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Assessment of genome-wide somatic copy number alterations in combination with driver mutation analysis in circulating DNA of colorectal cancer patients

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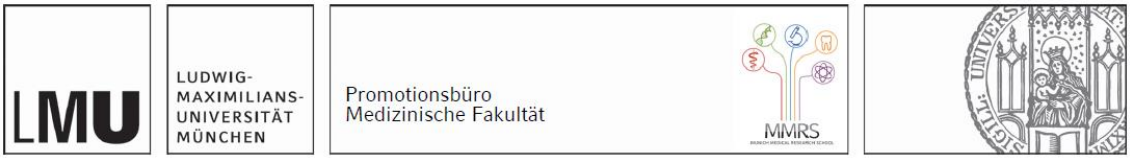

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List of abbreviations

AUC	Area under the curve
BloodPAC	Blood Profiling Atlas Consortium
BRAF	B-Raf Proto-Oncogene, Serine/Threonine Kinase
CEA	Carcinoembryonic antigen
cfDNA	Circulating free DNA
CRC	Colorectal cancer
CT	Computed tomography
CTC	Circulating tumor cell
ctDNA	Circulating tumor DNA
ddPCR	Droplet Digital PCR
DNA	Desoxyribonucleic acid
DPD	Dihydropyrimidine dehydrogenase
EGFR	Epidermal Growth Factor Receptor
FDA	U.S. Food and Drug Administration
KRAS	KRAS Proto-Oncogene, GTPase
LB	Liquid biopsy
LIFE-CNA	LIquid biopsy Fragmentation, Epigenetic signature and Copy Number Alteration
LOB	Limit of blank
LOD	Limit of detection
LOQ	Limit of quantification
miRNA	MicroRNA
ML	Machine learning
MMR/MSI	Mismatch repair / microsatellite instability
MRD	Molecular residual disease
MSI	Microsatellite instable
MSS	Microsatellite stable
NGS	Next generation sequencing
NRAS	NRAS Proto-Oncogene, GTPase
NSCLC	Non-small cell lung cancer
PBMC	Peripheral blood molecular cell
PCR	Polymerase chain reaction
PPV	Positive predictive value
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
SCNA	Somatic copy number alteration
TNM	Tumor, node, and metastasis
UICC	Union for International Cancer Control
VAF	Variant allele frequency
WGS	Whole-genome sequencing

List of publications

Paper I:

Hallermayr, A.; Benet-Pagès, A.; Steinke-Lange, V.; Mansmann, U.; Rentsch, M; Holinski-Feder, E.; Pickl, J.M.A. Liquid Biopsy Hotspot Variant Assays: Analytical Validation for Application in Residual Disease Detection and Treatment Monitoring, *Clinical Chemistry*, Volume 67, Issue 11, November 2021, Pages 1483–1491, https://doi.org/10.1093/clinchem/hvab124[1]

Paper II:

Hallermayr, A.; Steinke-Lange, V.; Vogelsang, H.; Rentsch, M.; de Wit, M.; Haberl, C.; Holinski-Feder, E.; Pickl, J.M.A. Clinical Validity of Circulating Tumor DNA as Prognostic and Predictive Marker for Personalized Colorectal Cancer Patient Management. *Cancers*, Volume 14, Issue 3, February 2022, Pages 851, https://doi.org/10.3390/cancers14030851[2]

Apendix A: Paper III:

Hallermayr, A.; Wohlfrom, T.; Steinke-Lange, V.; Benet-Pagès, A.; Scharf, F.; Heitzer, E.; Mansmann, U.; Haberl, C.; de Wit, M.; Vogelsang, H.; Rentsch, M.; Holinski-Feder, E.; Pickl, J.M.A. Somatic copy number alteration and fragmentation analysis in circulating tumor DNA for cancer screening and treatment monitoring in colorectal cancer patients. J Hematol Oncol 15, 125 (2022). https://doi.org/10.1186/s13045-022-01342-z[3]

(Apendix A: Paper III had not been published at the time of submission of the PhD Thesis.)

Your contribution to the publications

1.1 Contribution to paper I: Liquid Biopsy Hotspot Variant Assays: Analytical Validation for Application in Residual Disease Detection and Treatment Monitoring

Paper I is a first authorship of the PhD candidate.

A.H. established the method in the laboratory and conducted all experiments performed within the scope of this study.

Following the experimental procedures, A.H. analyzed the raw-data and performed statistical analysis of measurement results required for the analytical validation of the method.

Further, A.H. interpreted the data in terms of variability between the different assays and meaning of the established performance characteristics for clinical interpretation.

J.P. designed the study and supervised the work performed by the PhD candidate. She provided critical feedback throughout the whole process and thereby helped in shaping it.

E.H.-F. provided financial and technical resources to enable conduction of the study.

V.S.-L. was responsible for obtaining a positive ethics vote for patient recruitment.

M.R. contributed to patient recruitment.

A.H., A.B.-P., and J.P. wrote the manuscript as a team-effort.

All authors revised the manuscript and provided critical feedback in terms of analysis and results interpretation.

1.2 Contribution to paper II: Clinical Validity of Circulating Tumor DNA as Prognostic and Predictive Marker for Personalized Colorectal Cancer Patient Management

Paper II is a first authorship of the PhD candidate.

A.H. established and analytically validated the laboratory methodology as described in Paper I of this PhD thesis. Further, the PhD candidate established reference methods used in Paper II and Apendix A: Paper III of this PhD thesis.

A.H. performed all experimental procedures and analyzed the raw-data.

Following the experiments A.H. analyzed and interpreted the data. The PhD candidate performed statistical analysis to evaluate the potential and differences of biomarkers for disease monitoring and compared results to clinical findings in all study participants.

J.P. and V.S.-L. designed the study.

J.P. supervised the work performed by the PhD candidate and provided critical feedback throughout the process of this study and thereby helped in shaping it

E.H.-F. provided financial and technical resources to enable conduction of the study.

V.S.-L. was responsible for obtaining a positive ethics vote for patient recruitment.

M.R., H.V., M.d.W., and C.H. contributed to patient recruitment.

A.H. and J.P. wrote the manuscript as a team-effort.

All authors revised the manuscript and provided critical feedback in terms of analysis and results interpretation.

1.3 Contribution to paper III: Somatic copy number alteration and fragmentation analysis in circulating tumor DNA for cancer screening and treatment monitoring in colorectal cancer patients (Apendix)

Apendix A: Paper III is a first authorship of the PhD candidate, that had not been published at the time of submission of the PhD Thesis.

A.H. established, planed and performed all experimental procedures in the laboratory. A.H. and A.B.-P. developed the experimental procedures.

E.H. provided the Plasma-Seq pipeline. J.P. and A.H. researched the literature to identify the features that could improve sensitivity of data analysis.

F.S. helped setting up the Plasma-Seq pipeline at MGZ and supported initial tests to determine the required coverage for LIFE-CNA analysis.

A.H. developed and tested the bioinformatics analysis workflow for sequencing data. T.W. and the A.H. improved this workflow and selected the machine learning features as a team-effort. They created the final bioinformatics pipeline that can be used in clinical practice.

A.H. analyzed and interpreted the data obtained for each sample by comparing it to the clinical findings in each study participant.

In addition, A.H. performed statistical analysis to evaluate the performance characteristics of the method.

U.M. provided input to statistical analysis and machine learning.

J.P. and V.S.-L. designed the clinical study.

J.P. supervised the work performed by the PhD candidate and provided critical feedback throughout the process of this study and thereby helped shaping the research.

E.H.-F. provided financial and technical resources to enable conduction of the study.

V.S.-L. was responsible for obtaining the positive ethics vote for patient recruitment.

M.R., H.V., M.d.W., and C.H. contributed to patient recruitment.

A.H. and J.P. wrote the manuscript as a team-effort.

All authors revised the manuscript and provided critical feedback in terms of analysis and results interpretation.

2. Introductory summary

2.1 Background

2.1.1 Colorectal Cancer (CRC)

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer death worldwide. With an incidence of 1,931,590 and a mortality of 935,173 cases in 2020, survival rates decrease with advancing tumor stage at diagnosis[4]. Currently, nationwide screening programs and increased uptake of colonoscopies have been associated with a decrease in CRC incidence[5,6]. Moreover, hereditary and highly modifiable environmental risk factors, such as obesity and smoking, associated with CRC development, are well known and preventing them might further reduce the annual incidence of CRC[78].

2.1.1.1 Pathogenesis

CRC is considered to originate from a cancer stem cell triggered by the inactivation of tumor suppressor genes and the simultaneous activation of oncogenes, as a result of the accumulation of genetic and epigenetic alterations in DNA[9,10]. The development of CRC is estimated to take about 10-15 years[5].

Distinct pathways have been described for the development of CRC, each characterized by defined genetic and epigenetic events. The adenoma-carcinoma pathway (approximately 70-90% of CRCs) is initiated by a somatic variant in the *APC* gene followed, by the activation of *RAS* or loss of function of *TP53*, ultimately leading to the formation of microsatellite stable (MSS) CRC[11]. The serrated neoplasia pathway (10-20% of CRC cases), is initiated by somatic variants in *RAS* and *RAF* leading to epigenetic instability and microsatellite stable (MSS) or instable (MSI) CRC[5]. Beyond these two most prevalent manifestations, other subtypes of CRC are defined based on somatic variants in *POLE* or mismatch repair (MMR) deficiency[5].

2.1.1.2 Screening and diagnosis

Prognosis of CRC patients is most favorable if the disease is detected at an early stage. But, unfortunately, diagnosis at an advanced stage is frequent due to the largely asymptomatic course of the disease. To increase the detection rate of CRC at an early stage that is still curable, screening of individuals at average and high risk of developing CRC is recommended[12]. Colonoscopies and imaging methods are the most common techniques used for detection as well as accurate locoregional and distant staging of the disease[5,13].

Methods based on histological examination of tumor tissue removed during surgery or biopsy (i.e. pathological staging, subtyping, grading and evaluation of lymphatic, perineural, and venous invasion) are performed for risk stratification of each CRC patient[5,14].

2.1.1.3 Molecular biomarkers for precision medicine

Biomarkers are molecular targets that can be used for early cancer detection and to determine individualized treatment strategy of CRC[15]. They can be divided into diagnostic, prognostic or predictive classes. Thus, biomarkers provide patterns that are used at different stages of the dis-

ease to determine progression, recurrence, as well as therapeutic efficacy[5,12,14,16]. The most validated prognostic molecular markers in CRC to support adjuvant decision making are first mismatch repair / microsatellite intability (MMR/MSI) status and second dihydropyrimidine dehydrogenase (DPD)[12,14,17–20]. Furthermore, determination of baseline concentration of carcinoembryonic antigen (CEA), a high molecular weight glycoprotein in plasma, is used as biomarker to predict early recurrence in post-operative patients despite its limited accuracy[12,14,21,22].

Somatic variants in *BRAF* and *KRAS* genes are found in ~10% and ~34% of CRC patients, respectively, and are associated with poor prognosis in terms of overall survival[23–26]. These variants do not provide supportive information for adjuvant decision making[12,20] and are therefore only analyzed in patients with metastatic CRC. Several studies have shown promising results for combined treatment with *BRAF* and *EGFR* inhibitors[27,28] in CRC patients carrying the *BRAF* p.V600E somatic variant. In addition, activating mutations of the *KRAS* and *NRAS* genes are associated with resistance to treatment with monoclonal antibodies against *EGFR*[29,30]. Consequently, guidelines for the management of patients with metastatic CRC recommend testing for somatic variants in *BRAF*, *KRAS* and *NRAS* genes as predictive and prognostic markers[14]. To improve the management of CRC patients, as discussed in depth in Paper I and Paper II of this thesis, current research is focusing on highly sensitive analysis of these well-defined somatic variants from liquid biopsy (LB), allowing real-time monitoring to improve the management of CRC patients[1,2,14,22].

2.1.1.4 Treatment and follow-up

Depending on a patient's disease stage and risk of recurrence, different treatment and follow-up regimens are recommended in CRC (Figure 1)[12,14,16].

Localized CRC

Localized CRC is completely resected following diagnosis[12,31–35]. The risk of recurrence is estimated based on molecular markers and pathological stage according to the tumor, node, and metastasis (TNM) classification of the Union for International Cancer Control (UICC)[36,37]. Depending on the individual recurrence risk CRC patients either directly enter follow-up or are treated with adjuvant chemotherapy[12].

Extensive follow-up during the first five years after curative treatment is critical for the early detection of recurrence, occurring in 30% to 50% of patients treated for localized CRC[12,38–40]. Regular physical examination, determination of CEA concentration, CT (computed tomography) scans of the chest, abdomen, and pelvis, and colonoscopies are recommended[12,41,42].

Metastatic CRC

The optimal treatment strategy for each patient with metastatic CRC is discussed in multidisciplinary tumor boards based on clinical examinations, blood counts, liver and kidney function, CT or MRI scans, molecular tumor markers, and the patient's general health[14,43,44]. Depending on patient fitness and resectability of metastatic disease treatment consists of (I) immediate resection with or without perioperative chemotherapy with curative intent, (II) intensive treatment to either allow secondary resection or to reduce symptoms associated with tumor burden, (III) less intensive treatment with the objective of disease control, or (IV) palliative treatment[14,45–50]. To further refine treatment strategies, somatic variant in *RAS* and *BRAF* should be evaluated in patients with non-resectable metastases[14,27–30]. Eventually, therapy should be changed to second-line treatment upon detection of progressive disease[14].

Follow-up of metastatic CRC patients is prolonged to 10-years to confirm R0 resection[51]. Monitoring of treatment in patients with first, second, or third line therapy is performed with imaging and analysis of CEA concentration[14,52].

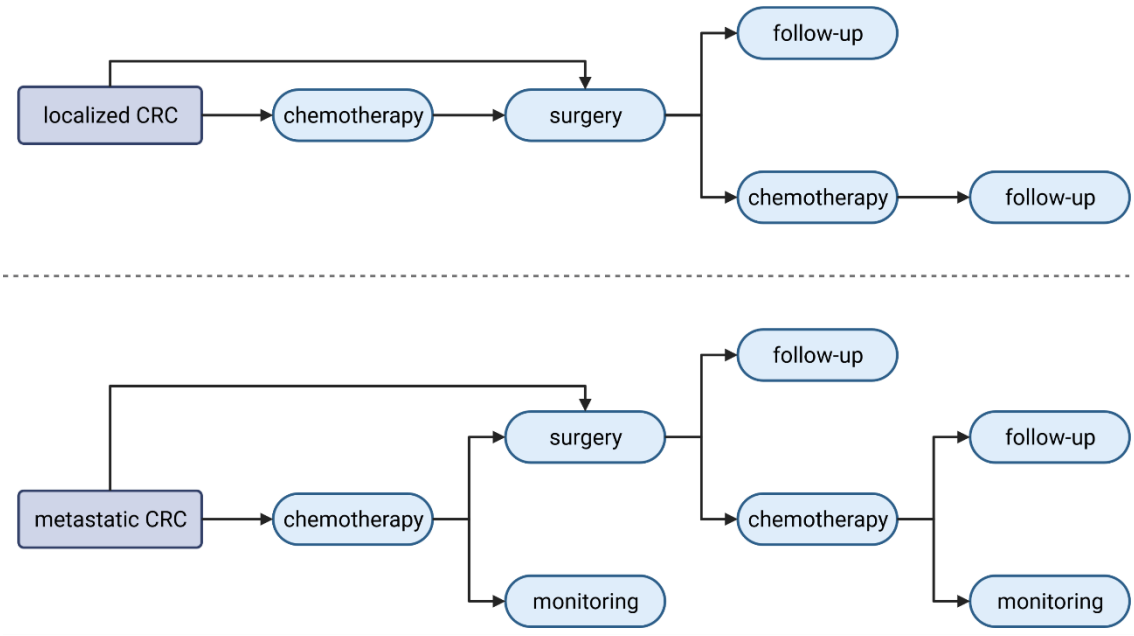

Figure 1 Treatment strategies and follow-up in CRC patients.[53]

2.1.2 Liquid biopsy

Precision oncology is a promising tool to improve the prognosis and treatment outcome of patients with solid tumors[54–57]. A variety of prognostic and predictive molecular tumor markers are analyzed at the time of diagnosis for risk stratification, during treatment to predict response or resistance, and in follow-up for the detection of residual disease and recurrence[14,58–60]. Traditionally, these markers are analyzed in tumor tissue obtained by invasive procedures[61–63]. Moreover, tissue biopsies represent only a single tumor site and thus do not necessarily represent the entire tumor heterogeneity[64–66]. As mentioned in chapter “Molecular biomarkers for precision medicine” analysis of circulating tumor markers present in LB is a promising tool in the field of precision oncology to overcome the limitations associated with tissue biopsies[57,67]. Since only a simple blood collection is required, LB can be used for serial monitoring of patients. Besides the primarily analyzed blood, circulating tumor markers are also present in other biological fluids such as urine, saliva and cerebrospinal fluid[57,68–71]. In addition, LB enables more detailed coverage of intra-tumor heterogeneity as well as clonal evolution[72,73].

2.1.2.1 Tumor derived analytes in liquid biopsy

A variety of tumor derived analytes, including circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), exosomes, and microRNA (miRNA) can be obtained from LB[74-77]. All of these markers have been investigated with respect to their potential for screening, diagnosis, prognosis, and prediction of treatment outcome in cancer patients. While, ctDNA profiles are highly concordant with the molecular profile of primary tumors and metastases, CTCs contain information on the single cell level, and exosomes represent the physiological and pathological stage of viable cells of origin [57,78–82]. However, methods for isolation and analysis of CTCs, exosomes, and miRNAs still lack standardization which prevent the translation[57,67,78,79,81–88]. Because of the great potential

for implementation into clinical practice this work is focused on ctDNA, the easiest accessible marker from LB.

2.1.2.2 Circulating tumor DNA

Dying cells from all tissues are releasing circulating free DNA (cfDNA) into the circulation, yet cfDNA from hematopoietic cells accounts for the largest proportion[89,90]. The ctDNA content released from tumor tissue can range from <0.01% to the majority of total cfDNA and represents a wide range of tumor sites, including metastasis[74,91]. Accordingly, ctDNA reflects intratumor heterogeneity, subclonal mutations and molecular subtypes with distinct genomic signatures[72,92–94]. In addition, the ctDNA fraction serves as a marker for total tumor burden and consequently provides useful information for assessing response or resistance to treatment[95–97]. Indeed, the detection of molecular residual disease (MRD) in form of post-surgery ctDNA has been identified as a strong prognostic marker for disease recurrence and poor prognosis[22,98,99].

Although ctDNA shows great potential as predictive and prognostic tumor marker, several considerations should be taken into account. To overcome the short half-life of cfDNA of <2 h it is important to collect blood samples in specific cfDNA stabilizing blood collection tubes[95]. The frequently low ctDNA fraction requires further highly sensitive and specific assays that need to be carefully analytically validated to enable clinical interpretation, as discussed in detail in Paper I of this thesis[1,100–102]. Also, clonal hematopoiesis may lead to biological false positives impairing the detection of ctDNA based on somatic variants in tumor tissue[103]. Therefore parallel analysis of ctDNA and genomic DNA from peripheral blood molecular cells (PBMCs) is recommended as specified in Paper II of this thesis[2,103–105].

Various approaches have been described for ctDNA detection and quantification, ranging from targeted analysis of hotspot variants to whole genome sequencing (WGS), with sensitivity decreasing as genome coverage increases[57,106–108]. As highlighted in Apendix A: Paper III of this thesis, increasing sensitivity of untargeted ctDNA analysis could be an important step to support disease surveillance in cancer patients without prior knowledge of somatic variants and could even, provide a tool for cancer screening[3,109,110].

2.1.3 New Technologies

2.1.3.1 Targeted ctDNA analysis

Key biomarkers in liquid biopsies, such as ctDNA, are present at very low levels and thus require highly sensitive methods for their accurate detection. With the advances made in ctDNA analysis toward clinical practice in recent years, numerous highly sensitive methods for the detection of ctDNA have been concurrently described. These methods range from the targeted analysis of specific hotspot variants to the untargeted detection of ctDNA (i.e. genome-wide ctDNA profiles rather than targeted variants)[106–108]. Initially, ctDNA analyses focused on PCR-based detection of somatic hotspot variants present at very low variant allele frequencies (VAFs) of <0.1%[95,107]. Meanwhile, the focus is shifting towards ultra-deep targeted next generation sequencing (NGS) to simultaneously detect multiple regions with frequent somatic variants instead of focusing on a single variant[111,112]. Recently, several companion diagnostics tests have been approved by the FDA (U.S. Food and Drug Administration) to guide decision making for targeted therapies in various solid cancers[113]. Moreover, in Germany, health insurance covers the costs of testing for

EGFR and *PIK3CA* variants in non-small cell lung cancer (NSCLC) and breast cancer, respectively[114–116]. Consequently, as the work in Paper I of this thesis shows, there is a very strong focus on determining the limitations and improving the accuracy of the current technologies to detect low frequency profiles of markers[1].

