

Evaluation of circulating cell-free DNA as a molecular monitoring tool in patients with metastatic cancer

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Abstract. The clinical decisions made when treating patients with metastatic cancer require knowledge of the current tumor extent and response to therapy. For the majority of solid tumors, a response assessment, which is based on imaging, is used to guide these decisions. However, measuring serum protein biomarkers (i.e. tumor markers) may be of additional use. Furthermore, tumor markers exhibit variable specificity and sensitivity and cannot therefore be solely relied upon when making decisions regarding cancer treatment. Therefore, there is a clinical requirement for the identification of specific, sensitive and quantitative biomarkers. In recent years, circulating cell-free DNA (cfDNA) and mutation-specific circulating cell-free tumor DNA (cftDNA) have been identified as novel potential biomarkers. In the current study, cfDNA and cftDNA were compared using imaging-based staging and current tumor markers in 15 patients with metastatic colorectal, pancreatic or breast cancer. These patients were treated at the

Third Medical Department of Paracelsus Medical University Salzburg (Austria). The results of the current study demonstrated a statistically significant correlation between the concentration changes of cfDNA and cftDNA and response to treatment, which was assessed by imaging. A correlation was not indicated with current clinically used tumor markers, including carcinoembryonic antigen, carcinoma antigen 15-3 and carcinoma antigen 19-9. The present study also indicated a correlation between cfDNA and cftDNA and the tumor volume of metastatic lesions, which was not observed with the current clinically used tumor markers. In conclusion, cfDNA and cftDNA exhibit the potential to become novel biomarkers for the response assessment following cancer treatment, and may serve as a tool for the estimation of tumor volume. The current study further supports the increasingly important role of cfDNA and cftDNA as new monitoring tools for use during cancer therapy.

Introduction

Recurrent gene mutations are found in the majority of cancer types. Genotyping tumor tissue for somatic genetic alterations, which leads to an accurate diagnosis of the tumor type, guides treatment decisions and/or predicts the response to therapy, has become common practice in medical oncology (1-3). Currently, tissue samples obtained via surgery or biopsy are the gold standard for use in this analysis.

For the treatment of patients with metastatic cancer, knowledge of tumor mass dynamics and response to therapy are important. Currently, imaging techniques, including CT and positron emission tomography (PET)-CT scanning are most commonly used for this purpose (4-7). Additionally, serum protein biomarkers, which are often referred to as tumor markers, are used in clinical practice to assess tumor dynamics and treatment response over time (8,9). However, the currently used tumor markers do not always accurately reflect the actual disease burden, and false positive results are sometimes seen in benign conditions such as inflammation (10,11). Therefore,

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Abbreviations: cfDNA, circulating cell-free DNA; cftDNA, circulating cell-free tumor DNA; PET, Positron Emission Tomography; FFPE, formalin fixed with paraffin embedded; CEA, Carcinoembryonic antigen; CA15-3, Carcinoma Antigen 15-3; CA19-9, Carcinoma Antigen 19-9

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these markers cannot be solely relied upon when estimating actual tumor mass, and need to be interpreted together with imaging results (12,13). Furthermore, for a number of tumor types, no reliable serum tumor marker has been identified.

There is an urgent clinical requirement for the identification of reliable tumor-specific biomarkers for the management of patients with metastatic cancer due to a number of reasons: i) Repetitive imaging studies can lead to a relevant radiation exposure; ii) the differentiation between residual viable tumor tissue and fibrotic tissue following neoadjuvant chemo- and/or radiationtherapy is often challenging, and iii) the differentiation between actual tumor progression and pseudoprogression, using current immunooncological approaches, can also be challenging in daily clinical practice (14).

