.8	0.2	11.2	
	CA 15-3	NC	13	22.9	10.7	103.0	0.438
		PR	20	23.4	9.8	73.0	
		CR	10	26.3	15.7	54.3	
Cycle 6	ALU 115	NC	13	15.7	0.05	4059.5	0.385
		PR	26	32.1	0.9	204.5	
		CR	9	12.1	5.0	83.7	
	ALU 247	NC	13	19.7	0.01	2507.3	0.593
		PR	26	38.3	0.3	304.7	
		CR	9	15.5	6.9	84.5	
	DNA Int 1	NC	13	1.1	0.1	1.6	0.285
		PR	26	1.0	0.3	1.7	
		CR	9	1.3	1.0	1.5	
	DNA Int 2	NC	13	0.3	0.09	1.2	0.385
		PR	26	0.3	0.07	0.5	
		CR	9	0.7	0.3	1.1	
	CEA	NC	13	1.6	0.5	4.5	0.738
		PR	25	1.7	0.2	14.0	
		CR	9	1.6	0.4	2.6	
	CA 15-3	NC	13	27.3	13.9	81.4	0.548
		PR	25	34.8	19.5	87.9	
		CR	9	23.4	16.0	48.8	
Cycle 2 compared with pretherapeutic values (%)	ALU 115	NC	13	133.0	−98.1	607.4	0.227
		PR	23	37.4	−98.9	1453.6	
		CR	10	28.2	−84.0	108.9	
	ALU 247	NC	13	143.2	−97.2	412.3	0.369
		PR	23	80.7	−99.8	1916.2	
		CR	10	34.1	−82.4	161.3	
	DNA Int 1	NC	13	13.4	−34.1	63.1	0.278
		PR	23	16.6	−83.5	111.8	
		CR	10	18.8	−21.0	148.0	
	DNA Int 2	NC	13	−52.9	−77.9	67.9	0.515
		PR	23	−58.9	−94.3	12.6	
		CR	10	−22.3	−72.9	98.6	
	CEA	NC	12	2.5	−33.3	33.3	0.546
		PR	21	0.0	−28.6	50.0	
		CR	9	0.0	−30.8	66.7	
	CA 15-3	NC	12	24.7	−7.0	48.4	0.642
		PR	20	20.6	−21.0	52.8	
		CR	9	24.4	4.8	199.0	
Cycle 6 compared with pretherapeutic values (%)	ALU 115	NC	13	109.8	−99.8	5100.6	**0.033**
		PR	26	109.4	−94.8	2744.7	
		CR	9	−39.4	−87.8	175.0	
	ALU 247	NC	13	123.9	−99.9	4223.4	**0.071**
		PR	26	125.6	−98.9	2279.5	
		CR	9	−16.3	−77.8	193.0	
	DNA Int 1	NC	13	−2.0	−89.4	89.1	0.182
		PR	26	−4.5	−78.1	82.7	
		CR	9	26.7	−17.0	85.4	
	DNA Int 2	NC	13	−58.5	−91.8	55.1	0.125
		PR	26	−67.6	−94.9	−22.8	
		CR	9	−30.7	−67.9	89.7	
	CEA	NC	12	−2.9	−66.7	216.7	1.00
		PR	25	40.0	−81.2	900.0	
		CR	8	−1.8	−87.2	166.7	
	CA 15-3	NC	12	19.3	−34.9	158.1	0.787
		PR	25	73.8	−80.1	209.8	
		CR	8	39.9	−48.0	76.6	

positive Her2/neu receptor status was found for 53.9% in the CR group, 34.4% in the PR group and 15.0% in the NC group.

3.2. Predictive value of pretherapeutic levels of DNA and conventional tumor markers

Concerning pretherapeutic levels of ALU 115, no differences were observed between CR patients (median 16.6 ng/ml) and NC patients (14.3 ng/ml; $p = 0.126$). Similar results were obtained for ALU 247 levels (CR: 19.2 ng/ml; NC: 14.4 ng/ml; $p = 0.294$), for DNA integrity 1 (CR: 0.8; NC: 1.1; $p = 0.117$) and DNA integrity 2 (CR: 0.7; NC: 0.8; $p = 0.178$). In addition, no difference was observed between response groups for conventional tumor markers CA 15-3 (CR: 18.1 U/ml; NC: 19.0 U/ml; $p = 0.824$) and CEA (CR: 0.7 ng/ml; NC: 1.5 ng/ml; $p = 0.105$), too (Table 2).