2.1.3.2 Untargeted ctDNA analysis

Although the clinical utility of detecting hotspot variants in ctDNA for companion diagnostics has been demonstrated, the utility of targeted ctDNA analysis is limited to cancer patients who present such variants in their tumor tissue. In contrast, untargeted tests based on genome-wide approaches could be useful for a much wider range of cancer patients. Current research is thus focused on improving the sensitivity of untargeted ctDNA detection. In this context, several features have been described that can be used to detect ctDNA in total cfDNA based on WGS[106,110,117–120].

The presence of somatic copy number alterations (SCNAs) is the most extensively studied ctDNA feature based on shallow WGS. However, not all CRC patients present SCNAs in their tumor tissue and the detection of SCNAs is not sensitive enough in many cases (i.e. ctDNA fraction from 5% to 10% required)[106,121,122]. Nevertheless, as discussed in Apendix A: Paper III of this thesis, the performance of untargeted approaches is substantial[3]. Recently, increased sensitivity for ctDNA detection down to a fraction of <1% based on global and regional ctDNA fragmentation and epigenetic signatures was described [110,117–119,123].

To summarize, clinical utility has only been demonstrated for hotspot variant-based detection of ctDNA to guide treatment decisions, although untargeted ctDNA analysis would be beneficial for all cancer patients regardless of the presence of somatic variants[124,125]. The progress in untargeted ctDNA analysis could be an important step to support disease surveillance in cancer patients without prior knowledge of somatic variants and could even, provide a tool for cancer screening[109,110].

2.1.3.3 Machine learning classifiers

With the rapid development of new technologies for ctDNA detection and the accumulation of massive amounts of data, there is an increasing focus on computational methods, including machine learning (ML) classifiers. ML enables the combined analysis of multiple cfDNA features to increase the sensitivity and specificity of ctDNA detection[126]. Recent studies revealed that ML classifiers can support the identification of individuals with cancer and even the categorization of the tissue of origin based on genome-wide fragmentation profiles or DNA methylation[110,117,119]. Using such classifiers enhanced sensitivity of ctDNA detection for fractions down to <1%, and cancer could be predicted up to four years before diagnosis[110,117,127].

In particular, WGS-based methods generate large amounts of data that cannot be conveniently analyzed for the presence of ctDNA on the basis of minute differences between healthy individuals and cancer patients. ML classifiers trained to assign samples based on defined feature sets, such as cfDNA fragmentation, could provide highly sensitive and specific results that can support clinical interpretation[117,119]. However, as discussed in detail in Apendix A: Paper III of this thesis, the high variability of biological features and the complexity of biological processes involved in cfDNA fragmentation may also lead to false positive predictions[3,128–130]. Therefore, large training sets and external validation of such ML classifiers are critical, as is careful consideration of their intended use, in order to use them in clinical practice[131].

2.2 Rationale and Methods for this project

The aim of this PhD project was to develop and validate robust approaches using novel tumor markers (i.e. ctDNA) for detection of residual disease and monitoring of cancer patients in clinical practice. To this end, two independent methods were developed and analytical and clinical validity was established:

(1) Targeted detection and quantification of ctDNA based on hotspot variant analysis using Droplet Digital PCR (ddPCR) to guide treatment decisions and for disease monitoring.
(2) Untargeted detection of ctDNA based on fragmentation, epigenetic signature and somatic copy number alterations using a whole genome sequencing approach for early detection and treatment monitoring.

2.2.1 Analytical validation as foundation for ctDNA analysis in the clinic

Comprehensive characterization of the analytical performance of clinical laboratory tests is important to understand their potential and limitations in terms of correct interpretation of results to ensure that they are 'fit for purpose'. Different scenarios, such as residual disease detection or monitoring of cancer patients, with different expected ctDNA fractions require different ctDNA detection and quantification capabilities[91]. Furthermore, comparability of liquid biopsy test results from different laboratories is essential to ensure robust interpretation[57,101,132].

In order to obtain meaningful results, thorough validation of ctDNA analysis methods is crucial to allow detection (qualitative test) and quantification (quantitative test) of ctDNA[102]. As defined in 'A standardized framework for the validation and verification of clinical molecular genetic tests'[133] and in 'Recommended Principles and Practices for Validating Clinical Molecular Pathology Tests'[134], sensitivity, precision in terms of the positive predictive value (PPV), specificity, reproducibility, and limit of detection (LOD) are required for analytical validation of a qualitative test. For the validation of a quantitative test, limit of blank (LOB), limit of detection (LOD), limit of quantification (LOQ), precision, trueness, and linearity are required[133,134]. These parameters have been confirmed by recently published recommendations from the Blood Profiling Atlas Consortium (BloodPAC) for the analytical validation of ctDNA NGS assays to be introduced into the clinic[100].

In the context of this PhD project, two cutoffs for the detection (LOB) and quantification (LOQ) of ctDNA were defined. The LOB represents the relevant cutoff for discriminating between background noise and the presence of ctDNA and is determined based on replicate measurements of normal controls. Therefore, the LOB is the basis for treatment decisions based on the presence of actionable variants and for the detection of molecular residual disease. The LOQ represents the cutoff above which ctDNA can be reliably quantified with distinct confidence intervals and is determined based on replicate measurements of positive controls with defined ctDNA levels. Therefore, changes in ctDNA content can only be interpreted above the LOQ in terms of disease monitoring. Depending on the intended use of a method for ctDNA analysis, either only the LOB or both, the LOB and the LOQ of an analytical method need to be established. Validation and reporting of these cutoffs is the foundation for clinical decision making (Figure 2).

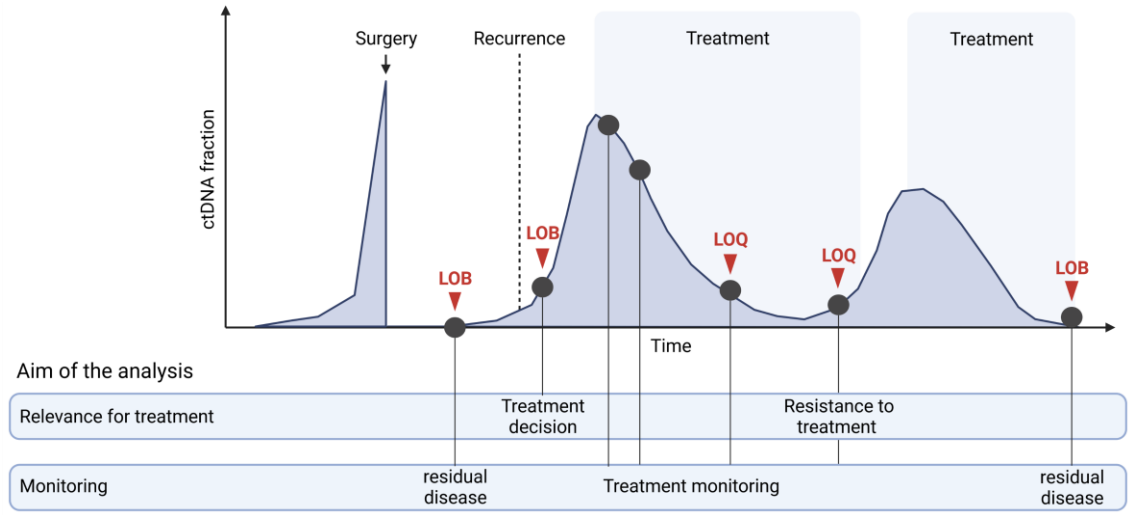

Figure 2 ctDNA fraction throughout the course of disease.
Depending on the clinical application either the LOB or the LOQ represent the critical cutoff. The LOB represents the cutoff for ctDNA detection which is critical for residual disease detection or companion diagnostics. The LOQ represents the cutoff for ctDNA quantification which is required for monitoring with regard to treatment response or resistance.[53]

2.2.2 Clinical validation of ctDNA analysis for disease monitoring in CRC

The clinical utility of ctDNA analysis has so far only been demonstrated for the detection of hotspot variants to guide treatment decisions[124,125]. In order to make further applications of ctDNA analysis feasible for clinical practice, in this PhD project, in addition to analytical validation of targeted approaches for ctDNA analysis, the clinical validity for residual disease detection and disease monitoring in CRC patients was determined.

A study cohort of 66 CRC patients and 80 healthy controls was included. Plasma samples from CRC patients were collected at baseline and at several time points during treatment and follow-up. CRC patients with localized CRC were included before primary surgery or neoadjuvant chemotherapy. Metastatic CRC patients were included either prior to initiation of first-line treatment or prior to surgery. A total of 54 patients were analyzed for evidence of MRD following surgery. Response or resistance to chemotherapy or immunotherapy was assessed in a total of 196 plasma samples of 16 CRC patients.

Based on the presence or absence of a *KRAS* p. G12[A/C/D/R/S/V], and *KRAS* p. G13D variant or *BRAF* p.V600E variant in tumor tissue, patients' plasma samples were analyzed using ddPCR for targeted ctDNA detection and/or LIquid biopsy Fragmentation, Epigenetic signature and Copy Number Alteration analysis (LIFE-CNA), a WGS based approach. In total, 124 plasma samples of 22 CRC patients were analyzed using ddPCR and 198 plasma samples of 50 CRC patients were analyzed using LIFE-CNA, as described in detail in Paper II and Apendix A: Paper III of this PhD thesis (Figure 3)[3].

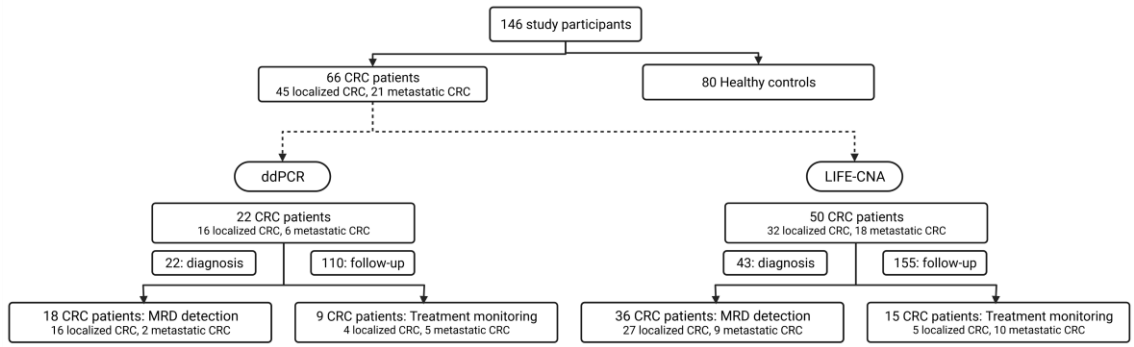

Figure 3 Study cohort.[53]

2.2.3 Targeted hotspot variant analysis using Droplet Digital PCR

As described in the introduction, a variety of technologies are available for the detection of ctDNA. For instance, ddPCR is a targeted analysis for the detection of specific hotspot variants. DdPCR allows discrimination between two different alleles by dividing the sample into up to 20,000 droplets, specifically amplifying the fragment of interest in each droplet, and identifying the tumor variant in the wild-type background with labelled competitive TaqMan probes[135,136]. This enables the detection of specific targeted variants down to VAFs of <0.1%[135].

The high sensitivity and low turnaround time make ddPCR suitable for companion diagnostics to guide treatment decisions, although it is only applicable for the detection of specific variants. Indeed, prognostic value has been demonstrated for only a few specific somatic variants[102,137]. Numerous studies have shown that ddPCR is a suitable method for the detection of tumor variants occurring in low VAFs in plasma. However, none of these studies validated their method with respect to all required assumptions or parameters for quantitative tests. While Riva et al. and Bidard et al. focused only on a cutoff based on the total number of positive droplets to discriminate between positive and negative results, Milosevic et al. established all required performance characteristics for quantitative tests assuming normally distributed results[138–140].

As described in detail in Paper I of this PhD thesis, five ddPCR assays targeting the most frequent actionable variants in NSCLC and CRC were validated according to the guidelines for quantitative test validation[1,133,134,141,142]. Striking differences in the performance of the different ddPCR assays were observed. While no false positive signals were observed in WT controls for the *EGFR* p.L858R and the *EGFR* exon 19 deletions screening assays, several false positive signals were detected with the *KRAS* p.G12/p.G13 screening assay, resulting in LOBs of ~0% and 0.11% measured VAF, respectively. Similarly, the established LOQs varied between the different assays, with the lowest for the *KRAS* p.G12/p.G13 screening assay being a VAF of 0.41% and the highest for the *EGFR* exon 19 deletions screening assay being a VAF of 0.7%. Furthermore, a guideline was developed for clinical interpretation of the results based on the LOB as cutoff for ctDNA detection and the LOQ as cutoff for ctDNA quantification. The importance of distinct cutoffs for ctDNA detection and quantification for clinical interpretation, in conjunction with the striking differences in assay performance, demonstrate the importance of independent analytical validation of each assay to provide meaningful reports in clinical practice[1].

To demonstrate the clinical validity of the LOB as cutoff for ctDNA detection and the LOQ as cutoff for ctDNA quantification, a total of 124 plasma samples collected from 22 CRC patients were analyzed with ddPCR as described in Paper II of this PhD thesis[2]. It was demonstrated that specific detection of residual disease and recurrence was feasible based on the LOB in three patients

post-surgery and one patient up to 2.5 months before clinical evidence of recurrence. Furthermore, based on VAF quantification above the LOQ, changes in ctDNA levels could be correlated with the actual course of disease in all of seven patients with ctDNA detected at baseline. Consistent with previous studies, this study also suggests that ctDNA is a more sensitive tumor marker compared with standard CEA and can provide valuable information for treatment monitoring and follow-up[2,22]. When using ctDNA analysis for predicting residual disease and recurrence and for treatment monitoring, accurate validation of the LOB as cutoff for ctDNA detection and the LOQ as cutoff for ctDNA quantification is critical. Therefore, in case of using ctDNA as an additional marker for residual disease and recurrence detection, and treatment monitoring, the LOB- and LOQ-based approach can be easily implemented to provide accurate results[2].

A large proportion of cancer patients will already benefit from highly sensitive and cost-effective ddPCR, provided that the most frequent hotspot variants are detected. Still, the analysis of only specific hotspot variants limits the benefits of this method to patients who actually present with such a somatic variant. To extend the benefits of ctDNA analysis to a larger number or even all cancer patients, thoroughly validated methods with equal high sensitivity for the untargeted ctDNA analysis (see chapter “Untargeted analysis using LIFE-CNA”) are needed. Such methods would also require distinct cutoffs for the detection and quantification of ctDNA to allow accurate clinical interpretation.

2.2.4 Untargeted analysis using LIFE-CNA

Untargeted analysis of ctDNA can be based on multiple cfDNA features, such as somatic copy number alterations (SCNAs), cfDNA fragmentation and epigenetic signatures. The potential to identify SCNAs based on shallow WGS of cfDNA was first published by Heitzer et al. in 2013[106]. In 2016, Ulz and Belic et al. described that also focal SCNAs can be identified using the same approach[143]. However, SCNA analysis based on shallow WGS only enables detection of ctDNA levels of >5% to 10%[106,122,144], limiting its advantages to late stage cancer patients, who typically have higher levels of ctDNA[91].Recently, multiple studies focused on cfDNA fragmentation pattern as result of nucleosome occupancy to detect ctDNA. Characteristically cfDNA shows a peak at 167 bp corresponding to DNA wrapped around one nucleosome plus linker DNA[119,145]. Consequently, Fragmentation of cfDNA reflects the nucleosome footprint and thus the chromatin profile of the cells of origin[117,120,123,146]. However, epigenetic dysregulation in cancer causes changes in nucleosome footprints and therefore enrichment of shorter or longer cfDNA fragments originating from open or closed chromatin, respectively[147]. Mouliere, Chandrananda, Piskorz, and Moore et al. described in 2018 that enriched tumor content in short cfDNA fragments from 90 to 150 improved SCNA detection when limiting WGS data to this fragment range[118]. One year later, Cristiano, Leal, Phallen, and Fiskel et al. identified significant differences in the regional fragmentation profiles of cancer patients compared to healthy controls correlating to chromatin profiles of the tissue of origin[117]. In addition to effects on cfDNA fragmentation nucleosome occupancy also leads to differences in sequencing depth, creating distinct WGS coverage profiles from where presence of ctDNA can be inferred[148]. In 2021, Peneder and Stütz et al. combined global and regional fragmentation as well as coverage in cancer specific chromatin signatures obtained from deep WGS data and successfully detected ctDNA independently of the somatic mutational profile[119].

In the scope of this PhD project LIFE-CNA was developed, an approach using WGS for ctDNA detection based on the combined analysis of fragmentation profiles and epigenetic signatures as

described by Peneder and Stütz et al.[119], and SCNA analysis focusing on focal events as described by Heitzer et al.[106] and Ulz and Belic et al.[143] (Figure4).

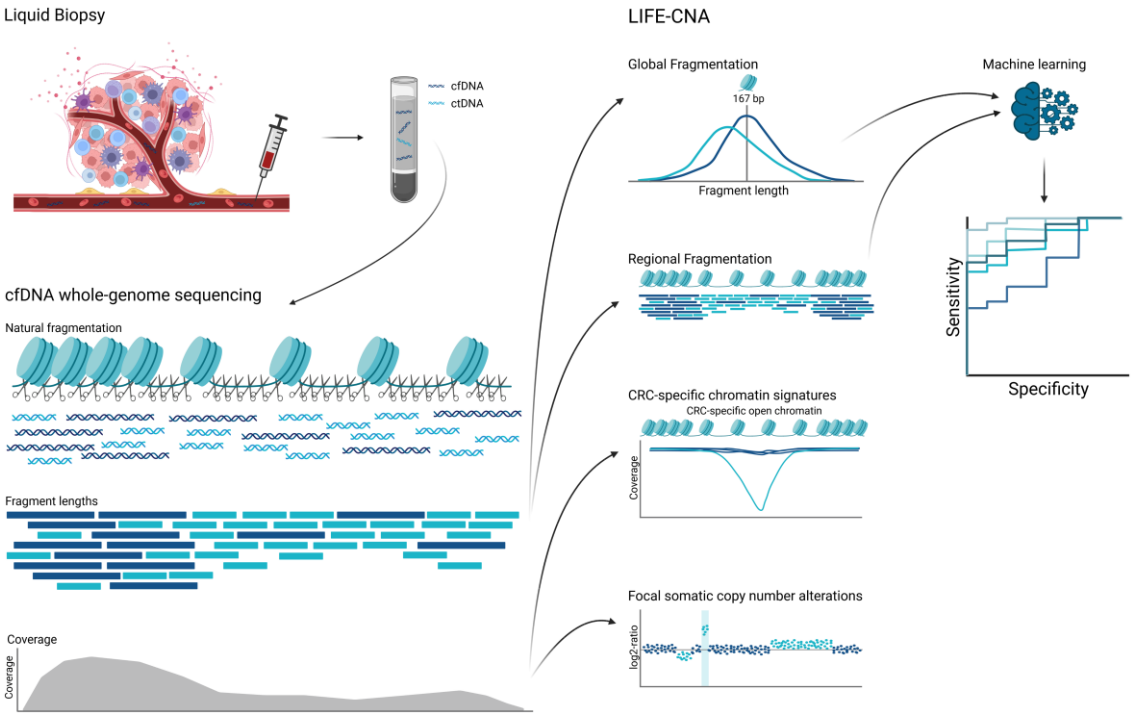

Figure 4 Overview of LIFE-CNA.
Fragment lengths obtained from paired-end WGS provide information on global and regional fragmentation. WGS coverage provides information on chromatin signatures and somatic copy number alterations. A machine learning algorithm is used to predict ctDNA based on global and regional fragmentation.[53]

As described in detail in Apendix A: Paper III of this PhD thesis, 259 plasma samples from 50 CRC patients and 61 healthy controls were analyzed for analytical and clinical validation of LIFE-CNA[3]. Distinct cutoffs or significance tests to reliably detect ctDNA were established for the analyzed cfDNA features based on 55 healthy controls. To evaluate whether ctDNA detection based on cfDNA fragmentation can be improved with the use of machine learning (ML), three ML classifier were trained based on the global and regional fragmentation of 63 control samples (55 healthy controls and 8 samples collected from patients in remission >6 weeks post-treatment with no evidence of recurrence) and 134 ctDNA positive samples (collected from CRC patients with clinically evident tumor burden). With these ML classifiers a sensitivity of up to 73% at 95% specificity with a receiver operating characteristic (ROC) area under the curve (AUC) of up to 95% was achieved to correctly identify ctDNA positive patients. However, the analysis of six external healthy controls, showed low specificity for the ML classifiers based on regional fragmentation and the meta-learner. Indicating the need of a larger training set and external validation to avoid overfitting before ML classifiers could be implemented into clinical practice.

For clinical validation, LIFE-CNA was evaluated for its ability to detect ctDNA at diagnosis, to predict residual disease and recurrence, and for treatment monitoring. Regional fragmentation showed the highest sensitivity for ctDNA detection at diagnosis for both localized (78%: 21/27) and metastatic CRC (81%: 13/16), followed by CRC-specific chromatin signatures that correctly identified ctDNA in 26% (7/27) of localized and 50% (8/16) of metastatic CRC. Both, SCNAs and

global fragmentation were identified to be mainly useful for metastatic CRC, with the lowest sensitivity observed for global fragmentation with 31% (5/16). Although SCNAs provide only information in CRC patients with high ctDNA levels, they have the highest clinical impact for disease monitoring, as specific focal amplification, such as *KRAS* amplification, might provide relevant information to guide treatment decisions[149].