In recent years, circulating cell-free DNA (cfDNA) has been indicated as a potential novel biomarker, largely due to the progression of sequencing technologies, including next generation sequencing and digital PCR (15). It has also been indicated that fragments of 'normal' DNA and circulating cell-free tumor DNA (cftDNA) enter the bloodstream via tumor-cells (16-18), cells undergoing apoptosis or necrosis or via the active release of DNA (19). Cell-free DNA can be detected in small amounts in healthy human plasma (3,20,21). However, higher concentrations of cfDNA are detected in the plasma of patients with cancer, due to tumor cell necrosis, apoptosis or its active release by tumor cells (3,22-24). cftDNA can reflect the mutations located in the primary tumor, including oncogene or tumor-suppressor gene mutations or gene-rearrangements (21,25,26), and can potentially be used to predict tumor burden more accurately than the protein biomarkers currently used (8,27-29).

The purpose of the current study was to evaluate cfDNA and cftDNA and the correlation with serum protein tumor markers and imaging results during therapy of patients with metastatic colorectal cancer (CRC), pancreatic cancer (PC) or breast cancer (BC).

Materials and methods

Patients and sample acquisition. The current study was approved by the Local Institutional Research Ethics Committee (415-E/1469/11-2013) and all patients provided written informed consent prior to blood sampling and tumor tissue analysis. Formalin-fixed paraffin-embedded (FFPE) tissue samples were obtained during surgery and analyzed at the Institute of Pathology, Paracelsus Medical University (Salzburg).

Blood sampling was performed between April 2012 and December 2013. Plasma samples were prospectively collected from 15 patients who were diagnosed and treated at the Department of Internal Medicine III, Salzburg Cancer Research Institute, Paracelsus Medical University Salzburg (Salzburg, Austria).

Patients were recruited consecutively within the study period. The only inclusion criterion was the diagnosis of metastatic CRC, PC or BC. Patients were considered for analysis if they had an adequate amount of sampling time-points available. All patients received at least one course of palliative systemic therapy. Patients with CRC most commonly received 5-FU based regimens in combination with Oxaliplatin or

Irinotecan. Patients with PC were most commonly treated with Gemcitabine based regimens and patients with BC were mainly treated with Taxans (Paclitaxel or Docetaxel).

The response to treatment was assessed using CT scans that were performed at 8-12 weeks intervals as indicated by the physician. The response was defined according to the Response Evaluation Criteria in Solid Tumors (RECIST) (30-33).

Isolation of DNA from FFPE tissues. Genomic DNA was extracted from 3-7 sequential sections (10 μ m) of the primary tumor FFPE specimens. A Proteinase K tissue digestion was performed in a 1.5 ml micro centrifuge tube containing 3-7 sections of paraffin-embedded tissue, and incubated at 70°C overnight to dissolve the tissue. DNA was then extracted using a Maxwell DNA LEV tissue DNA kit (Promega Corporation), according to the manufacturer's protocol, and eluted using 50 μ l elution buffer. The Maxwell® 16 Instrument purifies DNA using silica-clad paramagnetic particles, which provide a mobile solid phase that optimizes the capture, washing and elution of the target material. The quality of extracted DNA was examined using agarose gel electrophoresis and ethidium bromide staining, and concentrations were evaluated using photometry (NanoDrop 1000 Spectrophotometers; Thermo Scientific Inc.).

Direct sequencing of FFPE samples. Primary tumor samples were analysed using PCR amplification and Sanger sequencing. A number of genes were analysed, including KRAS, NRAS, BRAF, Tp53, NOTCH, EGFR, PTEN and PI3K, which are commonly mutated in cancer (34-37). BigDye® Sequencing Master Mix was used to perform the sequencing reaction according to the manufacturer's protocol. The samples were analysed on a capillary sequencer ABI 3100-Analyser (Applied Biosystems; Thermo Fisher Scientific, Inc.). Oncogenic mutations were identified in all 15 FFPE samples.