3.3. Early estimation of therapy response by intratherapeutic levels of DNA and conventional tumor markers

Before onset of the second cycle of chemotherapy, levels of ALU 115 and 247 were higher in both response groups when compared to pretherapeutic levels (medians ALU 115: CR: 20.0 ng/ml; NC: 28.1 ng/ml; ALU 247: CR: 25.0 ng/ml; NC: 30.8 ng/ml). However, differences between the response groups were not significant for ALU 115 ($p = 0.227$) and ALU 247 ($p = 0.369$). Similarly, neither absolute levels of DNA integrity 1 ($p = 0.336$) and DNA integrity 2 ($p = 0.403$) nor of conventional tumor markers CA 15-3 ($p = 0.438$) and CEA ($p = 0.291$), nor relative changes of markers levels from courses 1 to 2 could discriminate between the extreme response groups (Table 2).

Before onset of the sixth cycle of chemotherapy, i.e. shortly before surgery, absolute levels of ALU 115 and 247 were once again lower. While ALU 115 levels in the CR group (12.1 ng/ml) were only slightly lower than in the NC group (15.7 ng/ml; $p = 0.385$), the relative changes from courses 1 to 6 were significantly different in both groups showing a median decrease of 39.4% in the CR group and a median increase to 109.8% in the NC group ($p = 0.033$) (Fig. 1). Interestingly, PR patients also had increasing values (109.4%), but without a significant difference from CR patients ($p = 0.117$)

Similar results were obtained for ALU 247. While levels in the CR group (15.5 ng/ml) were not different from those in the NC group (19.7 ng/ml; $p = 0.593$), there was an almost significant difference of the relative changes from courses 1 to 6 between both groups showing a median decrease of 16.3% in the CR group and a median increase to 123.9% in the NC group ($p = 0.071$). All other markers, such as DNA integrity 1 and 2, CEA and CA 15-3, showed no statistically significant

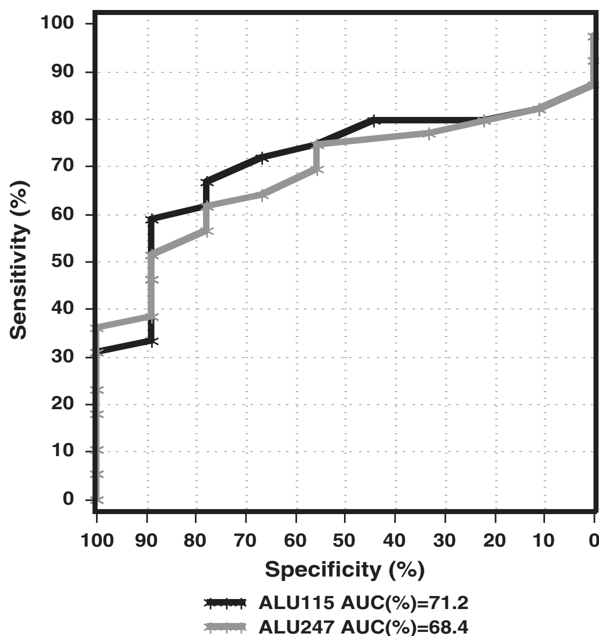

Fig. 2. ROC-curves for the estimation of complete remission (CR) versus partial remission (PR) and no change of disease (NC) for relative changes of ALU 115 and ALU 247 from courses 1 to 6.

difference, neither at cycle 6 nor when comparing cycle 6 values with pretherapeutic values.

For the comparison of the CR group with the NC and PR group regarding changes from pretherapeutic values to cycle 6, an area under the ROC-curve (AUC) of 71.2% and 68.4% was reached for ALU 115 and Alu 247, respectively. Sensitivities for CR detection at 90% specificity were 33% for ALU 115 and 36% for ALU 247 (Fig. 2).

4. Discussion

In recent years, many studies have been carried out investigating the potential of cfDNA and DNA integrity as new tumor biomarkers. Several of them showed elevated levels of cfDNA in both serum and plasma of patients suffering from breast, colorectal, lung or testicular cancer [15,25,26,36–38]. However, the diagnostic value for the individual patient is limited by the fact that benign pathologies that are relevant for the differential diagnosis of a suspicious finding are associated with elevated levels for cfDNA in some cases as well [19–23].