When evaluating LIFE-CNA for its ability to detect residual disease, false-positives were observed with all analyzed cfDNA features in patients within the first six weeks post-surgery. In addition to the complexity and variability of chromatin structure, there are several hypotheses that could provide possible explanations for the observed low specificity of post-surgery ctDNA detection based on cfDNA fragmentation. Namely, acute epigenetic changes have been described after surgery, and shorter cfDNA fragments have been associated with low-molecular-weight heparin treatment[128–130]. Nonetheless, decreasing numbers of false-positives more than six weeks post-treatment in combination with the high sensitivity of ctDNA detection at diagnosis indicate suitability of LIFE-CNA for recurrence monitoring and cancer screening.

Further LIFE-CNA was assessed for its eligibility for treatment monitoring by correlating changes in all analyzed cfDNA features to response or resistance to treatment. Based on changes in global and regional fragmentation, the coverage in CRC-specific active chromatin, and SCNAs, response or resistance to treatment could be predicted in 77% (10/13) or 100% (5/5) of cases, respectively.

Overall analytical and clinical validity of LIFE-CNA by combining global and regional fragmentation, coverage in CRC-specific active chromatin and analysis of SCNAs based on distinct cutoffs or significance tests for ctDNA detection was established. Apendix A: Paper III provides evidence that LIFE-CNA is a highly sensitive, untargeted approach for early detection and treatment monitoring in CRC patients, regardless of their mutational status, that could even be translated to all types of solid tumors[3]. Further, the cost-effectiveness of single sample analysis is much higher than ddPCR-based targeted analysis of hotspot variants because of the required ~6 fold genome coverage for disease monitoring in all CRC patients. In summary, LIFE-CNA is a tool with the potential to be implemented into clinical practice for CRC patient management.

2.2.5 Comparison of both ctDNA analysis approaches

Both methods described above provide high sensitivity for ctDNA detection. While ddPCR (see chapter: Targeted hotspot variant analysis using Droplet Digital PCR) can be used for targeted analysis of hotpot variants, LIFE-CNA (see chapter: Untargeted analysis using LIFE-CNA) allows the untargeted analysis of all cancer patients, regardless of their somatic mutational profile. With the shortest turnaround time and lowest cost for single sample analysis, ddPCR is the method that seems most reasonable within the current diagnostic workup for companion diagnostics in cancer patients. However, once ctDNA testing is introduced to clinical practice on a wider scale, LIFE-CNA may provide valuable information by enabling early detection and untargeted disease monitoring in follow-up samples of CRC patients. To select the ideal ctDNA analysis method, cancer patients should be stratified based on the presence of known hotspot variants, the clinical intent as well as the sensitivity, turnaround time, and cost-effectiveness of the two different ctDNA analysis methods.

With respect to early detection or residual disease and recurrence detection, the LOB of an assay is critical as a cutoff for ctDNA detection. For ddPCR, the LOB is defined as the measured VAF above which variants are detected with 95% specificity, thus avoiding technical false positives. In

case of LIFE-CNA, distinct 95% specificity cutoffs or significance tests for all cfDNA features are used to detect ctDNA.

Focusing on treatment monitoring, clinically meaningful reports can be generated by reliable VAF quantification above the LOQ using ddPCR. In case of LIFE-CNA, such a cutoff for ctDNA quantification cannot be established because of missing reference materials and the analysis of multiple cfDNA features in parallel. However, when follow-up samples are analyzed, treatment response can be reliably predicted once all features, including SCNAs, chromatin signatures, and fragmentation profiles, can no longer be distinguished from healthy controls. In addition, resistance to treatment can be predicted if one or more of these features newly emerge in follow-up samples (Figure 5).

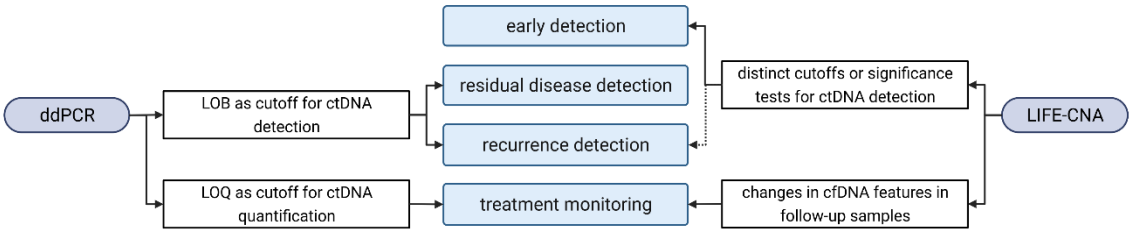

Figure 5 Clinical applicability of both established ctDNA analysis approaches.[53]

2.3 Contribution of this PhD thesis

In conclusion, within the scope of this PhD project, two robust methods for ctDNA analysis have been established that can be used in clinical practice for different scenarios. Each of the methods summarized above has its distinct advantages and limitations. Therefore, it is critical to consider the clinical situation when deciding which ctDNA analysis offers the best diagnostic options for a patient. Targeted analysis of only specific hotspot variants with ddPCR provides the ideal foundation to guide treatment decisions when the most common actionable variants need to be addressed. Residual disease and recurrence detection based on the LOB as cutoff for ctDNA detection and treatment monitoring in follow-up samples using the LOQ as cutoff for ctDNA quantification can be performed for patients with known hotpot variants by ddPCR. For patients without knowledge of the somatic mutational profiles, LIFE-CNA enables the highly sensitive untargeted detection of ctDNA based on multiple cfDNA features that could even support early detection of CRC. By tracking changes in global and regional fragmentation, coverage in CRC-specific active chromatin and SCNAs in follow-up samples, LIFE-CNA further allows clinical interpretation in terms of response or resistance to treatment.

3. Paper I

Hallermayr, A.; Benet-Pagès, A.; Steinke-Lange, V.; Mansmann, U.; Rentsch, M; Holinski-Feder, E.; Pickl, J.M.A. Liquid Biopsy Hotspot Variant Assays: Analytical Validation for Application in Residual Disease Detection and Treatment Monitoring, *Clinical Chemistry*, Volume 67, Issue 11, November 2021, Pages 1483–1491, https://doi.org/10.1093/clinchem/hvab124[1]

Clinical Chemistry 67:11
1483–1491 (2021)

Liquid Biopsy Hotspot Variant Assays: Analytical Validation for Application in Residual Disease Detection and Treatment Monitoring

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BACKGROUND: Analysis of circulating tumor DNA (ctDNA) in plasma is a powerful approach to guide decisions in personalized cancer treatment. Given the low concentration of ctDNA in plasma, highly sensitive methods are required to reliably identify clinically relevant variants.

METHODS: We evaluated the suitability of 5 droplet digital PCR (ddPCR) assays targeting *KRAS*, *BRAF*, and *EGFR* variants for ctDNA analysis in clinical use.

RESULTS: We investigated assay performance characteristics for very low amounts of variants, showing that the assays had very low limits of blank (0% to 0.11% variant allele frequency, VAF) and limits of quantification (0.41% to 0.7% VAF). Nevertheless, striking differences in detection and quantification of low mutant VAFs between the 5 tested assays were observed, highlighting the need for assay-specific analytical validation. Besides in-depth evaluation, a guide for clinical interpretation of obtained VAFs in plasma was developed, depending on the limits of blank and limits of quantification values.

CONCLUSION: It is possible to provide comprehensive clinical reports on actionable variants, allowing minimal residual disease detection and treatment monitoring in liquid biopsy.

Introduction

In the era of precision medicine, liquid biopsy is a promising tool for identification of genetic tumor status and real-time monitoring of evolutionary tumor dynamics in plasma (1). Detection and quantification of pathogenic variants in key oncogenes including *KRAS proto-oncogene* (*KRAS*), *B-Raf proto-oncogene* (*BRAF*), and *epidermal growth factor receptor* (*EGFR*) may be used to detect therapy resistance, residual disease, and recurrence before clinical evidence, in different types of solid cancer (2–5). In liquid biopsy, appropriate methods including droplet digital PCR (ddPCR) are required to detect lowest amounts of variants in plasma, and to distinguish their signals from inherent background noise, since these are relevant for individual treatment decision (6, 7). For instance, it could be shown that the presence of tumor variants, even at very low variant allele frequencies (VAFs) of <1%, serve as indicator of residual disease (8) and response to *EGFR*-directed therapy (9, 10). When applying these methods for clinical interpretation, it is critical to understand and assess their performance characteristics at the lower end of the measurement scale (2, 11, 12). Two important performance characteristics are the limit of blank (LOB) and the limit of quantification (LOQ), each of which have a distinct definition according to guidelines (13). The LOB serves as cutoff for a sample to be defined as positive or negative for a variant. The LOQ is the lowest VAF that can reliably be detected and accurately quantified. Additionally, to accurate assessment of very low VAFs, further, it is important to acknowledge assay variability, which is expressed by measurement uncertainty or total measurement error.

Here, we provide a large study assessing the performance characteristics of various ddPCR assays, which cover clinically relevant variants in *BRAF*, *KRAS*, and *EGFR*. We clearly determined for each assay whether a result was truly negative, positive, and quantifiable, and evaluated further parameters including total measurement error, trueness, precision, and linearity. We further developed a guide for clinical interpretation

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of obtained VAFs, depending on the LOB and LOQ values for each assay, which were evaluated in the course of this work, to enable clinical interpretation of pathogenic variants in plasma for liquid biopsy-based residual disease and treatment monitoring.

Material and Methods

SAMPLES AND DDPCR ANALYSES

Information on reference sample generation and VAF determination, DNA extraction, and ddPCR protocols are provided in the Methods in the online Data Supplement.

ACCEPTANCE CRITERIA FOR REPORTING CLINICALLY RELEVANT LIQUID BIOPSY VARIANTS

Liquid biopsy samples with very low measured VAFs above the LOB were detected with ≥95% specificity, and are reported as mutant positive in compliance with CLSI guidelines on quantitative clinical tests (Supplemental Fig. 1, A and B) (13). Detection of lowest variant amounts is crucial when assessing ctDNA status after surgery for prognostic purposes. Detection of ctDNA in plasma is a strong indicator of residual disease (Supplemental Fig. 1, C) (8). Liquid biopsy samples with measured VAFs ≥limit of detection (LOD) were detected with ≥95% sensitivity, while measured VAFs ≥LOQ were detected with ≥95% sensitivity and acceptable measurement uncertainty (i.e., total error ≤50%) (Supplemental Fig. 1, A and B) (13, 14). Due to precise VAF measurements at LOQ, quantification at the LOQ and higher frequencies is possible. This enables identification of minimal but true changes in VAF abundances ≥LOQ, thereby providing information on tumor progression and response to treatment, respectively (2, 3, 8) (Supplemental Fig. 1, C).

LOB The LOB was determined by measuring the fractional abundance, which represents the positive relative droplet counts for the tumor variant in all droplets of all detected alleles, in at least 60 negative control (NC) measurements (Supplemental Table 1) (13). The value of the rank of the 95th percentile of sorted results from the NC samples was determined as the LOB (Supplemental Methods, Supplemental Fig. 1, B).

LOD Determination of the LOD is critical for identifying the clinically relevant parameter, the LOQ (see next). Measured VAFs at the LOD were detected with ≥95% sensitivity. To obtain the LOD, at least 60 measurements of low mutant VAF positive control (PC) samples were performed (13) (Supplemental Table 2). Distribution of fractional abundance was assessed using probability quantile–quantile plots. Bartlett's test was used to test the consistency of the standard deviation of the fractional abundances across samples with different mutant VAFs (i.e., if samples had equal variances) (Supplemental Table 3) (15). Depending on the result distribution and pooled standard deviations (SD_S) of each independent test assay, a parametric or nonparametric approach was selected to determine the LOD (Supplemental Methods).

LOQ In addition to criteria at the LOB (specificity ≥95%) and the LOD (sensitivity ≥95%), at the LOQ, the total error of these measurement results was required to be ≤50% with a 95% confidence interval to allow accurate VAF quantification during treatment monitoring (13, 14). The LOQ was determined by measuring the mutant VAFs in >40 replicate measurements according to guidelines (13).

LINEARITY

Confirmation of assay linearity was critical to ensure precise VAF quantification across the entire measurement scale (i.e., from LOQ to 100%). This way, the entire VAF range in plasma of cancer patients was covered (16) and under- or overestimation of VAFs was avoided. Linear range was established by VAF measurements of 7 PC samples in 2 to 3 replicates with evenly distributed mutant VAFs (from the lowest VAF determined by the LOQ up to 100% VAF) for each of the 5 assays (17) (Supplemental Table 5). Linearity was assessed using polynomial regression analysis for first-, second-, and third-order polynomials. To assess if the fractional abundance equaled the mutant VAF the difference between the best fitting polynomial and the ideal linearity ($y = x$) must have been ≤10%. Further, to assess whether replicate measurements used for the determination of linearity were representative, repeatability of these measurements was assessed by calculating the pooled SD (SD_r) (Supplemental Methods) (17).

TRUENESS, PRECISION AND TOTAL ERROR

Trueness, representing the closeness of measurement results to the true value, was indirectly determined by calculating the bias for replicate measurements of PC samples with mutant VAFs at the LOQ of each independent assay (Supplemental Methods). To provide reliable results, the bias should not exceed 10% leading to trueness ≥90% (14).

The precision of each ddPCR assay, representing the closeness of repeated measurement results, was determined in terms of repeatability and intermediate precision, considering intra- and interapproach precision. Both parameters were calculated for replicate measurements of PC samples with mutant VAFs at and above the LOQ of the intended assay (Supplemental Table 6).

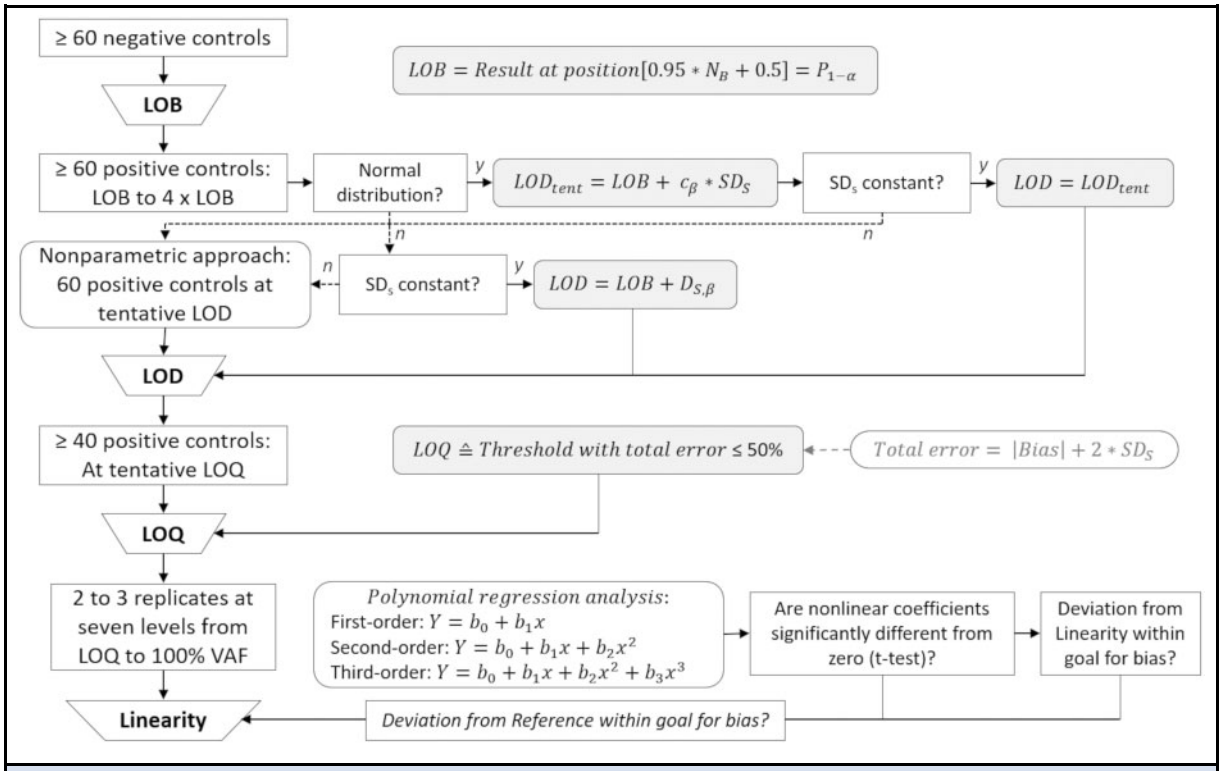

Fig. 1. Experimental design for the establishment of the limit of blank (LOB), limit of detection (LOD), limit of quantification (LOQ), and linearity for each of the ddPCR assays.
[N_B: Number of negative control results; $c_\beta = \frac{1.645}{1-\frac{1}{4n}}$ (95% confidence interval); SD_S: pooled standard deviation; $D_{S,\beta}$: Distance from the β percentile of the distribution of positive control results; b_i: regression coefficients; *y*: yes; *n*: no].

Repeatability, intermediate precision, and total error was calculated for 3 different VAF intervals: at the LOQ, from the LOQ to 10% mutant VAF, and from 10% to 100% mutant VAF. Repeatability was indirectly determined based on the SD_r (Eq. 6, Supplemental Methods) of all replicate measurement results obtained within one experiment. Intermediate precision was indirectly determined based on the intermediate SD (SD_{IP}, Eq. 10, Supplemental Methods). To provide reliable results, both SD_r and SD_{IP} should be less than 20% for mutant VAFs at the LOQ and less than 15% for mutant VAFs above the LOQ, leading to both repeatability and intermediate precision ≥80% for mutant VAFs at LOQ and ≥85% for mutant VAFs above LOQ (18).

The total error was calculated for a 95% confidence interval by combining bias with 2 times the pooled SD (SD_S) (13) (Supplemental Methods). The goal for the total error was set according to the acceptance criteria for trueness and precision and should therefore not exceed 50% for replicate measurements of PC samples at the LOQ and 40% for low, medium, and high mutant VAFs (14, 18).

Results

In this study we focused on 5 Bio-Rad ddPCR™ Mutation Detection Assays for analysis of *BRAF* p. V600E, *EGFR* p. T790M, *EGFR* p. L858R, *KRAS* p. G12[A/C/D/R/S/V], and *KRAS* p. G13D variants, and the 15 most frequent *EGFR* exon 19 small deletions, which are clinically relevant for patients with colorectal cancer and nonsmall cell lung cancer (4, 5). Determination of their key performance characteristics, LOB, LOD, LOQ, and linearity was performed in accordance with the current guidelines for analytical validation of quantitative tests (13, 17, 19, 20) (Fig. 1, online Data Supplement).

LOB OF ASSAYS

To determine the LOB, a nonparametric approach was used, since fractional abundance measured with ddPCR did not show a normal distribution. Accepting 95% specificity, the LOB was obtained by calculating the 95th percentile of obtained values. For *EGFR* p. L858R and *EGFR* exon 19 deletions assays, the LOB was confirmed

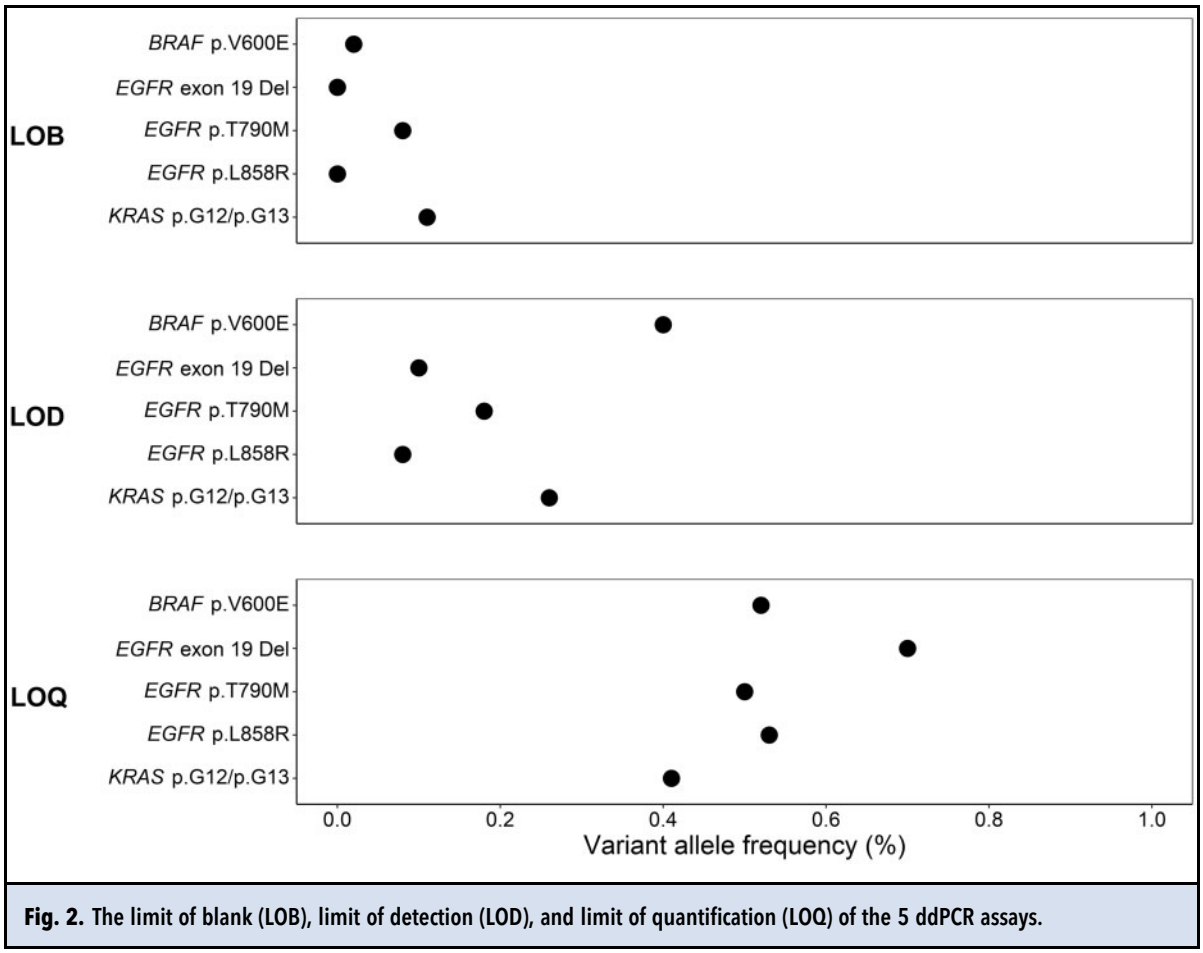

Fig. 2. The limit of blank (LOB), limit of detection (LOD), and limit of quantification (LOQ) of the 5 ddPCR assays.

at ~0.00%, for *BRAF* p. V600E at 0.02%, for *EGFR* p. T790M at 0.08%, and for *KRAS* p. G12/p.G13 at 0.11% mutant VAF (Fig. 2, Table 1, Supplemental Fig. 2). Taken together, these results showed that the background noise of the assays would be expected to be 0 or close to 0 for *EGFR* p. L858R, *EGFR* exon 19 deletion, and *BRAF* p. V600E assays, low for *EGFR* p. T790M assay and modest (but still acceptable) for *KRAS* p. G12/p.G13 assay. As mentioned above, measured VAFs > LOB are interpreted as "detected" in plasma, and indicate residual disease (8).