Plasma samples and DNA purification. Serial blood samples (8 ml each) were collected upon and following diagnosis. Mandatory blood sampling was performed on all patients at staging time-points and at intervals of 1-3 weeks between these points (depending on the frequency of clinical visits). All samples were processed within 30 min following blood collection and centrifuged once for 10 min at 1,500 x g. The resulting plasma sample was spun once more for 10 min at high speed (2,000 x g) in order to purify plasma from any remaining blood cells. The plasma was aliquoted and stored at -80°C.

Total nucleic acids were purified from 1 ml plasma using a modified phenol-chloroform extraction method, as previously described (38). A total of 108 serial plasma samples were obtained from 15 patients.

Identification of cfDNA somatic alterations in plasma. The specific mutations indicated in the primary analysis of FFPE samples (using Sanger sequencing) were used for every specific patient as a target for ultra-deep cfDNA sequencing.

By designing sequences that flank the target regions of interest, the specific PCR-products for ultra-deep sequencing were isolated. This process was used to prepare libraries for next generation sequencing. The primer sets that target the regions of

interest included adapter sequences for amplicon-based NGS analysis and had a mean coverage of 62,000x. The PCR-products were purified using Wizard SC Gel and a PCR Clean-Up system (Promega Corporation), according to the manufacturer's protocol for targeted re-sequencing (GATC Biotech AG).

Quantification of cfDNA and cftDNA in plasma. Human telomerase reverse transcriptase (hTERT) genomic amplification was used to quantify the total amount of cfDNA using reverse transcriptase-quantitative (RT-q)PCR. To quantify cfDNA, RT-qPCR was used, based on hTERT as the target (39,40). This system used two amplicon primers and a fluorogenic hybridization probe for amplifying hTERT. RT-qPCR was performed with a 20 μ l volume on a 7500 ABI detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Each run consisted of patient samples in duplicate, negative controls and a dilution of a standard TaqMan controlled human genomic DNA (Roche Diagnostics; 0.2 μ g/ μ l). The amount of cftDNA was calculated by multiplying the allelic fraction of the respective target gene with the total amount of cfDNA.

Analysis of serum tumor markers. Carcinoembryonic antigen (CEA), Carcinoma antigen 15-3 (CA 15-3) and carcinoma antigen 19-9 (CA 19-9) levels were analyzed during therapy at the same time-points as DNA acquisition. Analysis was performed on a Modular-E170 (CEA Ref: 11731629, CA 19-9 Ref: 11776193, CA 15-3 Ref: 03045838; Elektro Chemilumineszenz Immuno Assay; Roche Diagnostics) in cooperation with the University Institute of Medical and Chemical Laboratory Diagnostics (Salzburg, Austria).

cfDNA and cftDNA levels were correlated with CEA and CA19-9 levels in patients with colorectal and pancreatic cancer, and CA15-3 levels in breast cancer patients.

Volumetry of target lesions. In the current study, the tumor volume of two main metastatic target lesions of 5 CRC patients was analysed in the lung and liver. For segmentation, open-radART[®] (open-radART ion-ORAion Software Suite) was used to draw the boundaries of the tumor in each CT-slice. This segmentation produced a visual 3D-image and was used to analyse exact tumor-volume as described previously (41). Measurements of circulating cell free DNA were subsequently matched with the results of tumor-volumetry.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 6.01 (GraphPad Software, Inc.) and SPSS (IBM Corporation). Correlations were analysed using the Spearmans rank test. ANOVA tests followed by post hoc Tukey tests were used to compare multiple groups. $P < 0.05$ was considered to indicate a statistically significant result. All error bars represent the mean \pm standard deviation.

Results

Patient characteristics and DNA isolation. A total of 15 patients were included in the current study. A total of 6 patients had metastatic pancreatic cancer, 5 patients had metastatic colorectal cancer and 4 patients had metastatic breast cancer. The median age at diagnosis was 70 years. A total of 9 patients (6 patients with PC and 3 patients with CRC) exhibited

synchronous metastatic disease at diagnosis, and 6 patients developed metastasis during subsequent follow up. All patients had a median of three prior lines of palliative systemic therapy. The median overall patient survival was 93.1 weeks, from first diagnosis of metastatic disease for the whole cohort, and 183.3, 84.4 and 22.1 weeks for BC, CRC and PC, respectively. Patient characteristics are outlined in Table I.