Cancer development is known to be associated with high cellular proliferation which is initially counterbalanced by high rates of active apoptotic cell death and later by increasing rates of passive necrotic cell death when the tumor dedifferentiates and becomes invasive [29]. Therefore, high DNA Integrity Index values as relation of longer (non-apoptotic) to shorter DNA fragments (mirroring all cell death types) were supposed as relevant biomarkers for detection of aggressive cancers with poor prognosis [28]. Indeed, some studies reported a strong correlation of DNA integrity and cancer diagnosis [15,25,30], while others did not [31,32].

Recently, we compared the two most often used approaches for the calculation of DNA integrity in breast cancer patients. While ALU 115 and 247 levels were found to be significantly higher in locally confined breast cancer patients as compared with healthy controls, DNA integrity could not distinguish between the two groups [39]. It is noteworthy that the additionally tested, conventional tumor markers CA 15-3 and CEA were only informative for metastatic breast cancer detection but not for the locally confined disease [40].

Regarding therapy prediction and prognosis, several studies reported an association of high cfDNA levels with poorer overall and/or disease-free survival, e.g. for patients with breast, ovarian and prostate cancer [17,41–43]. In contrast, other studies could not confirm these findings in breast cancer patients concerning overall survival [24]. Regarding DNA integrity, Umetani et al. described a correlation between preoperative values and the presence of lymph node metastasis [15]. Another study found better survival rates in 105 patients with nasopharyngeal cancer undergoing radiotherapy if DNA integrity values decreased [44].

The predictive and prognostic value of cfDNA in patients undergoing chemotherapy has only been investigated in a small number studies to date showing mainly decreasing cfDNA levels as a marker for early treatment response, e.g. in lung cancer [45] or rectal cancer during neoadjuvant chemo-radiation therapy [46]. DNA integrity has not yet been addressed for this indication.

Here, a prospective observation study was conducted on a homogenous cohort of patients with locally confined breast cancer who were treated with neoadjuvant chemotherapy. This approach provided an excellent setting for the investigation of serum biomarkers measured at defined time points during treatment and the correlation with response to therapy objectified by immunohistochemistry at the time of tumor resection. As radiological staging after several months is currently used for evaluation of the macroscopic response to tumor therapy, new biochemical markers for a more efficient therapy monitoring and an early estimation of therapy response are urgently required [35].

While our results do not identify a marker for the prediction of therapy response before onset of chemotherapy nor at therapy cycle 2, there was a statistically significant difference for ALU 115 at cycle 6 when compared with pretherapeutic values. Indeed, ALU 115 was decreased in patients with complete tumor remission, whereas it was increased in patients with no change of disease stage when the tumor was removed. Corresponding with the small fragments, the longer ALU 247 showed a similar tendency. These results are in line with other findings of decreasing levels of circulating DNA and nucleosomes in patients with response to chemotherapy and with increasing values in non-responding patients [45,47–49]. Unfortunately, the DNA Integrity Index calculated by either formula did not indicate response to therapy when comparing patients with very favorable (complete remission) and non-favorable outcome (no change).

Of note, the currently used conventional tumor markers CA 15-3 and CEA were unable to distinguish between the response groups at any time point. This fact underlines the necessity to identify meaningful serum biomarkers for efficient therapy monitoring of breast cancer patients. In line with other studies, the Her2/neu status obtained before onset of therapy was indicative for therapy response. Earlier, higher rates of pathologically complete remission at the time of tumor removal were found for Her2/neu positive patients, even if Her2/neu-targeting antibodies (trastuzumab) were not included in the neoadjuvant treatment [50]. If it was included, rates of pathologically complete response in Her2/neu positive patients further increased by 16% [51].

It is obvious that the present study has a strongly explorative character. Diverse markers that were measured at different time points during therapy were tested on their ability to anticipate or indicate therapy response in a limited set of patients. While in this hypothesis-generating evaluation, no correction to multiple testing was performed, significant findings of this study have to be confirmed by independent validation studies. However, it has to be emphasized that this prospective single-center study included standardized serial blood drawings, controlled preanalytics and detailed clinical documentation and enrolled a homogenous group of breast cancer patients undergoing neoadjuvant therapy. This clinically and (pre-) analytically challenging approach could form the basis for further identification of new biomarkers relevant for response prediction and therapy monitoring in these patients.

5. Conclusion

Results of this prospective, exploratory study indicate circulating DNA markers ALU 115 and ALU 247 as two possible future biomarkers

for the investigation of response to neoadjuvant chemotherapy in breast cancer patients which has to be confirmed by future validation studies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2013.07.027.

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