Table 1. Limit of blank (LOB), limit of detection (LOD), and limit of quantification (LOQ) of 5 investigated assays.

	BRAF	*Del19*	*T790M*	*L858R*	*KRAS*
LOB	0.02	0.00	0.08	0.00	0.11
LOD	0.40	0.10	0.18	0.08	0.26
LOQ	0.52	0.70	0.50	0.53	0.41

BRAF, *BRAF* p. V600E assay; *Del19*, *EGFR* exon 19 deletions assay; *T790M*, *EGFR* p. T790M assay; *L858R*, *EGFR* p. L858R assay; *KRAS*, *KRAS* p. G12/p. G13 assay.

LOD OF ASSAYS

The LOD is the VAF at which 95% sensitivity was achieved (Supplemental Fig. 1, B). From a clinical point of view, the LOD is no cutoff for interpretation, in contrast to LOB and LOQ (13) (Supplemental Fig. 1, A and C). Actually, the LOD was a first step in identifying the clinically relevant LOQ. The *EGFR* p. L858R assay had the lowest LOD of all 5 assays (0.08%) followed by the *EGFR* exon 19 deletions assay (0.1%), the *EGFR* p. T790M assay (0.18%), the *KRAS* p. G12/p.G13 assay (0.26%), and was highest for the *BRAF* p. V600E assay (0.4%) (Fig. 2, Table 1, Supplemental Figs. 2 and 3), showing an up to 5-fold difference between the LODs across assays.

LOQ OF ASSAYS

The LOQ is the lowest mutant VAF that can be reliably quantified with acceptable measurement uncertainty in

addition to fulfilling the criteria at LOD, as the total measurement error is ≤50% (13, 14). The LOQ was lowest for *KRAS* p. G12/p.G13 (0.41%), followed by *EGFR* p. T790M (0.5%), *BRAF* p. V600E (0.52%), *EGFR* p. L858R (0.53%), and was highest for *EGFR* exon 19 deletions (0.7%) assays (Fig. 2, Table 1, Supplemental Fig. 2). Hence, the obtained LOQs differed up to 1.7-fold between assays. Changes in VAFs measured ≥LOQ over time during treatment are a direct indicator of treatment response or tumor progression (9, 10).

LINEARITY OF ASSAYS

Linearity describes the ability of a method to provide results that are directly proportional to the VAF of a mutant variant (17). Briefly, the coefficients of variation for these replicate fractional abundance measurements were 6.30% in case of the *EGFR* p. T790M, 12.23% for *EGFR* p. L858R, 7.75% for *EGFR* exon 19 deletions, 7.50% for *BRAF* p. V600E, and 9.35% for the *KRAS* p. G12/p.G13 assays. Allowing 10% bias, the measured fractional abundances reflected the mutant VAF from the LOQ to 100% for all intended assays, confirming that measured VAFs were neither under- nor overestimated throughout the entire measurement range. This is critical for direct comparison of VAFs measured during serial samplings in treatment monitoring to deduce accurate tumor burden chances over time (Supplemental Fig. 4).

TRUENESS, PRECISION, AND TOTAL ERROR OF ASSAYS

We further determined trueness at the LOQ, as well as precision and total error within the linear range from LOQ to 100% VAF for each ddPCR assay by using VAF intervals: LOQ [(x = LOQ)], from LOQ to 10% mutant VAF [(LOQ< x <10%)], and from 10% to 100% mutant VAF [(x ≥ 10%)] (Table 2, Supplemental Table 6). The total error combined bias and imprecision (Supplemental Methods, Eq. 11) and represented the 95% confidence interval around a measurement result. Knowledge of the total error is critical when analyzing longitudinal samples for treatment monitoring. At the LOQ, trueness was calculated for each independent ddPCR assay, and ranged from 88.14% to 96.75%. Trueness of the *EGFR* p. T790M assay did not meet the goal of ≥90%, still the total error of this assay met the goal of ≤50% at the LOQ. Repeatability at the LOQ ranged from 77.4% to 83.7%, intermediate precision ranged from 70.5% to 81.5%, and the total error ranged from 39.0% to 52.4%. Repeatability and intermediate precision of the *BRAF* p. V600E, the *EGFR* p. L858R, and the *KRAS* p. G12/p. G13 assays did not meet the goal for precision of ≥80%, still the total error of the *EGFR* p. L858R and the *KRAS* p. G12/p.G13 assays met this goal. The total error of the *BRAF* p. V600E assay resulted in 52.4%, and was only a little bit higher than the goal of ≤50% at the LOQ. In the interval LOQ to 10% mutant VAF, repeatability ranged from 87.2% to 93.0%, intermediate precision ranged from 89.0% to 94.5%, and the total error ranged from 26.2% to 37.8%. In the interval 10% to 100% mutant VAF, repeatability was obtained in a range from 98.1% to 99.7%, intermediate precision ranged from 98.8% to 99.1%, and the total error was obtained in a range from 7.6% to 16.5%.

As expected, repeatability and intermediate precision were higher for higher mutant VAFs than for lower mutant VAFs, which is accordingly reflected in the total error.

Table 2. Trueness, precision, and total error of 5 independent ddPCR assays.

	BRAF	*Del19*	*T790M*	*L858R*	*KRAS*
Trueness (%)	90.4	96.6	88.1	96.8	95.0
Repeatability (%): x = LOQ	78.5	81.7	83.7	78.9	77.4
Repeatability (%): LOQ < x < 10%	90.5	87.2	90.7	92.3	93.0
Repeatability (%): x ≥ 10%	99.7	98.9	99.0	98.9	98.1
Intermediate precision (%): x = LOQ	72.7	81.5	80.4	75.8	70.5
Intermediate precision (%): LOQ < x < 10%	94.2	89.0	91.8	93.2	94.5
Intermediate precision (%): x ≥ 10%	99.0	99.1	99.1	99.0	98.8
Total error (%): x = LOQ	52.4	39.0	47.3	44.5	46.3
Total error (%): LOQ < x < 10%	36.3	37.8	32.2	34.1	26.2
Total error (%): x ≥ 10%	14.9	8.6	16.5	7.6	10.9

LOQ, limit of quantification; *BRAF*, *BRAF* p. V600E assay; *Del19*, *EGFR* exon 19 deletions assay; *T790M*, *EGFR* p. T790M assay; *L858R*, *EGFR* p. L858R assay; *KRAS*, *KRAS* p. G12/p. G13 assay.

APPLICATION OF LOB AND LOQ THRESHOLDS IN CLINICAL USE

As an example of the application of LOB and LOQ thresholds in clinical use, follow-up circulating-free DNA (cfDNA) samples of 2 patients with colorectal cancer having either the *KRAS* p.G13D variant or the *BRAF* p. V600E variant present in tumor tissue were analyzed at different time points throughout the course of disease (Fig. 3). The analytical validation performed within this study enabled precise differentiation between negative and positive results (LOB) and quantification of the VAF (above the LOQ). Using the liquid biopsy, we observed tumor progression (Fig. 3, A) and response to treatment (Fig. 3, B).

Figure 4 provides an overview at which time points during treatment measurement of mutant variants by liquid biopsy is of clinical relevance. In clinical practice, determination of residual disease after surgery with liquid biopsy assays improves classification into patients at high- and low-risk, especially for borderline patients. Here, the detection of VAFs above or below the LOB, is an indicator of the presence or absence of residual disease, respectively (Supplemental Fig. 1, A) (21). Similarly, during follow-up, liquid biopsy enables a more accurate determination of prognosis. Thus, follow-up intervals or new therapies can be planned more precisely. During chemotherapy, the liquid biopsy can provide an indication of treatment response or tumor progression by assessing tumor burden during therapy by monitoring VAFs ≥LOQ (Supplemental Fig. 1, A).

Discussion

In this study, we evaluated the ability of 5 ddPCR-based assays to reliably detect and quantify clinically relevant *BRAF*, *EGFR*, and *KRAS* tumor variants in ctDNA. We performed validation of the *BRAF* p. V600E, *EGFR* exon 19 deletions, *EGFR* p. T790M, *EGFR* p. L858R, and *KRAS* p. G12/p.G13 assays, and confirmed the reliability and accuracy of variant detection and quantification for clinical use. Thereby, we focused on international guidelines for quantitative tests (13, 14, 17, 19, 20). We further provide a guide for how to apply the determined detection and quantification limits in the liquid biopsy reports on residual disease and monitoring approaches and highlight the clinical meaning of measured VAFs (Fig. 4).

Although other studies have applied ctDNA-based ddPCR for the detection of tumor variants occurring at low VAFs in plasma, they did not address all the parameters required for VAF quantification. The most detailed validation protocol for ddPCR-based liquid biopsy assays was provided by Milosevic et al. (22), but there were some limitations: first, they analyzed mutant copies/mL plasma for determining the performance parameters, instead of the fractional abundance of mutant alleles in a background of wild type alleles, which is the

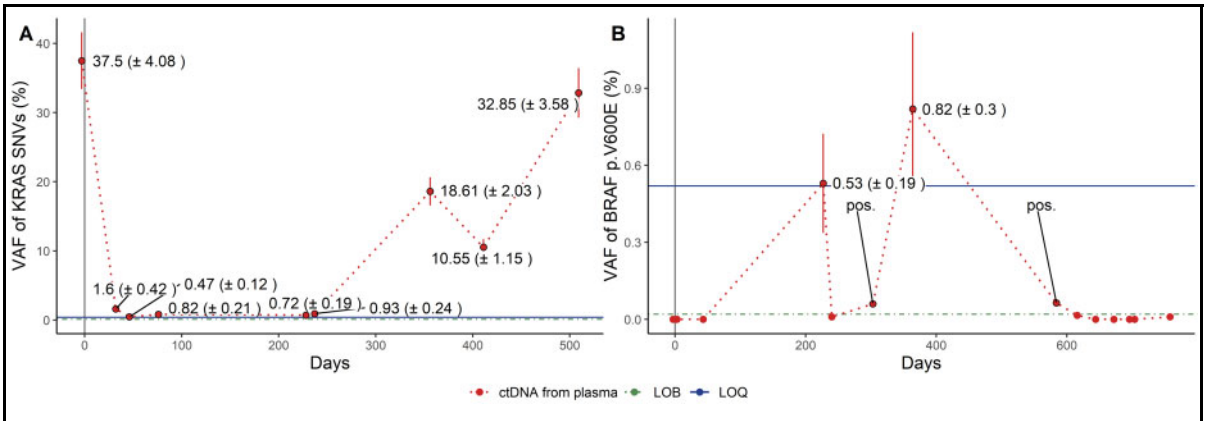

Fig. 3. Disease monitoring in 2 patients with colorectal cancer. Several samples were analyzed throughout the course of disease of 2 patients with colorectal cancer. Day 0 indicates the start of therapy. Measured mutant variant allele frequencies (VAFs) greater than the limit of quantification are labeled with the respective VAF and 95% confidence interval. Measured mutant VAFs between the limit of blank and the limit of quantification are labeled with "pos." to emphasize that the mutant variant was detected but the VAF could be quantified. (A), the variant allele frequency (VAF) of *KRAS proto-oncogene* (*KRAS*) single nucleotide variants (SNVs) analyzed in 9 circulating-free DNA (cfDNA) samples from a patient with *KRAS* p. G13D mutant variant. Plasma samples from this patient were collected over a period of 1.5 years. (B), the VAF of *B-Raf proto-oncogene* (*BRAF*) p. V600E was analyzed in 11 cfDNA samples from a patient with the respective variant present in tumor tissue. Plasma samples of this patient were collected over a period of 2.5 years. LOB, limit of blank; LOQ, limit of quantification.

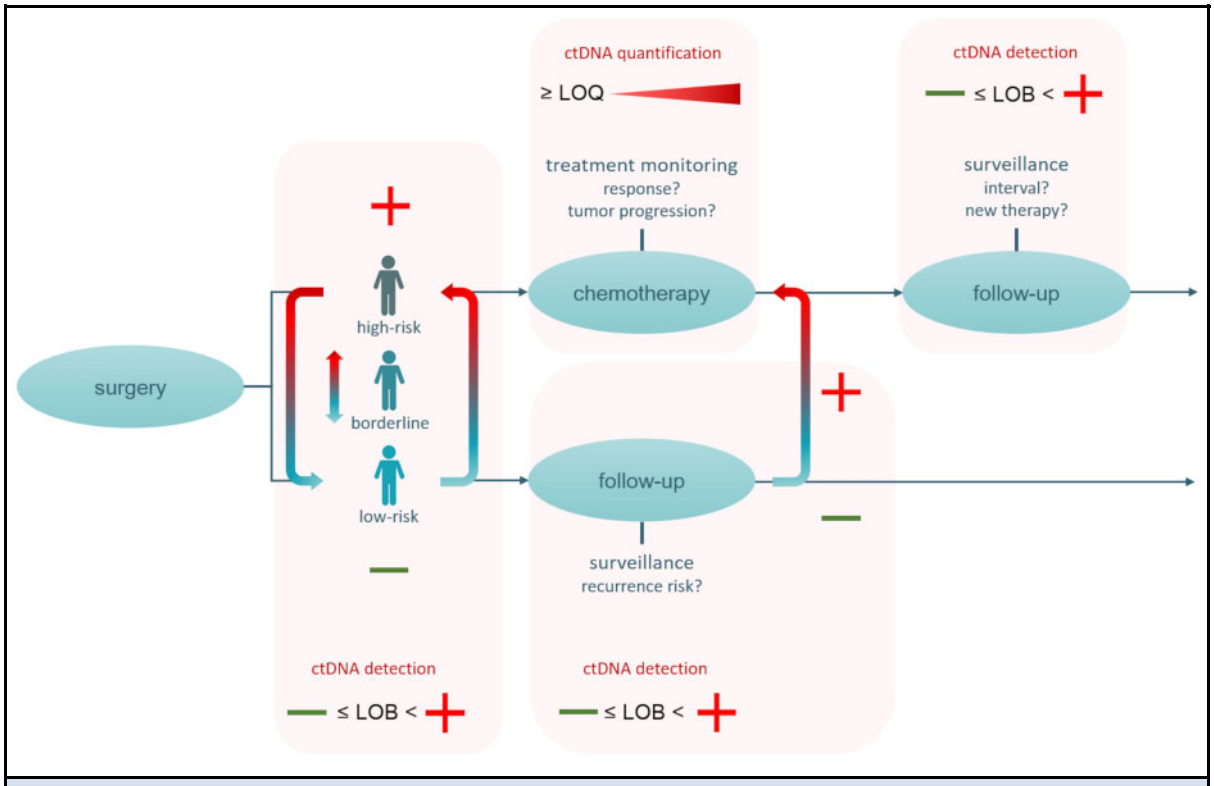

Fig. 4. Guide for clinical interpretation of circulating tumor DNA (ctDNA) analysis results, dependent on determined limit of blank (LOB) and limit of quantification (LOQ). An exemplary course of disease with several clinical decisions to be made is shown. During treatment and follow-up of patients with cancer, both ctDNA detection (negative ≤LOB, positive >LOB) and ctDNA quantification (possible ≥LOQ) of mutant variants supports clinical decisions for treatment.

crucial clinical parameter for the interpretation of findings. Second, all performance parameters were obtained based on the assumption that data were normally distributed, which is not true for the LOB since no values less than zero can be obtained. Third, linearity determination was performed based on DNA concentration, but not on the mutant VAF, which should be reported in disease monitoring (23).

Our validation involved analysis of reference samples with known mutant VAFs from 0% to 100% (Supplemental Methods) to stratify its detection and quantification. We observed substantial differences between the 5 ddPCR assays in the assessment of the LOB. While the *EGFR* exon 19 deletions, the *BRAF* p. V600E and the *EGFR* p. L858R assays produced none or almost no false positive signals, the *EGFR* p. T790M and the *KRAS* p. G12/p. G13 assays generated numerous false positive signals (i.e., LOB of 0.08% and 0.11%, respectively). Also, differences in LOD and LOQ were observed between the 5 ddPCR assays. The *EGFR* p. L858R and *EGFR* exon 19 deletions assays revealed the lowest LOD (0.08% and 0.1%, respectively), which combined with a LOB of zero indicates the capacity of both assays to detect true positive signals at very low VAFs. In contrast, the *BRAF* p. V600E assay showed the highest LOD (0.4%) despite an LOB of 0.02%. We found that the fractional abundance measured by the *BRAF* p. V600E assay was lower than the mutant VAF of the PC sample used, which could explain the higher LOD of this assay. Finally, the *KRAS* p. G12/p. G13 and the *EGFR* p. T790M assays showed intermediate LOD thresholds (0.26% and 0.18%, respectively), which combined with the high LOB (0.11% and 0.08%, respectively) indicate that it is possible to reliably detect relatively low VAFs, although both assays will accumulate false positive signals. Consequently, we conclude that having a high sensitivity is not directly associated with the ability to detect variants at low VAFs.

The lowest LOQ was determined at a mutant VAF of 0.41% for the *KRAS* p. G12/p. G13 assay, while the highest LOQ was determined at a mutant VAF of 0.7% for the *EGFR* exon 19 deletions assay. These results again emphasize that the sensitivity of an assay

(expressed by the LOD) is not an indicator of the assay's suitability for the quantification of variants at low VAFs. To the best of our knowledge, this has not been emphasized in other publications using ddPCR liquid biopsy assays. Thus, the differences in LOB, LOD, as well as LOQ across assays highlight the need for determining these performance characteristics for each single mutant variant assay individually to enable precise clinical interpretation for each variant. Notably, many available assays covering multiple mutant variants provide only mean values across all variants, which does not for differences in performance across mutant variants (24–26). For instance, Woodhouse et al. determined an LOB of a next-generation sequencing panel at 0.013% VAF, although the exact LOB was either higher or lower for most variants (26). This leads to false positive or false negative results on mutant variant detection and hence interpretation on residual disease status.

Since mutant VAFs will vary during disease progression and/or treatment (27), we addressed whether precise measurement results could be repeatedly obtained for the 5 assays (expressed by trueness, precision, and linearity). Trueness and precision were determined as the variability in the total error of replicate measurements of reference samples with known mutant VAFs from the LOQ to 100% VAF (Supplemental Methods). This is crucial since wrong error estimation would lead to misinterpretation of the VAF during disease monitoring. The highest precision was detected within the interval from 10% to 100% mutant VAF (>98% for all 5 assays) and the lowest was detected at the LOQ values (>70.5% for all 5 assays). Overall, quantification of the mutant VAF within the acceptable confidence interval is recommended by the European Medicines Agency guidelines for bioanalytical method validation (18), and was possible in all 5 assays.

Finally, all 5 assays showed linearity of the measurements. Measured fractional abundances were directly proportional to the actual mutant VAFs, meaning the mutant VAF of the samples can be reliably determined. These important findings verify that direct comparison of measurements performed at different time points during treatment of the patient is feasible. Yet, besides the analytical variability, the biological variability might also influence the results obtained. Therefore, knowledge of potential confounding factors, such as exact sampling time, is critical, since for example an increase in cfDNA concentrations has been shown during and after surgery (28). To minimize the effect of biological variability, analysis of follow-up samples from a patient should ensure correct interpretation of results.