Analysis of KRAS, NRAS, BRAF, Tp53, NOTCH, EGFR, PTEN and PI3K, revealed one common somatic mutation within Tp53, KRAS and NOTCH1 in every primary tumor sample. Five distinct mutations were revealed in KRAS, Tp53 and NOTCH1 (Fig. 1). These mutations were subsequently detected and quantified, in the respective matched plasma samples, using targeted re-sequencing. The median baseline plasma concentration of cfDNA was 340.5 pg/ μ l (range, 31.8-3160.8 pg/ μ l). The median concentration of cftDNA was 180.29 pg/ μ l (range, 0.011 pg/ μ l-1754.6 pg/ μ l).

Correlation of quantitative levels of cfDNA, cftDNA and established tumor markers. cfDNA and cftDNA concentrations were compared during the course of treatment. Following analysis of all available plasma samples ($n=108$), a modest overall correlation was observed between the amount of cfDNA and cftDNA over time (Spearman correlation coefficient 0.7536; $P < 0.001$; Fig. 2A).

The results of the analysis indicated that a large number of samples did not contain detectable amounts of cftDNA, while exhibiting small amounts of cfDNA. It was suggested that this may be due to a discordant expression in samples drawn in the days following treatment. We therefore focused on samples drawn at defined staging time-points.

When plasma samples, which were obtained at staging time-points were analysed, the correlation between cfDNA and cftDNA was strong (Spearman correlation coefficient 0.9221; $P < 0.0001$; Fig. 2B). No correlation was observed between cfDNA and cftDNA in samples drawn in between staging time-points (Spearman correlation coefficient 0.0325 $P = 0.2113$; Fig. S1). This correlation was also demonstrated when analysing the BC (Spearman correlation coefficient 0.9335; $P < 0.001$), PC (0.9158; $P = 0.002$) and CRC (0.563; $P = 0.004$) subgroups separately (Fig. S2).

Whether the quantity of cfDNA levels of established biomarkers and if cftDNA correlated with levels of established biomarkers was assessed according to cancer subtype. In the colorectal cancer group, a significant correlation was indicated between cfDNA and CEA (0.8962, $P = 0.039$) and cftDNA and CEA (0.9554; $P < 0.001$).

In the PC group, a significant correlation was exhibited between cfDNA and CEA (0.8895; $P = 0.002$; Fig. S3), but no significant correlation was observed between cftDNA and CEA (0.7235; $P = 0.074$; Fig. S3). No correlation was indicated between cfDNA or cftDNA with CA 19-9 ($P = 0.192$; $P = 0.724$; Fig. S3).

In the BC group, no correlation was observed between cfDNA and CA 15-3 (0.2526, $P = 0.527$) or cftDNA and CA15-3 (0.4623; $P = 0.702$; Fig. S3).

Correlation between cfDNA, cftDNA and tumor burden. CfDNA and cftDNA concentrations and serum tumor markers were correlated with volumetric measurements of selected

Table I. Baseline patient characteristics.

Characteristic	Number of patients (n=15)	Percentage of patients (%)
Sex		
Female	8	53.3
Male	7	46.7
Median age at diagnosis (years; range)	70 (47-82)	
Median follow up (months; range)	6 (2-8)	
Cancer type		
Breast cancer	4	26.6
Colorectal cancer	5	33.3
Pancreatic cancer	6	40.1
Primary metastatic disease	9	60
Median prior lines of palliative therapy (range)	3 (1-6)	
Median overall survival (range; weeks)	93.1 (15.8-196.9)	
Breast cancer	183.3 (149.9-196.2)	
Colorectal cancer	84.4 (55.5-187.3)	
Pancreatic cancer	22.1 (12.3-63.9)	

BC, breast cancer; CRC, colorectal cancer; PC, pancreatic cancer.