In the future, it will be important to prove the clinical validity of each applied liquid biopsy assay. Therefore, longitudinal patient samples should be analyzed using the assays validated within this study and results should be compared to clinical evidence of recurrence and status of disease progression. In this context, numerous studies suggest that generally predictions about residual disease and tumor progression based on liquid biopsy assays reflect the actual disease status (Fig. 4) (2, 3, 6, 21, 29).

Taken together, the hotspot variant-specific ddPCR assays are well designed genetic tests to reliably detect and quantify *EGFR* exon 19 deletions, *EGFR* p. L858R, *EGFR* p. T790M, *BRAF* p. V600E, and *KRAS* p. G12/p. G13 variants at very low VAFs. ddPCR is a fast and cost-effective method for the analysis of tumor variants, which can be easily implemented into clinical practice. We demonstrate that analytical validity is essential and needs to be independently established for all clinical grade assays to identify assay-specific performance thresholds. Overall, with our findings we are able to provide a comprehensive actionable report for patients and enable disease monitoring.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: ctDNA, circulating tumor DNA; ddPCR, droplet digital PCR; LOB, limit of blank; VAF, variant allele frequency; LOQ, limit of quantification; NC, negative control; LOD, limit of detection; PC, positive control; cfDNA, circulating-free DNA; SNV, single nucleotide variant

Human Genes: *KRAS*, KRAS proto-oncogene, GTPase; *BRAF*, B-Raf proto-oncogene, serine/threonine kinase; *EGFR*, epidermal growth factor receptor.

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A. Hallermayr, A. Benet-Pagès, and J.M.A. Pickl wrote the manuscript; A. Hallermayr performed the experiments, analyzed, and interpreted the data; V. Steinke-Lange was responsible for obtaining a positive ethics vote and initiated sampling; M. Rentsch contributed to patient recruitment; U. Mansmann, M. Rentsch, and E. Holinski-Feder revised the manuscript; J.M.A. Pickl designed the study and supervised the work. All authors reviewed and agreed to the content of the final manuscript.

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4. Paper II

Paper II:

Hallermayr, A.; Steinke-Lange, V.; Vogelsang, H.; Rentsch, M.; de Wit, M.; Haberl, C.; Holinski-Feder, E.; Pickl, J.M.A. Clinical Validity of Circulating Tumor DNA as Prognostic and Predictive Marker for Personalized Colorectal Cancer Patient Management. *Cancers*, Volume 14, Issue 3, February 2022, Pages 851, https://doi.org/10.3390/cancers14030851[2]

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Article

Clinical Validity of Circulating Tumor DNA as Prognostic and Predictive Marker for Personalized Colorectal Cancer Patient Management

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Simple Summary: CtDNA analysis is a promising tool in liquid biopsy for the detection of tumor recurrence and progression, and is increasingly adopted into clinical practice. Still, guidelines for the accurate clinical interpretation of ctDNA analysis results are largely lacking, especially for tumor mutant variants detected at very low frequencies. Here, we show that cutoff determination for the detection and quantification of low-frequency mutant variants enables the accurate prediction of residual disease, tumor recurrence and progression, even before clinical evidence. CtDNA analysis using these cutoffs outperformed cfDNA and CEA level measurements. With these findings, we highlight the need to thoroughly validate each liquid biopsy assay and define the assay-specific limit of blanks (LOB) and limit of quantifications (LOQ) of *BRAF* p.V600E and *KRAS* p.G12/p.G13 assays for clinical interpretation. Our approach enables accurate clinical interpretation to support clinical decision making.

Abstract: Circulating tumor DNA (ctDNA) is a promising liquid biopsy (LB) marker to support clinical decisions in precision medicine. For implementation into routine clinical practice, clinicians need precise ctDNA level cutoffs for reporting residual disease and monitoring tumor burden changes during therapy. We clinically validated the limit of blank (LOB) and the limit of quantification (LOQ) of assays for the clinically most relevant somatic variants *BRAF* p.V600E and *KRAS* p.G12/p.G13 in colorectal cancer (CRC) in a study cohort encompassing a total of 212 plasma samples. We prove that residual disease detection using the LOB as a clinically verified cutoff for ctDNA positivity is in concordance with clinical evidence of metastasis or recurrence. We further show that tumor burden changes during chemotherapy and the course of disease are correctly predicted using the LOQ as a cutoff for quantitative ctDNA changes. The high potential of LB using ctDNA for accurately predicting the course of disease was proven by direct comparison to the routinely used carcinoembryonic antigen (CEA) as well as the circulating free DNA (cfDNA) concentration. Our results show that LB

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using validated ctDNA assays outperforms CEA and cfDNA for residual disease detection and the prediction of tumor burden changes.

Keywords: ctDNA; cfDNA; residual disease; monitoring; colorectal cancer

1. Introduction

CfDNA is released from both tumor and normal cells into the circulation [1,2]. The presence and proportion of tumor-specific ctDNA in the entirety of cfDNA can be used as surrogates for tumor presence and overall tumor burden, and are analyzed through the measurement of tumor-specific mutant variants [3–5]. Besides ctDNA, other LB analytes including circulating tumor cells (CTCs) have also been investigated for clinical application. Whereas CTCs allow the extraction of detailed information at the single cell level [6,7], numerous studies have shown that ctDNA profiles are highly concordant with the molecular profile of primary tumors and metastases [8,9], and are considered to have a higher likelihood to enter clinical application [10]. Since CTCs and ctDNA are analytes present in LB, the advantages of LB, such as non-invasiveness and ease of repeatability, enable CTC and ctDNA analysis independently of the patient's condition and at any desired time point [11–13]. In certain clinical courses of patients with non-small cell lung cancer (NSCLC) and breast cancer, LB using ctDNA analysis is already recommended to guide therapeutic decisions [14,15], and is even covered by health insurance. Furthermore, LB is expected to enter the clinical routine for CRC patient management in the near future, once clinical utility is proven for the following applications: (1) residual disease detection after surgery in CRC to facilitate decision on adjuvant therapy, (2) recurrence monitoring and (3) real-time monitoring of treatment response during chemotherapy.

Encouraging results of LB studies in these applications are highly desirable, as current standard procedures in CRC patient management, including CEA measurements, harbor several shortcomings. For example, the determination of prognosis after surgery based on CEA status combined with clinical-pathological characteristics of the resected primary specimen has limited predictive accuracy [16]. Furthermore, treatment monitoring and follow-up using CEA measurements, computed tomography (CT) scans and colonoscopies do not show an overall benefit [17], and harbor limited accuracy in recurrence prediction [18,19].

Before the implementation of ctDNA analysis into routine clinical practice, the diagnostic sensitivity and specificity of obtained variant-specific ctDNA measurement results must be clarified. CtDNA is often present at <1% variant allele frequency (VAF) in plasma, especially after surgery and chemotherapy initiation [3,20,21]. At these low levels of ctDNA, variant detection may be interfered with by the intrinsic noise of assays and by non-tumor specific signals due to clonal hematopoiesis. Hence, it may be difficult for clinicians to correctly interpret obtained ctDNA measurement results in the clinical context. Clinicians require variant-specific cutoffs for ctDNA positivity, which accurately predict residual disease and recurrence [3,22,23]. Furthermore, clinicians need precise quantification of ctDNA levels for correlation with tumor progression [23]. According to the "Protocols for Determination of Limits of Detection and Limits of Quantitation" (CLSI guidelines) [24], for the establishment of clinical laboratory measurement procedures, these detection and quantification cutoffs of clinical assays are obligatory in a clinical report, and are not equal to the general limit of detection (LOD) of the method, which is the only parameter commonly determined by LB assay providers.

To enable correct clinical interpretation of ctDNA analysis results, we determined the LOB and LOQ for each variant-specific ctDNA assay and aimed to validate these as cutoffs for ctDNA positivity and ctDNA quantification for usage in the detection of residual disease, recurrence and tumor burden monitoring by analyzing a total of 124 plasma samples of 22 CRC patients. We further compared the clinical validity of ctDNA analysis results with cfDNA and CEA concentration measurements.

2. Materials and Methods

2.1. Study Design and Participants

A total of 212 plasma samples were collected from 29 CRC UICC stage I-IV patients and 80 healthy individuals aged 19 to 87 years from October 2018 until March 2021 (Figure 1, Supplementary Tables S1 and S2) [25]. Patients prior to the initiation of therapy (surgery or chemotherapy) were included. The inclusion criteria for healthy individuals were no evidence for tumor predisposition, no previously diagnosed tumor, and no pregnancy. Since both *KRAS* and *BRAF* are localized on autosomes, the sex of healthy individuals was not considered as a co-morbidity. To account for age differences between the overall younger healthy controls and older patients, lymphocyte genomic DNA (gDNA) was analyzed to exclude clonal hematopoiesis in case of ctDNA positive status, which might be more frequent in elderly individuals [26].

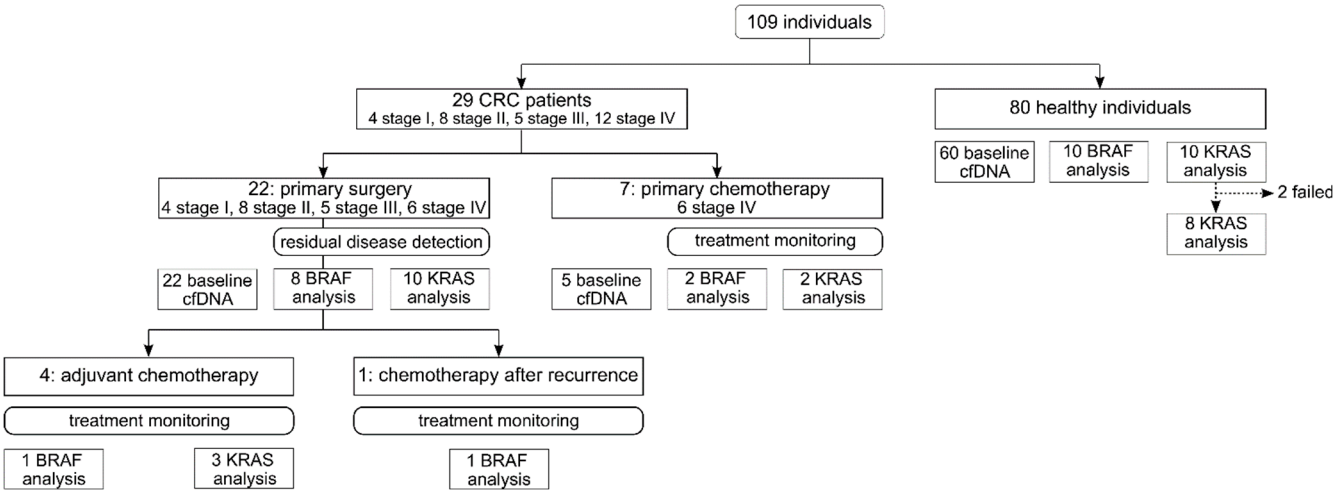

Figure 1. Study design. *BRAF* analysis indicates *BRAF* p.V600E analysis, and *KRAS* analysis represents analysis of one of the following variants: *KRAS* p.G12 [A/C/D/R/S/V] or *KRAS* p.G13D.

Twenty-two out of 29 CRC patients underwent primary surgery. Baseline plasma samples were collected up to 5 days prior to primary surgery, and four to 50 days after surgery (Supplementary Data—Residual disease). Eighteen out of 22 had known tumor variant status (8 *BRAF* p.V600E and 10 *KRAS* p.G12/p.G13). In these 18 patients, respective ctDNA analysis was performed in plasma prior to and after surgery for residual disease detection (Supplementary Figures S1–S18). Four out of 18 patients underwent adjuvant chemotherapy (1 *BRAF* p.V600E and 3 *KRAS* p.G12/p.G13), and 1/18 patient received chemotherapy after disease recurrence. Treatment monitoring by ctDNA analysis was performed in all five cases.

Seven out of 29 CRC patients were treated with primary chemotherapy. Baseline plasma samples were collected up to five days prior to chemotherapy. Monitoring samples were collected at several time points throughout chemotherapy (Supplementary Figures S19–S27). Four out of seven had known tumor variant status (2 *BRAF* p.V600E and 2 *KRAS* p.G12/p.G13). Treatment monitoring using ctDNA analysis was conducted in all four cases.

ctDNA analysis was conducted in 18/29 patients (87 plasma samples) pre- and postoperatively using the LOB as distinct cutoff for residual disease detection. Further treatment was monitored in 9/29 patients (76 plasma samples) to assess whether the LOQ as a cutoff for ctDNA quantification can be used to reliably predict a response or resistance to chemotherapy.

Sample collection and preparation are described in the Supplementary Methods. This study was approved by the ethics commission of the Bavarian Medical Association (No. 17059) and was registered with the German registry for clinical trials (trial registration ID: DRKS00012890).

Neither clinicians nor patients were informed about the results. All participants provided informed written consent prior to blood and tissue specimen collection.

2.2. Sample Preparation and Droplet Digital PCR

Information on DNA extraction and ddPCR protocols is provided in the Supplementary Methods.

2.3. Droplet Digital™ PCR

Droplet Digital PCR (ddPCR) was performed using the single Probe ddPCR *BRAF* p.V600E assay (Bio-Rad, Hercules, CA, USA, #dHsaMDV2010027) and the *KRAS* p.G12/p.G13 screening kit (Bio-Rad, #1863506) on the QX200 system (Bio-Rad) according to the manufacturer's instructions (see also Supplementary Methods). The *KRAS* p.G12/p.G13 screening kit (Bio-Rad) provides a positive signal if one of seven variants is present, but cannot specify which of the seven analyzed variants is actually present. To obtain reliable results, 20 to 30 ng of cfDNA was analyzed per reaction. Gating was performed based on mutant variant and wild-type (WT) control samples, and variant populations were identified using the QX Manager software (Bio-Rad, v.1.1). ctDNA was quantified in terms of the mutant VAF, which describes the abundance of detected mutant alleles within all detected alleles and is calculated as follows:

$$\text{VAF } (\%) = \frac{N_{mut}}{N_{mut} + N_{WT}} \times 100 \quad (1)$$

Equation (1). Variant allele frequency. VAF: variant allele frequency; N_{mut}: number of mutant alleles; N_{WT}: number of wild type alleles.

Samples with VAFs > LOB were defined with ctDNA positive status, and samples with VAFs > LOQ harbored quantifiable ctDNA VAFs.

2.4. Determination of Cutoffs for ctDNA Positive Status and Quantifiable ctDNA

The LOB and LOQ of ddPCR assays were determined with 95% confidence intervals in accordance with CLSI guidelines [24]. As the determination of the LOB is based on the detection of false positive results in negative controls, the 95% confidence interval at this threshold describes 95% specificity of the assays. Accordingly, ≥60 WT controls were measured with each assay to determine the LOB at 0.02 and 0.11% VAF for *BRAF* pV600E and *KRAS* p.G12/p.G13 assays, respectively. Furthermore, ≥40 replicates of positive controls containing the targeted variant with a VAF of the tentative LOQ were measured to determine the LOQ with achieving at least 80% precision and 90% trueness, at 0.52 and 0.41% VAF for *BRAF* pV600E and *KRAS* p.G12/p.G13 assays, respectively. Cutoffs were validated for 20 to 30 ng of input DNA [27].

2.5. Determination of Cutoff for Elevated cfDNA Concentrations

Previous studies described elevated plasma cfDNA concentrations in CRC patients [28–31]. To test whether plasma cfDNA concentration can add useful information to the biomarker portfolio in CRC, plasma cfDNA concentrations of 60 healthy individuals were compared to those in 128 samples from 29 CRC patients, which were quantified using the High-Sensitivity NGS Fragment Analysis Kit (Agilent, Santa Clara, CA, USA, #DNF-474-0500) on the Fragment Analyzer system (Agilent) (Supplementary Tables S2 and S3). According to CLSI guidelines [24], a minimum sample size of 60 is required for establishing the LOB with a 95% confidence interval. Therefore, including 60 healthy individuals for establishing the LOB as a cutoff for elevated cfDNA concentration is considered robust. With a median plasma cfDNA concentration of 2.5 ng/mL in healthy individuals and 11.6 ng/mL in CRC patients at baseline, significantly higher cfDNA concentrations were observed in CRC patients (p-value: 2.64×10^{-11}, Wilcoxon test) (Supplementary Figure S28). With 95%

specificity, a cutoff at 5.6 ng/mL cfDNA was established to differentiate between CRC patients and healthy individuals, as follows:

$$\text{LOB} = \text{Result at position}[0.95 \times N_B + 0.5] = P_{1-\alpha} \quad (2)$$

Equation (2). Determination of the cfDNA cutoff. LOB: limit of blank; N_B: number of negative control measurements; $P_{(1-\alpha)}$: percentile at the level of $1 - \alpha$.

Linear interpolation between the results of the next lower and the next higher rank position was used to determine the cfDNA cutoff [24].

2.6. CEA Analysis

CEA levels in plasma were determined using the Human CEA ELISA Kit (Biorbyt, Cambridge, UK, Cat# orb438561) in accordance with the manufacturers' instructions.

2.7. Statistical Analysis

Differences between the cfDNA concentration of healthy individuals and CRC patients were determined using a Wilcoxon test. Differences in ctDNA VAF, cfDNA and CEA concentrations depending on time in the course of the disease were calculated using a Kruskal–Wallis test. Bonferroni correction was used to adjust p-values for multiple testing. Using a priori power and sample size analysis, the minimum required sample sizes for a power of 0.8 were determined to be 18 and 9 per group for the Wilcoxon test and the Kruskal–Wallis test, respectively (G*Power version 3.1.94, https://gpower.software.informer.com/3.1/, accessed on 3 January 2022). *p*-values < 0.05 were considered statistically significant. All statistical analyses were performed using the stats R package in R version 4.0.3 (https://www.r-project.org/, accessed on 3 January 2022).

3. Results

3.1. Cutoff Validation for ctDNA Status and Quantifiable ctDNA VAFs in Reference Materials

Precise cutoff definition of positive ctDNA status in a plasma sample is a prerequisite for the ctDNA-based detection of residual disease and recurrence, and was determined previously using well-characterized WT control reference materials according to CLSI guidelines [24]. In our study, samples are defined as ctDNA-positive when ctDNA VAFs of *BRAF* p.V600E or *KRAS* p.G12/p.G13 variants are detected above the respective LOB (at 0.02 and 0.11% VAF, respectively, Figure 2 in blue) [27]. In the case of *KRAS* p.G12/p.G13 variants, the assay cannot differentiate between the targeted variants, and therefore a singular cutoff was validated.

Exact definition of the cutoff, from which ctDNA VAFs can be quantified, is required for monitoring quantitative tumor changes during treatment, and was defined previously using positive control reference materials according to CLSI guidelines [24,27]. This cutoff is defined as LOQ. In this study, quantitative ctDNA VAFs can be assessed from samples with *BRAF* p.V600E and *KRAS* p.G12/p.G13 ctDNA VAFs above LOQ (at 0.52 and 0.41% VAF, respectively).

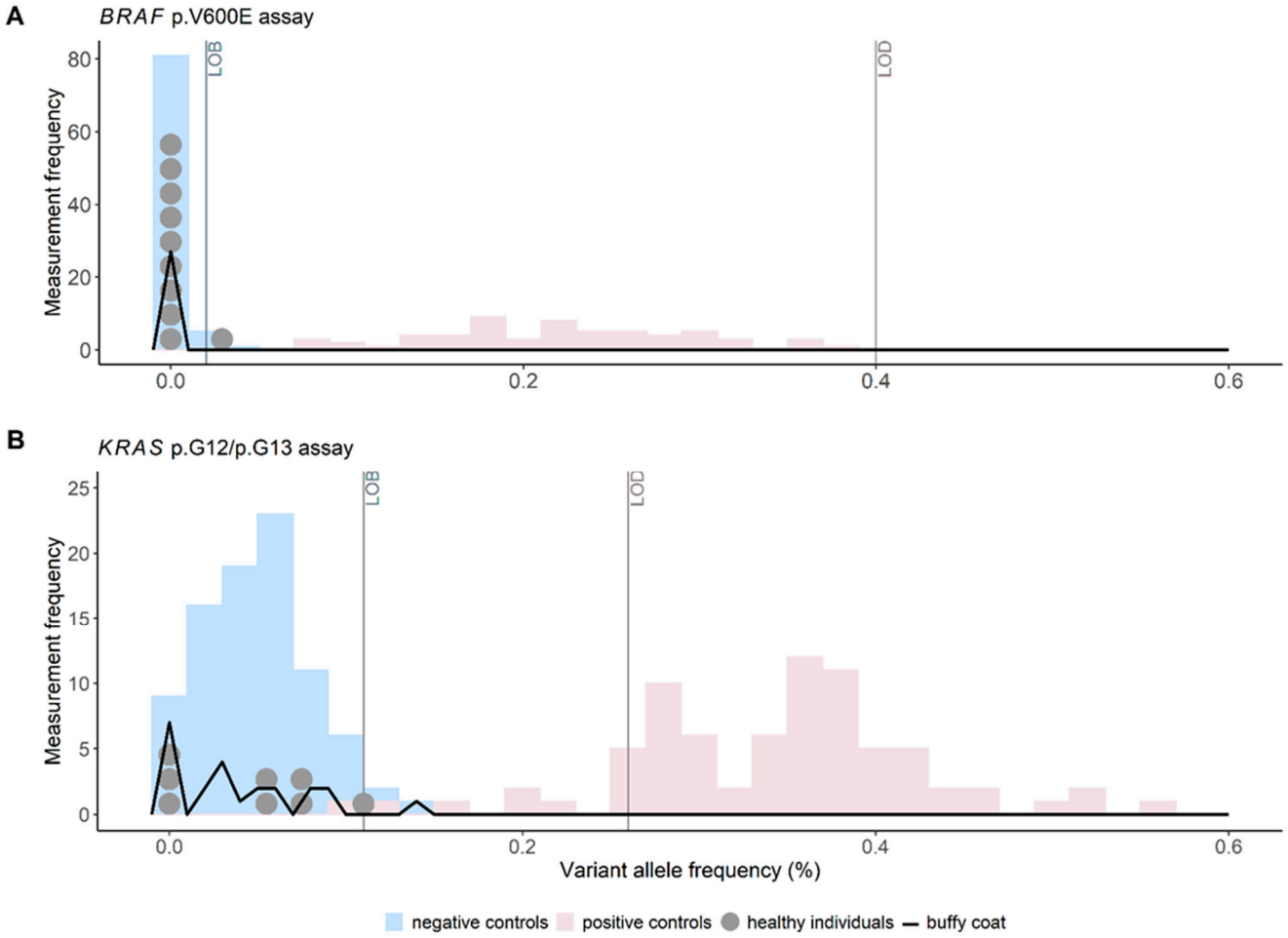

Figure 2. Cutoff for positive ctDNA status (i.e., LOB) is clinically verified by ctDNA measurements of healthy control samples (grey dots), as ctDNA signals of healthy controls are generally below the set ctDNA positivity cutoff of (**A**) *BRAF* p.V600E and (**B**) *KRAS* p.G12/p.G13 assays. Clonal hematopoiesis does not interfere with true tumor-derived positive ctDNA status, as ctDNA signals from buffy coat-containing lymphocyte DNA are generally below LOB (black line). Histogram of measurement results of negative (blue) and positive control (pink) reference material measurements allow definition of the LOB and LOD, respectively, as described in the analytical validation [27].

3.2. Cutoff Verification for ctDNA Status in Plasma of Healthy Controls

To clinically verify the variant-specific ctDNA positivity cutoff, previously validated using reference materials, ten healthy control samples were analyzed with *KRAS* p.G12/p.G13 and *BRAF* p.V600E assays, respectively (Figure 2).

Notably, as two *KRAS* p.G12/p.G13 analyses failed, the results of the measurement of eight *KRAS* p.G12/p.G13 considered. None (zero out of eight) and 10% (1/10) of the healthy control samples exceeded the previously validated ctDNA positivity cutoffs for the *KRAS* p.G12/p.G13 and *BRAF* p.V600E assays, respectively. In detail, the single measured VAF of the healthy control sample exceeding the cutoff of the *BRAF* p.V600E assay was 0.03%. By definition, a maximum of 15% of the healthy control samples may exceed the cutoff for verification [24]. Since clinical cutoff verification was conducted in the same setting as analytical validation and the criteria for cutoff verification are met, the ctDNA positivity cutoff was clinically verified for both *KRAS* p.G12/p.G13 and *BRAF* p.V600E assays (Figure 2, grey dots). Consequently, cutoffs could be used for residual disease and recurrence analysis.

3.3. Positive ctDNA Status Is Tumor Specific

The number of non-tumor-derived positive ctDNA signals in plasma samples of CRC patients must be thoroughly determined. To that the detected ctDNA signals in the plasma of CRC patients are non-tumor-specific and actually a result of clonal hematopoiesis [32], *BRAF* p.V600E and *KRAS* p.G12/p.G13 analyses were performed on lymphocyte gDNA of all CRC patients with ctDNA-positive plasma samples (Figure 2, black line). Zero out of 27 *BRAF* and only 1/23 *KRAS* signals were detected above the cutoff for ctDNA positivity (i.e., >LOB) in these samples. As in this single case of a positive lymphocyte gDNA signal, the VAF was lower than in the plasma sample (0.14 vs. 1.48% (±0.39%)), the respective plasma was still considered as ctDNA-positive. Overall, these results indicate that clonal hematopoiesis did not perturbate ctDNA results, and that positive plasma ctDNA status was indeed tumor specific.

3.4. At Baseline, Elevated cfDNA Concentration Outperform ctDNA Positive Status and CEA Levels

Using the ctDNA positivity cutoffs, positive ctDNA status was observed in 9/18 patients at baseline. Positive ctDNA status rate increased from early to late UICC stage (i.e., zero out of four stage I, four out of eight stage II, three out of four stage III, and two out of two stage IV) (Figure 3, Supplementary Methods—Residual disease). These observations are in line with previous studies showing that higher tumor stages are expected to release more ctDNA into circulation [22,28–30].

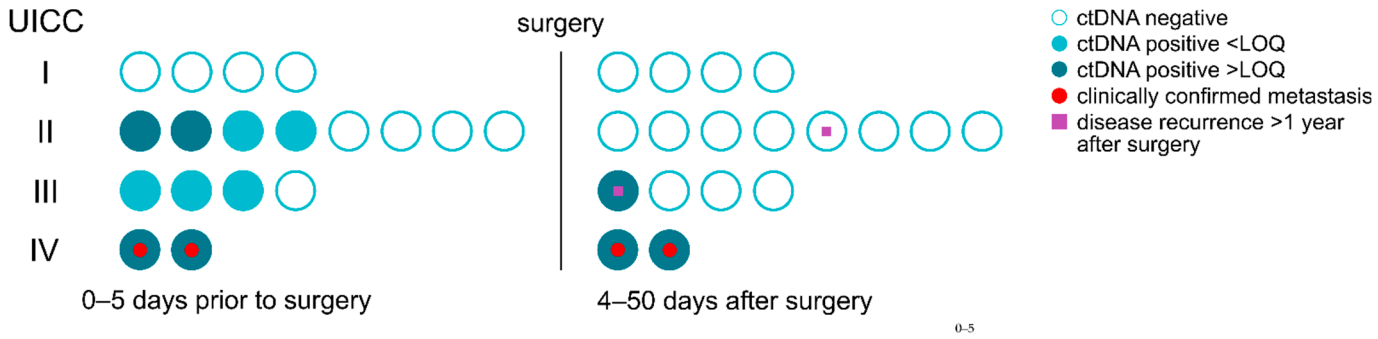

Figure 3. Determination of ctDNA positivity status in CRC stage I–IV patients for residual disease detection.

As a reference biomarker to ctDNA, the commonly used CRC marker CEA was measured in the plasma of CRC patients. Elevated CEA levels (in CRC patients compared to healthy individuals) were defined according to the literature as values above 2.5 and 5 ng/mL in non-smokers and smokers, respectively [33,34]. At baseline, CEA levels were elevated in only 2/18 patients (i.e., zero out of four stage I, zero out of eight stage II, one out of four stage III, and one out of two stage IV).

For the analysis of total cfDNA, a clinical cutoff of 5.6 ng/mL cfDNA in plasma was established for elevated cfDNA concentration (in CRC patients compared to healthy individuals) (Methods, Supplementary Figure S1). At baseline, elevated cfDNA concentrations were observed in 12/18 patients (i.e., two out of four stage I, four out of eight stage II, four out of four stage III, and two out of two stage IV). Since both ctDNA and elevated plasma cfDNA concentration are detected with 95% specificity, results can be compared and indicate that cfDNA is a better singular diagnostic marker at baseline than ctDNA and CEA. The combination of the three markers did not improve the detection rate of CRC at baseline.

3.5. Residual Disease and Recurrence Are Predicted by ctDNA Positive Status

To test the association of ctDNA-positive status in plasma with residual disease detection after surgery, VAFs of *BRAF* p.V600E and *KRAS* p.G12/p.G13 variants were

measured in cfDNA from plasma of 18 CRC patients prior to surgery (i.e., baseline sample) and four to 50 days after surgery (Figure 3). All 18 patients underwent locoregional R0 resection.

At baseline, the mutant variants were detected in half of the CRC patients (i.e., VAF > LOB, 9/18): one out of fourstage II, one out of one stage III and one out of one stage IV patients with *BRAF* and three out of four stage II, two out of three stage III and one out of one stage IV patients with *KRAS* variants. As expected, the nine patients with negative ctDNA status prior to surgery were also negative after surgery. These CRC patients with negative ctDNA status did not have metastasis from baseline up to 50 days after surgery, indicating high diagnostic specificity of ctDNA results. Notably, one of these patients later had clinically recurrent disease twice, which could be predicted by ctDNA detection up to three months prior to clinical evidence (Figures 3 and 4A, LB-CRC-07).

Of the nine patients with positive ctDNA status at baseline, three remained ctDNA-positive after surgery (one out of three stage III, and two out of two stage IV patients; all > LOQ), i.e., molecular residual disease (MRD), defined by the presence of ctDNA in plasma, could be identified. The two stage IV patients with MRD had clinically confirmed metastasis. The stage III patient with MRD had no confirmed metastasis, but was classified as a high-risk patient and therefore received adjuvant chemotherapy, which resulted in the disappearance of ctDNA during the course of treatment. This patient had recurrence more than one year after surgery and ~six months after the completion of adjuvant chemotherapy (Figures 3 and 4B, LB-CRC-25). Although disease recurrence could not be predicted around one month prior to clinical evidence in one patient, overall, these results indicate the potential of ctDNA analysis as a complementary marker for MRD and recurrence detection.

3.6. Residual Disease and Recurrence Are More Reliably Predicted by Positive ctDNA Status Than by Elevated cfDNA Concentration and CEA Levels

To estimate the predictive accuracy of positive ctDNA status for residual disease and recurrence detection, results were compared to cfDNA and CEA. MRD was detected in 3/18 patients, and recurrence occurred in 2/18 patients treated with primary surgery. Two of the three patients with MRD had clinically confirmed metastasis. In one of these two patients, the cfDNA concentration was elevated, whereas CEA levels were within the normal range from baseline until one month after surgery. Accordingly, besides ctDNA, cfDNA but not CEA predicted MRD in this patient (LB-CRC-38, Supplementary Figure S17). In the second patient, cfDNA concentration could not be assessed in the period of four to seven weeks after surgery. CEA levels were elevated at baseline and two weeks after surgery, indicating MRD (LB-CRC-09, Supplementary Figure S6). In the third patient with MRD, recurrence occurred more than one year after surgery and ~six months after completion of adjuvant chemotherapy. Plasma cfDNA concentration was elevated throughout the entire course of the disease. As no qualitative changes of the cfDNA marker were observed over time, neither MRD nor recurrence could be predicted by cfDNA measurement in this patient. CEA levels were elevated in this patient from baseline until two months after surgery, but were in the normal range one and seven months before recurrence. Therefore, CEA predicted MRD but not disease recurrence in this patient (LB-CRC-25, Figure 4B), analogous to ctDNA. In the second patient with recurrence, cfDNA concentration was within the normal range from baseline throughout the first year of follow-up. With the initiation of chemotherapy for the treatment of systemic nodal progression, cfDNA concentration was elevated, but not before or in parallel (zero days to 2.5 months) to clinically evident recurrence. CEA levels were within the normal range from baseline throughout the course of disease. Hence, in contrast to ctDNA, both biomarkers did not predict disease recurrence (LB-CRC-07, Figure 4A). Taken together, cfDNA predicted residual disease in one of the patients with MRD and CEA in two of them, but could not predict recurrence in any case.

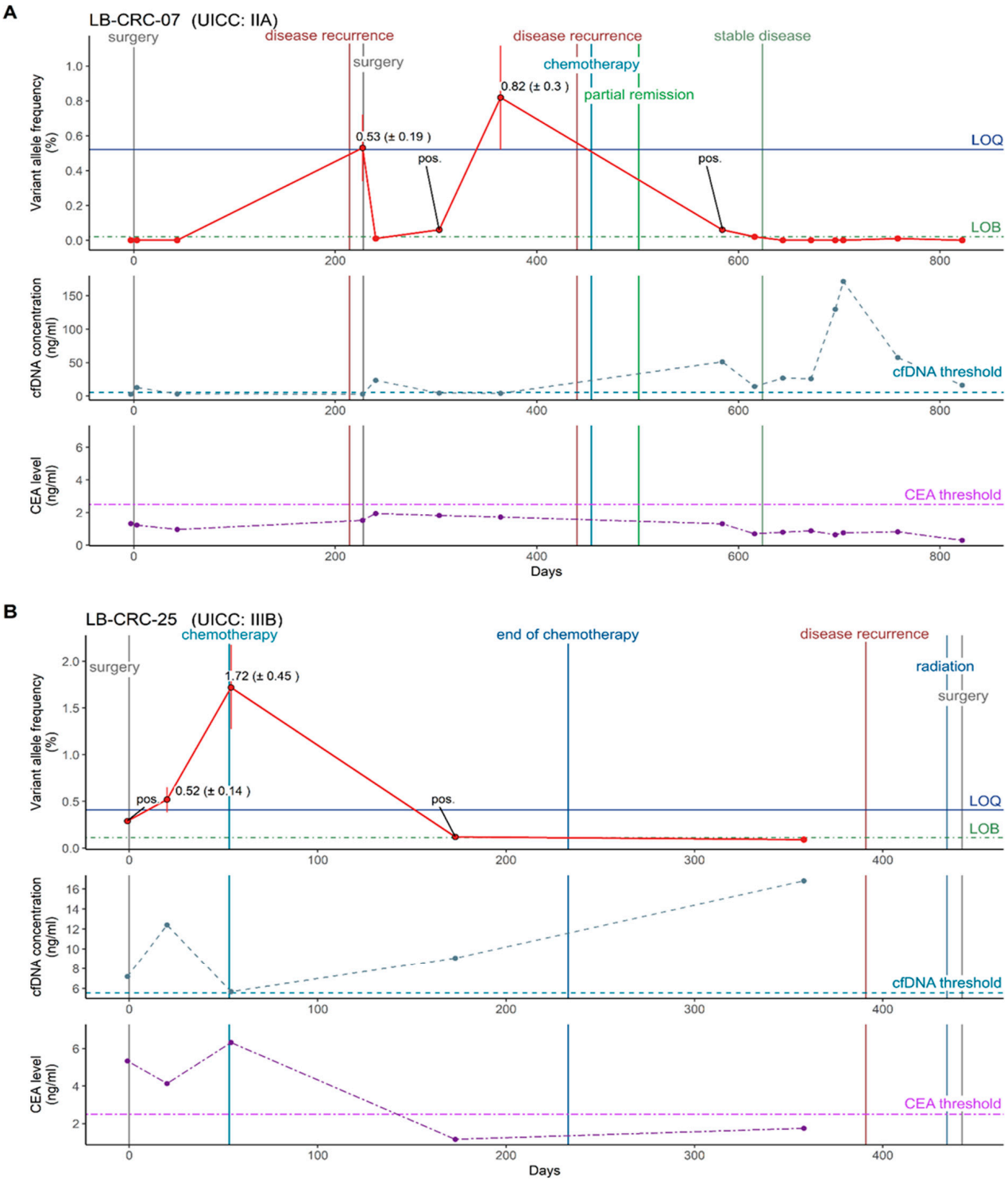

Figure 4. ctDNA (line 1), cfDNA (line 2) and CEA (line 3) analysis throughout the course of the disease in two CRC patients (**A**) LB-CRC-07 and (**B**) LB-CRC-25, with recurrence.

These results indicate higher sensitivity of ctDNA compared to cfDNA and CEA for detection of MRD and recurrence.

3.7. Chemotherapy Monitoring Possible through Precise ctDNA Quantification

In contrast to residual disease and recurrence detection, for which ctDNA-positive status in plasma is determined, quantitative changes in ctDNA VAF are analyzed for chemotherapy monitoring.

Treatment was monitored in nine CRC patients to assess whether quantitative changes in ctDNA VAF can predict response or resistance to chemotherapy.

Patients with primary chemotherapy: Four out of nine patients who received primary chemotherapy (LB-CRC-02, LB-CRC-30, LB-CRC-32, LB-CRC-43) were diagnosed with stage IV CRC (two out of four *BRAF* and two out of four *KRAS* variants). At baseline, ctDNA VAFs of these four patients ranged from 8.99 (±2.36%) to 47.75% (±7.11%), and significantly decreased within the first month of treatment (undetectable to 12.19% (±1.82%)), indicating a good response to chemotherapy. In all of these patients, decreases or increases in ctDNA VAF during the course of treatment were associated with a response or resistance to chemotherapy.

Patients with adjuvant chemotherapy: Four out of nine patients were treated with adjuvant chemotherapy following primary surgery (three out of four stage III with *KRAS* and one out of four stage IV with *BRAF* variants). MRD was detected in only two of those four patients after surgery and before the initiation of adjuvant chemotherapy. The decrease in ctDNA VAF from 1.72 (±0.45%) and 2.01% (±0.73%), respectively, to undetectable ctDNA was associated with a good response to treatment in both patients (LB-CRC-38, LB-CRC-25). In the remaining two patients, no MRD was detected after surgery and before initiation of adjuvant chemotherapy. For these patients, no positive ctDNA signals were detected in follow-up samples collected within one year (LB-CRC-18, LB-CRC-29).

Patient with palliative chemotherapy: In one out of nine stage II patients (*BRAF* variant) who received chemotherapy after diagnosis of systemic nodal progression more than one year after primary surgery (LB-CRC-07), ctDNA VAF decreased from 0.82% (±0.30%) to undetectable ctDNA alongside partial remission and stable disease.

Overall, the precise quantification of ctDNA VAF enabled the association of increasing ctDNA VAF to resistance and decreasing ctDNA VAF to response to chemotherapy in all patients.

3.8. Chemotherapy Monitoring by Precise ctDNA Quantification Outperforms cfDNA Concentration and CEA Levels

To evaluate the predictive accuracy of quantitative changes in ctDNA VAFs for predicting response or resistance to chemotherapy, results were compared to cfDNA and CEA. Before the initiation of chemotherapy, ctDNA was detected in seven out of nine patients with ctDNA VAFs ranging from 0.52 (±0.14%) to 47.75% (±7.11%). In all of these patients, decreases or increases in ctDNA VAFs during the course of treatment were associated with a response or resistance to chemotherapy.

Patients without quantifiable ctDNA before chemotherapy: The two patients without detectable ctDNA before and during chemotherapy (stage III, *KRAS* variants) showed increased cfDNA levels before the initiation of chemotherapy. While in one of these two patients cfDNA levels decreased to the normal range within one year (LB-CRC-18), the cfDNA levels in the second patient oscillated during chemotherapy and were elevated again one year after treatment initiation (LB-CRC-29). In both patients, CEA levels were within the normal range throughout chemotherapy. Therefore, cfDNA and CEA could not support prediction of response or resistance to chemotherapy in both patients (Supplementary Figures S22 and S24).

Patients with quantifiable ctDNA before chemotherapy: In one patient (stage II at diagnosis, *BRAF* variant), cfDNA concentration was elevated only after the initiation of chemotherapy, which was inconsistent with the clinical findings of partial remission and stable disease. CEA levels were within the normal range throughout the course of chemotherapy (LB-CRC-07, Figure 4A). One patient (stage III, *KRAS* variant) showed an elevated cfDNA concentration throughout the course of chemotherapy. CEA levels

were elevated before initiation and decreased to the normal range during the course of chemotherapy (LB-CRC-25, Figure 4B). The remaining five out of seven patients were diagnosed with stage IV CRC (three out of five *BRAF* variants, two out of five *KRAS* variants). In one of these patients, cfDNA concentration and CEA levels could only be assessed in samples collected following progressive disease. Both markers were elevated in all of these samples and correlated well with the clinical finding of progressive disease (LB-CRC-02, Supplementary Figure S20). In another stage IV patient, the cfDNA concentration was elevated before initiation, decreased to the normal range after two months of chemotherapy, and was elevated again approximately two months later, correlating well with clinical findings of stable and progressive disease. CEA levels were elevated throughout the entire course of disease (LB-CRC-30, Supplementary Figure S25). In the third stage IV patient, the cfDNA concentration was elevated in all but one sample throughout the course of chemotherapy. CEA levels were elevated before the initiation of chemotherapy and decreased to the normal range approximately 1.5 months after ctDNA became undetectable for the first time (LB-CRC-32, Supplementary Figure S26). In the remaining two stage IV patients, cfDNA concentration was elevated throughout the course of chemotherapy. In one of the two patients, CEA levels were within the normal range in all but one sample that did not harbor ctDNA, whereas in the second patient, CEA levels were within the normal range throughout chemotherapy (LB-CRC-38 and LB-CRC-43, Supplementary Figures S27 and S28). Taken together, cfDNA predicted response or resistance to chemotherapy in only two and CEA in only three of the patients for whom ctDNA VAFs could be correlated with response or resistance to chemotherapy.

These results indicate the higher sensitivity of ctDNA quantification compared to cfDNA and CEA for predicting response or resistance to chemotherapy and therefore suggest that ctDNA is a more suitable marker than cfDNA and CEA.