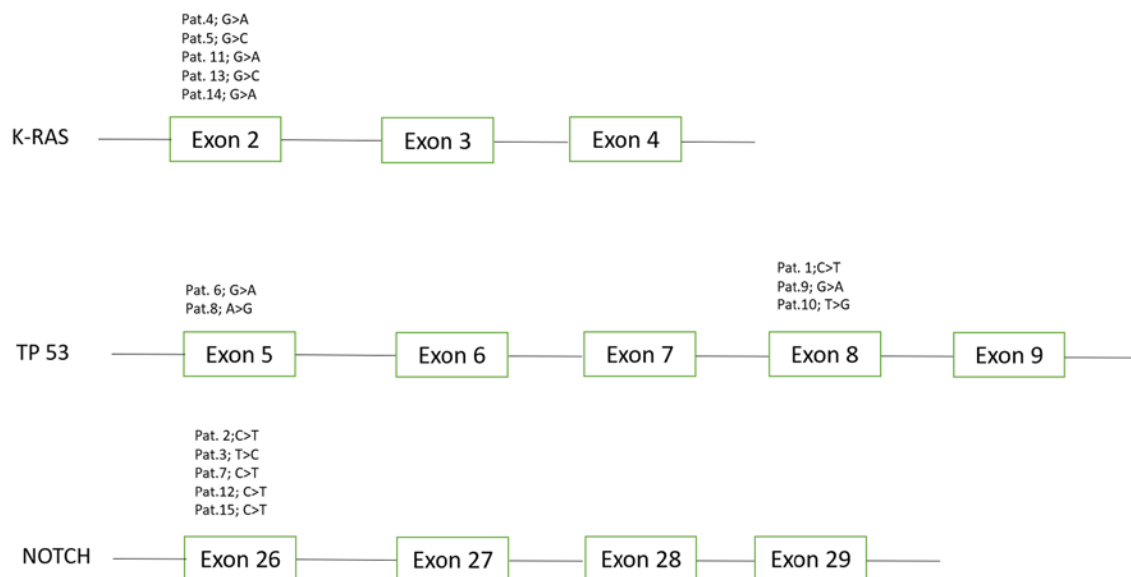


Figure 1. Overview of all detected mutations in the primary tumor sample. Mutations were identified in KRAS (5 patients), Tp53 (5 patients) and NOTCH 2 (5 patients).

metastatic target lesions in the liver and the lung of five patients with metastatic CRC. A significant correlation was demonstrated between tumor volume in the liver and cfDNA ($P=0.016$), and tumor volume in the lung and cfDNA ($P=0.003$).

The results of cftDNA and tumor volume analysis revealed a borderline significant correlation between tumor burden in the liver ($P=0.058$), and no correlation in the lung ($P=0.383$).

The results of the comparison of tumor marker levels of CA 19-9 and CEA with tumor volume, no correlation was indicated between tumor burden in the liver ($P=0.104$ for CA19-9; $P=0.873$ for CEA) or the lung ($P=0.789$ for CA 19-9; $P=0.052$ for CEA).

cfDNA, cftDNA and clinical response. The current study investigated how changes in cfDNA or cftDNA during treatment correlated with the response to therapy (assessed via imaging), and how cfDNA and cftDNA performed compared to currently used clinical biomarkers. Therefore, disease response assessed by CT imaging [partial response (PR), stable disease (SD) or progressive disease (PD) according to RECIST] was compared with concentration changes of cfDNA and cftDNA and tumor markers over time. The ratio of cf (t) DNA and tumor markers before and at the time of the respective staging CT was measured (i.e. $\text{cfDNA}_{\text{before staging}} (\text{pg}/\mu\text{l}) / \text{cfDNA}_{\text{staging}} (\text{pg}/\mu\text{l})$), and this ratio was correlated with