3.9. Significant Differences Depending on Time in Course of Disease in ctDNA VAFs, but Not in cfDNA Concentration and CEA Levels

To test whether the observed differences in ctDNA VAFs, cfDNA concentration and CEA levels depending on the course of disease are statistically significant, all three markers were compared between samples collected at baseline, during the course of disease with clinically evident tumor, and during follow-up after curative treatment. Indeed, significant differences in ctDNA VAFs were identified depending on sampling time (baseline, course of disease, after curative treatment) ($p = 1.9 \times 10^{-6}$, Figure 5A). In contrast, no significant differences were observed in cfDNA concentration or CEA levels ($p = 0.1$ and 0.12, respectively, Figure 5B,C). These results strongly suggest that accurate detection of positive ctDNA status and precise quantification of ctDNA VAFs using well-defined mutant-specific LOBs and LOQs reflect the generally increased tumor burden at baseline and during the course of disease, as well as the absence of tumors in follow-up samples after curative treatment ($p = 1 \times 10^{-4}$ and 6.8×10^{-6}, respectively). In contrast, cfDNA concentration and CEA levels do not an detect increased tumor burden or the absence of tumors with comparable sensitivity. These data are consistent with results for residual disease and recurrence detection and for chemotherapy monitoring.

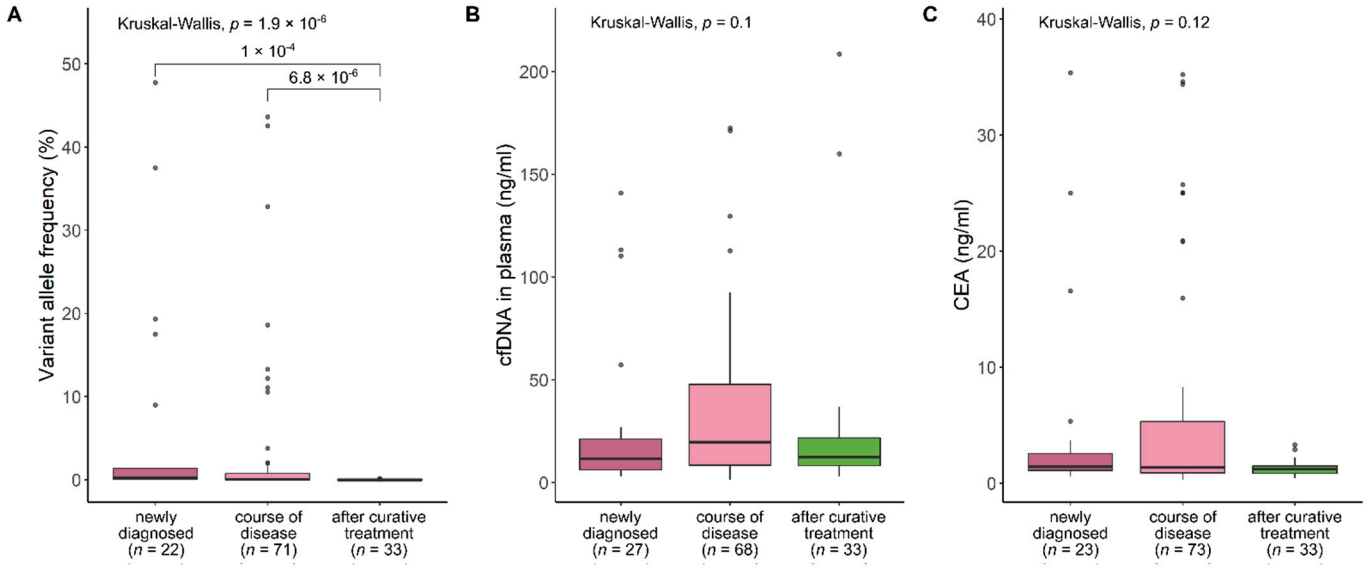

Figure 5. ctDNA VAFs, cfDNA concentration and CEA levels at different times in the course of the disease. (**A**) ctDNA quantification levels but not (**B**) cfDNA concentration and (**C**) CEA levels significantly differ at different times in the course of disease.

4. Discussion

CtDNA analysis enables the non-invasive and real-time assessment of tumor burden and mutant signatures in cancer patients, which may be used for residual disease, recurrence and tumor progression detection. For implementation into clinical routine, clinically relevant positive ctDNA signals at low levels must clearly be verified as being tumor-specific without interference from analytical measurement noise (technical artefact) or clonal hematopoiesis (biological artefact) [35]. Hence, clinicians need a defined cutoff for true tumor-specific ctDNA positivity. In this study, we defined and clinically verified a cutoff for ctDNA positivity according to the CLSI guidelines [24,27], and showed that only tumor samples but not healthy controls exceeded this cutoff. We further showed that clonal hematopoiesis did not interfere with positive ctDNA results in plasma samples. These results demonstrate that ctDNA analysis should be thoroughly validated, as with other clinical test procedures, and that obtained ctDNA signals above the defined ctDNA positive cutoff are indeed tumor-specific. This is an advantage over non-tumor-specific CEA and cfDNA markers in the plasma of CRC patients, which are generally present in healthy individuals.

We clinically validated the positive ctDNA status for the assessment of residual disease and recurrence. To validate LB for residual disease, we analyzed 18 CRC patients up to 50 days after primary surgery. We detected MRD in three of these patients, two of them having metastatic disease and one of them without clinical evidence of recurrence at the time of measurement. We found that positive ctDNA status is a better prognostic marker than the alternative liquid biopsy marker cfDNA and the commonly used CRC marker CEA. These results are in line with data from a pooled analysis of cohort studies showing that the prognostic accuracy of CEA and other commonly used markers is only ~50–60% [16]. In contrast, residual disease detection with postsurgery ctDNA status outperformed all routinely used markers with a prognostic accuracy of ~70%. The combination of ctDNA status with all standard clinical-pathological markers resulted in ~80% prognostic accuracy [16].

To validate LB for recurrence, we monitored patients throughout the course of disease and correlated signals above the ctDNA positivity cutoff with clinically evident recurrence. Our data suggest that ctDNA analysis may be meaningful in surveillance during follow-up, particularly as it outperformed the routinely used CEA marker. Furthermore, recurrence was detected by ctDNA up to three months before clinical evidence. These findings are con-

sistent with the results published by Reinert et al. and Wang et al. [36,37]. CtDNA analysis to support current approaches may be valuable, as a large meta-analysis including 19 studies showed that current follow up strategies including CEA, CT scans and colonoscopies do not improve overall survival, CRC-specific survival or relapse-free survival. Conversely, more intensive follow-ups are likely to increase harms and costs [38]. In contrast, non-invasive liquid biopsies are likely to add accuracy to follow-up measurements, thus enabling the detection of recurrence several months before standard measurements [16].

Besides the qualitative assessment of ctDNA positivity, it is necessary to define a ctDNA quantification cutoff at a low level, from which reliable and precise determination of ctDNA VAFs is possible. Precise quantification with 95% confidence interval from LOQ to 100% ctDNA VAF is a prerequisite for the reliable interpretation of decreases or increases [27]. The LOQ enables clinicians to identify true VAF changes, a prerequisite for tumor monitoring during chemotherapy. We used our previously defined LOQ as a cutoff for ctDNA VAF quantifiability, and show that changes in VAFs above LOQ actually reflect tumor progression and response to therapy in all cases, thereby outperforming cfDNA and CEA measurements. These data are in line with previous data investigating standard approaches including CEA for treatment monitoring. Specifically, a meta-analysis comprising 52 studies concluded that CEA should be supplemented by another method due to poor performance [39]. Our data, in line with others [36,37,40,41], suggest that ctDNA can add valuable information on tumor progression and the response to treatment.

Although cutoffs for ctDNA-positive status and ctDNA quantifiability are assay-specific, our results indicate that defining cutoffs is critical for sensitive and specific clinical interpretation of ctDNA analysis results. Cutoffs for other assays can be easily validated according to the protocol described here. Validation of specific cutoffs for ctDNA-positive status and ctDNA quantification is an essential step towards implementation into clinical practice.

Our data show that elevated cfDNA concentration may be a valuable supporting marker for diagnosis of CRC, as cfDNA levels were elevated in 12/18 CRC patients at baseline. Thereby, the detection rate was slightly higher when compared to positive ctDNA status (9/18 detected) and clearly more sensitive than CEA (2/18 detected). Taken together, both liquid biopsy markers, cfDNA concentration and ctDNA status and VAFs, may add value to clinical practice, with cfDNA concentration being a supportive diagnostic marker and ctDNA status and VAFs being informative of residual disease, recurrence and tumor progression.

Nevertheless, our study has some limitations that should be considered. First, the small sample size of CRC patients included represents a limitation. However, by analyzing a large number of plasma samples over a long period of time and by applying extensively validated cutoffs for residual disease detection and treatment monitoring, we were able to thoroughly characterize all of our patients by obtaining strong evidence regarding the potential of ctDNA analysis for disease monitoring. This strong evidence can be further supported by the concordance of our results with previously published data. Future studies on recurrence and treatment monitoring may use our approach of accurately defined cutoffs in a larger cohort.

Second, the inclusion of patients at all stages could reduce the power to identify relevant differences between these disease stages in CRC patients. However, our main goal is to demonstrate the importance of distinct cutoffs for the clinical interpretation of ctDNA analysis, regardless of disease stage. Therefore, the inclusion of all stages does not impact our findings.

Third, analyzing only the *BRAF* and *KRAS* hotspot variants limits the benefits of this method to patients carrying one of these variants in tumor tissue. Still, since approximately 10 and 34% of CRC cases harbor the *BRAF* and *KRAS* hotspot variants, respectively [42], a large proportion of CRC patients will benefit from this highly sensitive and cost-effective ctDNA analysis method. Moreover, testing for hotspot variants in these two genes is recommended for metastatic CRC patients, which may facilitate the clinical application of

BRAF and *KRAS* hotspot variants in LB [43]. To extend the benefits of ctDNA analysis to all CRC patients, validated untargeted ctDNA analysis would be required in the future. Furthermore, combining ctDNA with additional analytes present in LB might improve the patient management of more CRC patients. CTCs may represent a useful predictive and prognostic marker [44–47]. Recent advances in CTC-based technologies even enable genome-wide analysis of CTCs [6,7,48]. Furthermore, the differential expression of exosomal miRNAs such as miRNA-21 or miRNA-345 could be identified as potential prognostic markers in CRC [46,47,49–51]. Specific miRNAs such as miRNA-21 could even be identified as potential new targets for the treatment of drug resistance [46,47,52].

Fourth, the age difference of our control cohort and CRC patients might introduce potential bias due false positive signals in the older CRC cohort due to age-related clonal hematopoiesis. Therefore, we analyzed lymphocyte gDNA to exclude false positive signals originating from clonal hematopoiesis [26].

5. Conclusions

In conclusion, our LOB- and LOQ-based approach for ctDNA detection and quantification in CRC patients is an accurate approach and is easy to implement in clinical care for the prediction of residual disease, disease recurrence and treatment monitoring as a supplement to current approaches such as CEA.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers14030851/s1, Supplement: Supplementary Methods and Data; Table S1: Baseline characteristics of study participants, Table S2: Data of all samples collected from study participants, Table S3: Data used for the determination of the cutoff for elevated cfDNA concentrations, Figure S1: Residual disease detection in patient LB-CRC-03, Figure S2: Residual disease detection in patient LB-CRC-05, Figure S3: Residual disease detection in patient LB-CRC-06, Figure S4: Residual disease detection in patient LB-CRC-07, Figure S5: Residual disease detection in patient LB-CRC-09, Figure S6: Residual disease detection in patient LB-CRC-10, Figure S7: Residual disease detection in patient LB-CRC-11, Figure S8: Residual disease detection in patient LB-CRC-14, Figure S9: Residual disease detection in patient LB-CRC-18, Figure S10: Residual disease detection in patient LB-CRC-19, Figure S11: Residual disease detection in patient LB-CRC-23, Figure S12: Residual disease detection in patient LB-CRC-24, Figure S13: Residual disease detection in patient LB-CRC-25, Figure S14: Residual disease detection in patient LB-CRC-29, Figure S15: Residual disease detection in patient LB-CRC-37, Figure S16: Residual disease detection in patient LB-CRC-38, Figure S17: Residual disease detection in patient LB-CRC-40, Figure S18 Residual disease detection in patient LB-CRC-41, Figure S19: Monitoring of mutant variant, CEA levels and cfDNA concentration in patient LB-CRC-02, Figure S20: Monitoring of mutant variant, CEA levels and cfDNA concentration in patient LB-CRC-07, Figure S21: Monitoring of mutant variant, CEA levels and cfDNA concentration in patient LB-CRC-18, Figure S22: Monitoring of mutant variant, CEA levels and cfDNA concentration in patient LB-CRC-25, Figure S23: Monitoring of mutant variant, CEA levels and cfDNA concentration in patient LB-CRC-29, Figure S24: Monitoring of mutant variant, CEA levels and cfDNA concentration in patient LB-CRC-30, Figure S25: Monitoring of mutant variant, CEA levels and cfDNA concentration in patient LB-CRC-32, Figure S26: Monitoring of mutant variant, CEA levels and cfDNA concentration in patient LB-CRC-38, Figure S27: Monitoring of mutant variant, CEA levels and cfDNA concentration in patient LB-CRC-43, Figure S28: Measurements of plasma cfDNA concentration in healthy individuals and newly diagnosed CRC patients for establishment of a CRC-specific cutoff for cfDNA elevation.

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Apendix A: Paper III

Hallermayr, A.; Wohlfrom, T.; Steinke-Lange, V.; Benet-Pagès, A.; Scharf, F.; Heitzer, E.; Mansmann, U.; Haberl, C.; de Wit, M.; Vogelsang, H.; Rentsch, M.; Holinski-Feder, E.; Pickl, J.M.A. Somatic copy number alteration and fragmentation analysis in circulating tumor DNA for cancer screening and treatment monitoring in colorectal cancer patients. *J Hematol Oncol* 15, 125 (2022). https://doi.org/10.1186/s13045-022-01342-z

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RESEARCH

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Somatic copy number alteration and fragmentation analysis in circulating tumor DNA for cancer screening and treatment monitoring in colorectal cancer patients

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Abstract

Background: Analysis of circulating free DNA (cfDNA) is a promising tool for personalized management of colorectal cancer (CRC) patients. Untargeted cfDNA analysis using whole-genome sequencing (WGS) does not need a priori knowledge of the patient´s mutation profile.

Methods: Here we established LIquid biopsy Fragmentation, Epigenetic signature and Copy Number Alteration analysis (LIFE-CNA) using WGS with ~ 6× coverage for detection of circulating tumor DNA (ctDNA) in CRC patients as a marker for CRC detection and monitoring.

Results: We describe the analytical validity and a clinical proof-of-concept of LIFE-CNA using a total of 259 plasma samples collected from 50 patients with stage I-IV CRC and 61 healthy controls. To reliably distinguish CRC patients from healthy controls, we determined cutoffs for the detection of ctDNA based on global and regional cfDNA fragmentation patterns, transcriptionally active chromatin sites, and somatic copy number alterations. We further combined global and regional fragmentation pattern into a machine learning (ML) classifier to accurately predict ctDNA for cancer detection. By following individual patients throughout their course of disease, we show that LIFE-CNA enables the reliable prediction of response or resistance to treatment up to 3.5 months before commonly used CEA.

Conclusion: In summary, we developed and validated a sensitive and cost-effective method for untargeted ctDNA detection at diagnosis as well as for treatment monitoring of all CRC patients based on genetic as well as non-genetic tumor-specific cfDNA features. Thus, once sensitivity and specificity have been externally validated, LIFE-CNA has the potential to be implemented into clinical practice. To the best of our knowledge, this is the first study to consider multiple genetic and non-genetic cfDNA features in combination with ML classifiers and to evaluate their potential in both cancer detection and treatment monitoring.

Trial registration DRKS00012890.

Keywords: ctDNA, Colorectal cancer, Liquid biopsy, Whole-genome sequencing, Somatic copy number alterations, cfDNA fragmentation, Chromatin signatures

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Background

Liquid biopsy (LB) is a highly promising tool for personalized patient management [1–5]. An important LB marker is circulating tumor DNA (ctDNA), which represents the fraction of circulating free DNA (cfDNA) released by tumor cells [6]. A major challenge in ctDNA analysis is the very low fractions of ctDNA in total cfDNA (commonly < 5%) [6, 7]. Therefore, methods with high analytical sensitivity and specificity are required [8–10], but to date, mainly methods targeting frequent hotspot variants have been validated [11–14]. However, this approach limits the application of LB to patients with known genetic tumor profiles. To extend the advantages of LB to all cancer patients, highly sensitive untargeted methods are required.

A commonly used approach for untargeted ctDNA detection is shallow whole-genome sequencing (WGS) (i.e., < 1× coverage) to identify genome-wide somatic copy number alterations (SCNAs) [15] However, this approach requires ctDNA fractions of at least 5% to 10% that may be present in a subset of CRC samples only [15–17]. Various studies suggest that enrichment of the ctDNA fraction in cfDNA by size selection, tumor-specific fragmentation patterns, and epigenetic signatures can enhance ctDNA detection [18–21].

In this study, we developed LIquid biopsy Fragmentation, Epigenetic signature and Copy Number Alteration analysis (LIFE-CNA) as an untargeted approach to detect ctDNA with high sensitivity in plasma samples of colorectal cancer (CRC) patients as a diagnostic, predictive and prognostic marker. To enable detection of ctDNA fractions < 5%, we increased coverage from shallow WGS to ~ 6× and combined the Illumina DRAGEN CNV (copy number variation) workflow with the Plasma-Seq pipeline for copy number profiling [15, 22], a fragmentation pipeline, and LIQUORICE, a tool for the identification of coverage in open-chromatin regions [20]. With this workflow, we integrated detection of multiple cfDNA features, including focal SCNAs, cfDNA fragmentation patterns and chromatin signatures, and established machine learning (ML) classifiers for the highly sensitive detection of ctDNA. Using LIFE-CNA, we aimed to establish cutoffs for ctDNA detection to facilitate translation of untargeted LB analysis into clinical practice. We further evaluated whether ctDNA analysis using LIFE-CNA is able to predict response or resistance to treatment. For analytical validation and a clinical proof-of-concept of LIFE-CNA, 259 cfDNA samples from 50 patients with stage I-IV CRC and 61 healthy controls were analyzed. To the best of our knowledge, this is the first study combining SCNA and fragmentation profiles for disease monitoring and providing a complete analytically validated workflow showing a clinical proof-of-concept that can be easily implemented into clinical practice to support CRC patient management.

Methods

Study design and participants

A total of 259 plasma samples were collected from 50 patients with UICC stage I-IV CRC (7 stage I, 14 stage II, 11 stage III, 18 stage IV) and 61 healthy individuals aged 20 to 88 years from March 2018 until April 2022 (Additional file 1: Table S1, Figure S1; Additional file 2: Table S2) [23]. 55 healthy controls were included in the reference set. Six healthy controls were used for external validation of LIFE-CNA. 198 plasma samples from 50 CRC patients were collected at diagnosis and during follow-up. These samples were categorized according to the time of sample collection during the course of disease (Additional file 1: Methods, Table S1). The course of disease was monitored by colonoscopies and imaging during routinely scheduled follow-up examinations. 134 of the 198 plasma samples from CRC patients collected during the course of disease with clinically diagnosed tumor burden served as positive controls. To identify molecular residual disease (MRD) following surgery, baseline blood samples were collected up to eight days pre-surgery and follow-up samples were collected one day up to six weeks post-surgery in 33 patients. For treatment monitoring, plasma samples from 15 patients were collected at several time points throughout the course of disease.

The study was approved by the ethics commission of the Bavarian Medical Association (No. 17059) and is registered with the German registry for clinical trials (trial registration ID: DRKS00012890). Neither clinicians nor patients were informed about the results. All participants provided informed written consent prior to blood and tissue specimen collection.

Clinical sample collection and categorization, DNA extraction, droplet digital PCR, CEA analysis, library preparation and in silico dilutions

Information on sample collection and categorization, DNA extraction, droplet digital PCR (ddPCR), carcinoembryonic antigen (CEA) analysis, library preparation, and in silico dilutions are provided in the Supplementary Methods (Additional file 1).

Whole-genome sequencing bioinformatics analysis

Following paired-end sequencing with 2 × 101 bp reads on the NovaSeq 6000 system (Illumina, San Diego, California, USA), demultiplexing of samples was performed using BCL Convert (Illumina), and raw sequencing data were processed using the DRAGEN DNA Pipeline on the Illumina DRAGEN Bio-IT Platform (Illumina) v3.9. After adapter trimming, sequencing reads were aligned

to GRCh38/hg38. Duplicates and reads with a mapping quality < 30 were removed from analysis. A second bam file with 90–150 bp fragments only was generated for SCNA analysis. In all of the following analysis regions overlapping with ENCODE blacklist [24] and the UCSC gap track [25] were excluded.