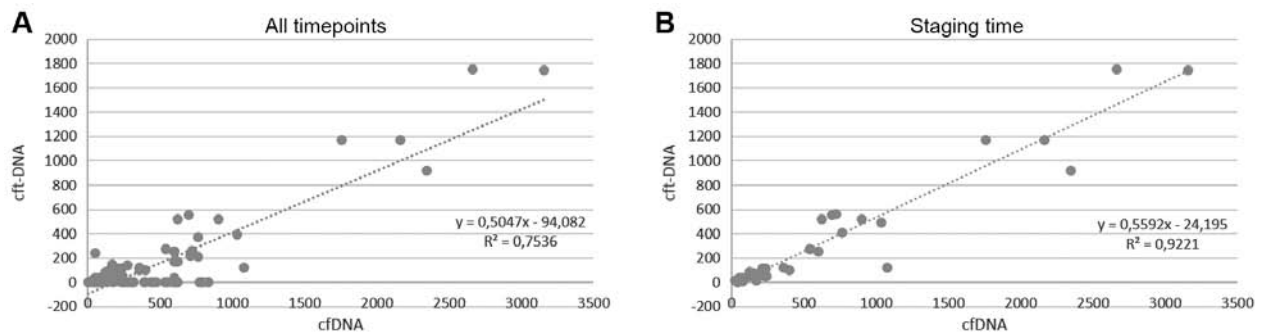


Figure 2. (A) Correlation between cfDNA levels (pg/ μ l) and cftDNA levels (pg/ μ l) for all available samples (n=108). (B) Correlation between cfDNA and cftDNA for samples obtained at staging time-points (n=38). cfDNA, circulating cell-free DNA; cftDNA, circulating cell-free tumor DNA.

the imaging result. A significant correlation was exhibited between the response assessed via imaging, and cfDNA and cftDNA (Fig. 3A). However, no correlation was observed between imaging results and tumor marker changes (Fig. 3B).

Subsequently, whether early changes in the ratio of cfDNA to cftDNA could predict treatment response was assessed. The ratio of cfDNA to cftDNA at time-points between treatment start and the first restaging CT were compared. However, the results did not demonstrate a significant correlation between treatment response and changes to the cfDNA/cftDNA ratio before imaging (Fig. S4). However, there was a marked trend, which was not statistically significant.

Discussion

In the current study, the potential role of cfDNA as a quantitative monitoring tool during cancer therapy in daily clinical practice was explored, and cfDNA was compared between imaging techniques and classical tumor markers that are currently used.

A total of 15 patients with three common tumor subtypes were assessed. The majority of patients had metastatic disease at diagnosis and the median OS observed in the respective cancer subtypes was in line with previously published cohorts (42-46).

Analyses were performed using mutations in commonly mutated genes, which were indicated by previously published data (21,36,47-54). The results of Sanger sequencing showed the presence of mutations within KRAS, Tp53 and NOTCH1 in the primary tumor sample. No other mutations were investigated. The overall concentration of cfDNA in our cohort was comparable to previously published reports (21,24,55).

A strong correlation was demonstrated between cfDNA and cftDNA from plasma samples obtained at staging time-points, compared to the correlation in all plasma samples or samples drawn in-between staging time-points.

In our practice, restaging time-points were often scheduled two to three weeks following the last application of systemic therapy (prior to the next scheduled application). Therefore, less fluctuations in cfDNA or cftDNA levels at these time-points were expected, presumably due to less tumor cell turnover. The data revealed that the time-point of sample acquisition was important for the interpretation of cfDNA or cftDNA levels, and should be further standardized in the future.

In contrast, it was observed that the changes in ratio between cfDNA and cftDNA in-between staging CTs may be

able to predict treatment response. However, due to the small sample size we were only able to see a trend, which was not statistically significant and therefore needs further investigation in future trials.

cfDNA was subsequently compared with tumor markers in colorectal cancer, and a correlation was indicated between CEA, but not CA 19-9. These results may be due to CEA being a more specific tumor marker in CRC than CA 19-9 (56-60).

In the pancreatic cancer group, a correlation was indicated between cfDNA and CEA, but not CA 19-9. A rise in cfDNA was observed when patients were examined in more detail, which correlated with disease progression upon imaging, but was not reflected by a rise in CA 19-9. Likewise, no correlation was demonstrated between cfDNA/cftDNA and CA 15-3 in the breast cancer group. However, a rise in cfDNA/cftDNA correlated with disease progression upon imaging, but was not reflected by a rise in CA 15-3. These observations support the potentially superior reflection of tumor dynamics with the use of cfDNA and cftDNA compared to classical biomarkers.

The results of the comparison of treatment response upon imaging demonstrated a stronger correlation between clinical staging and cfDNA and cftDNA than between classical tumor markers, further highlighting the potential of this new biomarker.

In the current study, tumor volumetric measurements were also compared during treatment with cfDNA/cftDNA, in comparison with classical biomarkers. A total of 5 patients with CRC who all had metastatic disease in the lung or the liver were assessed. These 5 patients were focused on due to the fact that volumetry of metastatic lesions can be performed more accurately in the lung and liver because of the better contrast between tumor and normal organ tissue. A strong correlation was observed between the amount of cfDNA and volume of the metastatic lesions. This correlation could not be demonstrated with classical biomarkers. cfDNA indicated a stronger correlation with the metastatic tumor burden than cftDNA. Possible explanations for this observation are the molecular heterogeneity of the tumor, clonal evolution during treatment or changes in the genetic background of the tumor, which were not detected by targeted resequencing.

The sample size of 15 patients in this study is relatively small, therefore further trials with higher patient numbers are required to confirm the reported findings. However, the correlation between cfDNA and cftDNA and the correlation

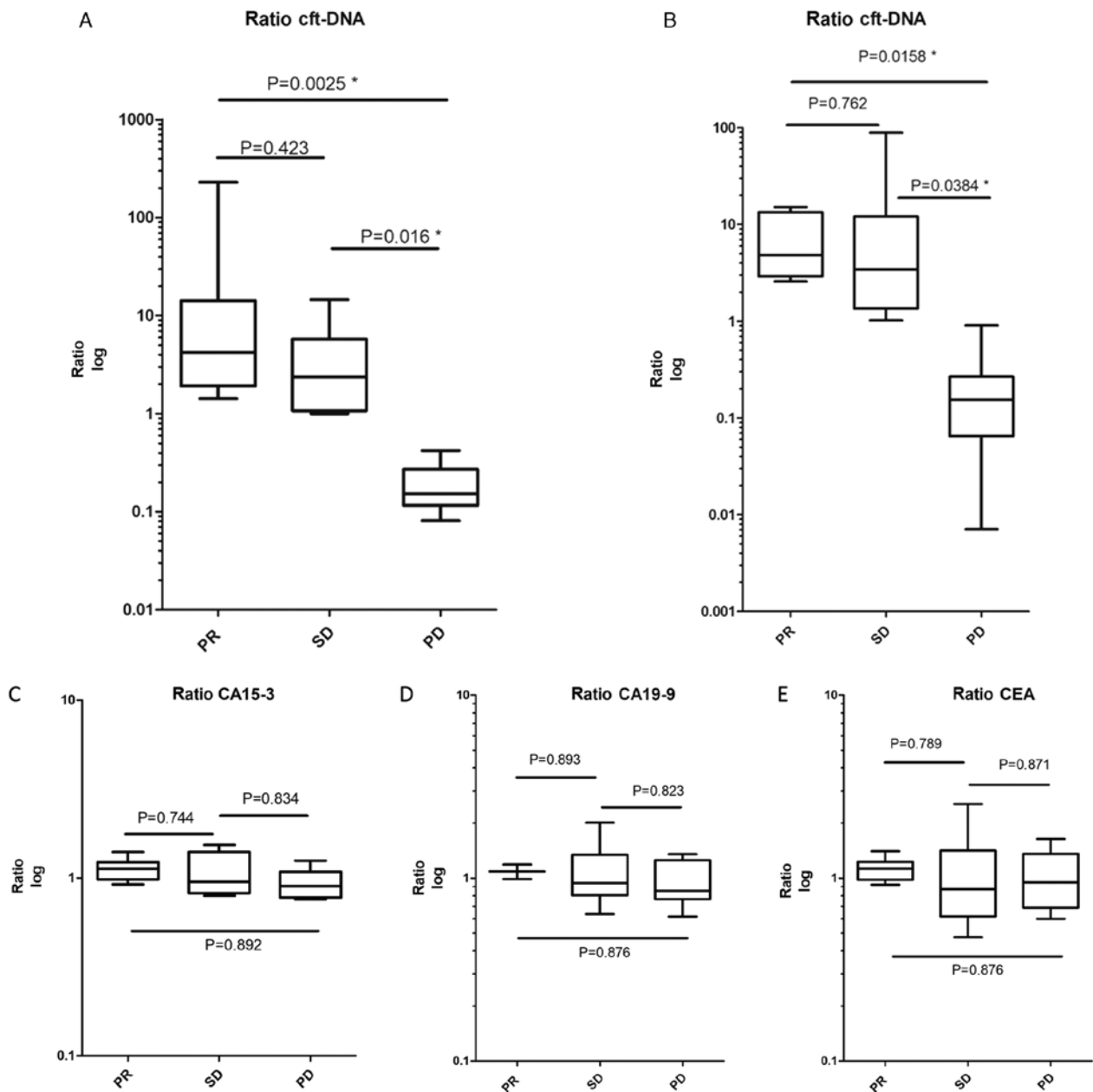


Figure 3. (A) Ratio of cfDNA before staging and cfDNA staging plotted against treatment response according to RECIST. (B) Ratio of cftDNA before staging and cftDNA staging plotted against treatment response according to RECIST. (C) Ratio of CA15-3 before staging and CA15-3 staging plotted against treatment response according to RECIST. (D) Ratio of CA19-9 before staging and CA19-9 staging plotted against treatment response according to RECIST. (E) Ratio of CEA before staging and CEA staging plotted against treatment response according to RECIST. cfDNA, circulating cell-free DNA; cftDNA, circulating cell-free tumor DNA; RECIST, Response Evaluation Criteria in Solid Tumors; CEA, Carcinoembryonic antigen; CA15-3, Carcinoma Antigen 15-3; CA19-9, Carcinoma Antigen 19-9.

between clinical staging and cfDNA/cftDNA in our study is significant despite the small patient number. Patients were selected with three different tumor types, which allowed investigation across different disease entities; however, this leads to a certain amount of heterogeneity of the data. Tumor volumetry was only available for the CRC group, so conclusions regarding the other two tumor types could not be made. We were not able to perform a fragment analysis of cfDNA due to the low concentration in the plasma samples. This should be implemented in future studies.

Overall, the results of the current study indicated that cfDNA and cftDNA outperformed currently used biomarkers

in predicting the response to therapy and quantifying tumor burden in a small patient cohort. Standards for the optimal time-point of sample acquisition for cfDNA analysis should be defined further in the future.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CH, ML and AE: Data analysis, review and writing. CH: Molecular analysis. CH, ML, LW and TMei: Data analysis, MM, DA, RG, GR and DN: Statistical analysis and interpretation, PS and TMei CT/PET-CT analysis, AE and RG supervised this work and assisted in preparing the manuscript. All authors have read and approved the final manuscript. All co-authors provided continuous intellectual guidance, repeatedly reviewed and edited the manuscript, and gave the final approval for submission.

Ethics approval and consent to participate

The current study was approved by the local institutional research ethics committee (permit no. 415-E/1469/11-2013) and all patients provided written informed consent prior to blood or tissue sampling.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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