Global and regional fragmentation analysis

Global and regional fragmentation of cfDNA was analyzed as described by Peneder et al. in 2021 [20]. Briefly, fragment length was determined using Picard CollectInsertSizeMetrics (version 2.26.6) and global fragmentation was derived as the fraction of fragments with distinct lengths. Regional fragmentation was established as the z-scored difference in the ratio of short (90–150 bp) to long (151–220 bp) fragments (S/L ratio) in 100 kb bins compared to the 55 healthy controls. Z-scores of the fragmentation of healthy controls were calculated by comparison to the other 54 healthy control samples. Data of genomic regions harboring SCNAs were excluded to avoid bias due to regionally enriched ctDNA. The computational analysis described by Peneder et al. in 2021 [20] was adapted that regions harboring SCNAs were identified based on the SCNA workflow, described below rather than ichorCNA. Furthermore, we used a significant enrichment of short fragments (90–150 bp) as indicator for ctDNA based on global, and significantly different z-scored S/L-ratios on at least one chromosome arm as indictor for ctDNA based on regional fragmentation.

Coverage in CRC-specific regions of interest

The LIQUORICE tool (v.0.5), developed by Peneder et al. in 2021 [20], was used to identify ctDNA based on significant coverage drops in CRC-specific transcriptionally active chromatin regions (epigenetic signatures). We analyzed the coverage in CRC-specific active chromatin regions, published by Chiara et al. in 2021 [26] and in universal DNase I hypersensitivity sites (DHS). The neighboring 20 kb of each region set weresplit into 500 bp bins to identify the mean coverage around the regions of interest. To correct for bias due to regionally enriched ctDNA, SCNAs, identified with the SCNA workflow, described below, were provided to LIQUORICE. Significant coverage drops compared to healthy controls in at least two of the analyzed region sets were considered as indicator for ctDNA.

Somatic copy number alterations

The CNV workflow provided with the Illumina DRAGEN Bio-IT Platform (Illumina) was performed based on 90–150 bp fragments, since higher sensitivity for SCNA calling was previously described for these short fragments [18, 20]. In detail, reads were counted in 50 kb bins, followed by GC bias correction and normalization based on a reference set containing data from 55 healthy control samples. Segmentation was performed by circular binary segmentation with disabled merging of two adjacent segments (merge-threshold = 0). Following the DRAGEN CNV workflow, SCNAs were identified according to the Plasma-Seq pipeline described by Heitzer et al. [15] applying chromosome specific thresholds (Additional file 1: Figure S3, Methods; Additional file 2: Tables S3 and S4).

Focal somatic copy number alterations

Focal SCNAs, identified within the Plasma-Seq pipeline were defined as described by Ulz and Belic et al. in 2016 [22]. SCNAs of < 20 Mb, overlapping with ≤ 100 genes of the COSMIC cancer gene census [27] with a higher or lower log2 ratio than the chromosome specific LOB compared to the neighboring 20 Mb were identified as focal SCNAs. In addition segments with a higher log2 ratio of 0.58 (~ three copies) compared to the neighboring 20 Mb were identified as focal amplifications, even if no gene of the COSMIC cancer gene census [27] overlapped.

Machine learning model for tumor detection

For ctDNA detection in samples collected from CRC patients, different machine learning (ML) classifiers were trained as described by Peneder et al. in 2021 [20]. Briefly, support vector machines, feed-forward neural networks, random forests and binomial generalized linear models with elastic-net regularization were trained and evaluated using 100 bootstrapping iterations with fivefold cross-validation in each training set.

We evaluated the performance of ML classifiers on the following feature sets: (i) Global fragmentation, (ii) regional fragmentation, and (iii) a meta-learner (Additional file 1: Methods; Additional file 2: Table S5) [18–20].

For each feature set the support vector machine was selected as best ML classifier to build a final ML model on the complete data.

Statistical analysis

Differences in global and regional fragmentation of healthy individuals and CRC patients were determined using a Mann–Whitney-U test. Bonferroni correction was used to adjust p-values for multiple testing. All statistical analyses were performed using statistical functions within the Python module SciPy v.1.8 (scipy.stats) with Python version 3.10.

Results

Tumor-specific global fragmentation pattern

To establish a comprehensive data set for LB analysis in all stages of CRC, we applied WGS with a median coverage of 6x (SD = 2.37) in 259 plasma samples of CRC patients ($n = 50$) and healthy controls ($n = 61$) (Additional file 2: Table S2). We first evaluated, whether the global fragmentation pattern of cfDNA may be a suitable marker for untargeted ctDNA detection. Fragmentation patterns are a result of various chromatin states that are associated with altered expression of tumor-associated genes [19, 21, 28, 29].

We compared the global fragmentation of cfDNA from CRC patients to cfDNA from healthy controls which typically present with a peak of ~ 167 bp corresponding to DNA bound by one nucleosome plus linker DNA [20] (Fig. 1A). We observed a significant enrichment of short fragments (90–150 bp) in CRC patient samples with clinically diagnosed tumor burden ($n = 134$) compared to healthy controls ($n = 55$) (Mann–Whitney-U test, p-value = $4.75*10^{-5}$) (Fig. 1B). When allocating CRC patient samples according to the course of disease, we observed a significant enrichment of short fragments during therapy (between surgery and adjuvant chemotherapy, or during chemotherapy before staging) ($n = 27$) (p-value = $1.48*10^{-4}$). A tendency (albeit not statistically significant) toward a higher proportion of short fragments could be identified in all other progression sample groups with clinically diagnosed tumor burden (Fig. 1C). When stratifying CRC patient samples collected at diagnosis according to their disease stage, we further observed a significant enrichment of short fragments in patients with stage IV CRC ($n = 16$) (p-value = $7.25*10^{-5}$) (Fig. 1D).

Interestingly albeit not statistically significant, we detected different fragmentation profiles due to enrichment of short fragments < 167 bp when analyzing samples from CRC patients in remission with no evidence

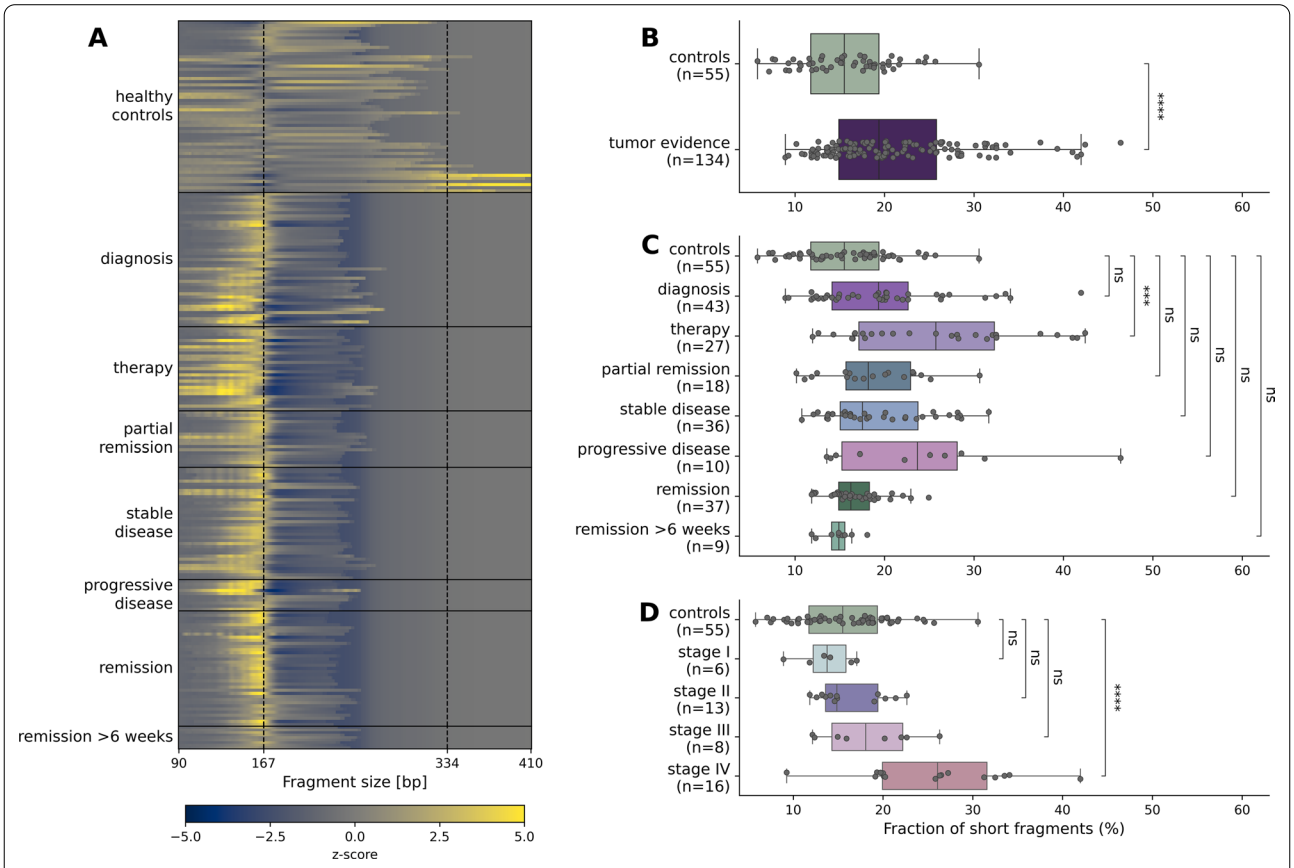


Fig. 1 Differences in global fragmentation between cfDNA from CRC patients and healthy controls. **A** Heat map showing enrichment or decrease in cfDNA fragments from 90 to 410 bp according to their length as z-scores of each sample compared to healthy controls. **B** Short cfDNA fragments (90–150 bp) are significantly enriched in samples collected from CRC patients with clinically diagnosed tumor burden. **C** Only for samples collected in the beginning of therapy a significantly enriched fraction of short fragments can be observed. **D** At diagnosis a significant enrichment in short fragments was only observed in patients with stage IV CRC. (ns: p-value ≤ 1; *: p-value ≤ $5*10^{-2}$, **: p-value ≤ $1*10^{-2}$, ***: p-value ≤ $1*10^{-3}$, ****: p-value ≤ $1*10^{-4}$)

of disease compared to healthy controls. When focusing on samples from CRC patients in remission more than six weeks post-treatment, we did no longer observe an enrichment of short fragments < 167 bp (Fig. 1A). The observed enrichment within the first weeks post-surgery is likely associated with the intake of low-molecular weight heparin, in accordance with previous findings [30, 31]. Taken together, our results indicate that cfDNA is more fragmented in CRC patients compared to healthy controls and can therefore support untargeted detection of ctDNA.

Tumor-specific regional fragmentation profiles

To assess whether regional fragmentation across the genome could serve as another non-genetic marker for ctDNA detection in CRC patients, we calculated the ratio of short (100–150 bp) to long (151–220 bp) fragments (S/L ratio) in 100 kb bins for each chromosome in CRC patients and healthy controls, as recently described [19, 20]. Notably, data of chromosome arms harboring SCNAs were excluded to avoid bias due to regionally enriched ctDNA. Compared to healthy controls, we observed distinct differences in the S/L ratio of CRC patients at diagnosis, during therapy, and with stable or progressive disease. In contrast, in CRC patients with partial remission or in remission, we did not observe such differences (Fig. 2A). Focusing on CRC patient samples with clinically diagnosed tumor burden, we observed a significant enrichment in short fragments on chromosome arms 1p and 15q, and significant enrichment of long fragments on chromosome arms 4p, 5p, 11p, 11q, 19q, 21p and 21q (Fig. 2B). Overall, we were able to detect ctDNA in 75% (100/134) of samples collected from CRC patients with clinically diagnosed tumor burden based on significantly different regional fragmentation on at least one chromosome arm.

The differences in regional fragmentation between CRC patients and healthy controls support recent findings identifying cfDNA fragmentation as independent biological feature representing chromatin profiles of the cells of origin [19, 20].

Combination of global and regional fragmentation analysis using machine learning

To test whether machine learning (ML) classifier based on global fragmentation and regional fragmentation in 5 Mb bins increase accurate detection of ctDNA, we trained four ML algorithms using 100 bootstrapping iterations with fivefold cross-validation (see Materials and Methods). For each iteration the prediction of the best model was stored and predictions for the two classifiers based on global and regional fragmentation were combined within a supervised meta-learner [20]. Samples collected from CRC patients with clinically diagnosed tumor burden ($n = 134$) served as positive cohort, and healthy individuals, including samples collected from patients in remission more than six weeks post-treatment without any known recurrence at a later time point ($n = 63$) served as control cohort for a better representation of biological variability (Additional file 1: Figure S1). All classifiers showed high prediction performance to distinguish cfDNA from CRC patients and healthy controls, with receiver operating characteristic (ROC) area under the curve (AUC) values of up to 94% and sensitivity at 95% specificity of up to 70% (Fig. 3A). Since our ultimate goal was to develop a workflow applicable in clinical practice, we trained a final model based on the best performing ML algorithm for each feature set. Evaluating the performance of ML classifiers using only the support vector machine, we observed ROC AUC values and sensitivity at 95% specificity of up to 95% and 75%, respectively (Fig. 3B). Eventually, we trained final ML models for both feature sets as well as the meta-learner including all data of CRC patients ($n = 134$) and controls ($n = 63$) without further subsetting. Applying these models with 95% specificity, ctDNA presence was correctly predicted in 36% (48/134) of samples based on global fragmentation (34/91 metastatic, 14/43 localized), and in 90% of samples based on regional fragmentation (121/134: 85/91 metastatic, 36/43 localized) and based on the meta-learner (120/134: 84/91 metastatic, 36/43 localized). However, also samples collected from patients in remission, especially within the first six weeks post-surgery were classified as ctDNA positive (Fig. 3C). These results in combination with the findings above indicate that the non-genetic cfDNA features analyzed within LIFE-CNA are not informative for the correct identification of ctDNA within the first six weeks post-surgery. However, the effects of surgery on cfDNA fragmentation seem to normalize after six weeks, indicating a potential use for recurrence monitoring starting at this time point.

CRC-specific active chromatin for ctDNA detection

We evaluated whether CRC specific chromatin signatures can be detected based on coverage changes using the LIQUORICE tool [20] and whether these chromatin signatures represent an independent marker for ctDNA detection. Specifically, we analyzed five sets of enhancer regions identified to be active in CRC including (i) active distal ChromHMM-defined [32] enhancer regions, (ii) CRC-specific gained enhancers identified by Hi-C [33], (iii) gained enhancers occupied by the transcriptional coactivators YAP/TAZ, (iv) highly conserved enhancers occupied by YAP/TAZ, and (v) active transcriptional start sites (TSS) in CRC [26]. In addition, we analyzed the coverage in universal DHS.

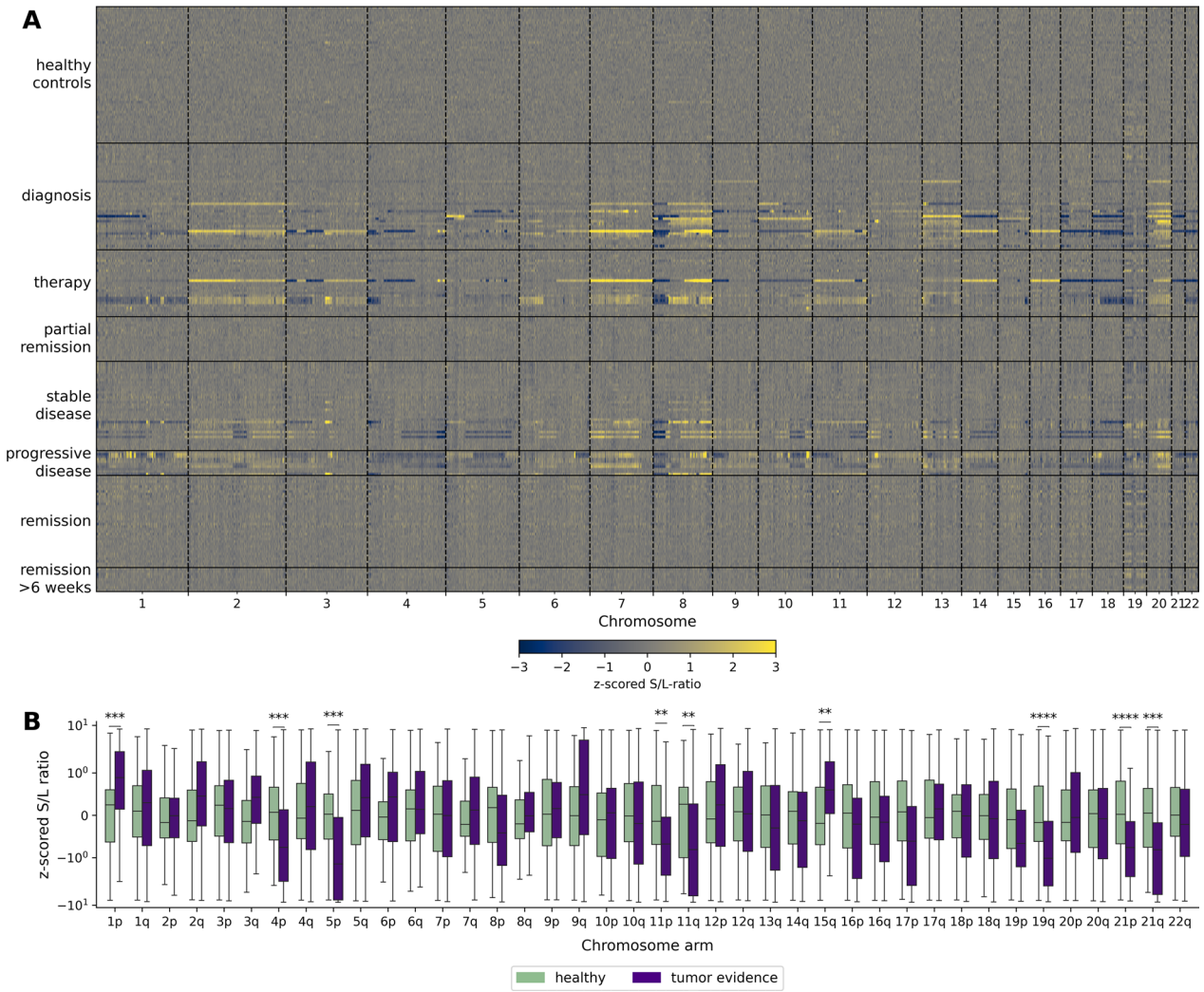
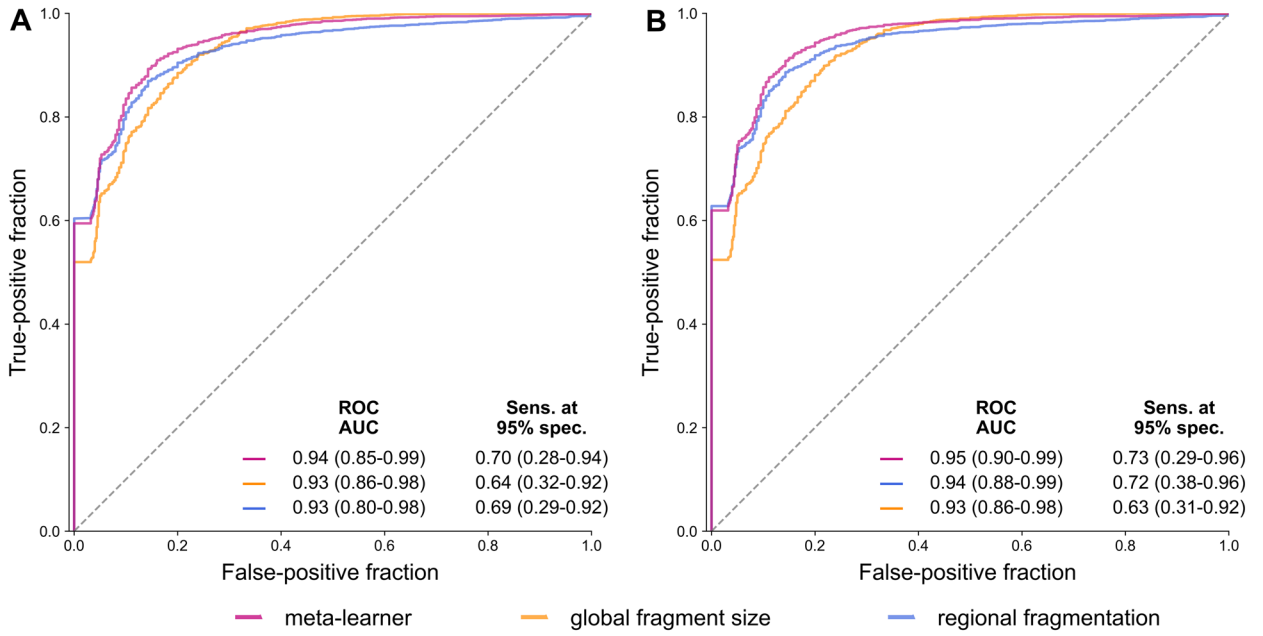


Fig. 2 Differences in regional fragmentation between cfDNA from CRC patients and healthy controls. **A** Heat map showing the z -scored of S/L-ratios in 100 kb bins of each sample compared to healthy controls. **B** Significant differences in z-scored S/L-ratios between samples collected from CRC patients with clinically diagnosed tumor burden and healthy controls were observed on multiple chromosome arms. (*: p-value $\leq 5*10^{-2}$, **: p-value $\leq 1*10^{-2}$, ***: p-value $\leq 1*10^{-3}$, ****: p-value $\leq 1*10^{-4}$)

In total, we observed significantly stronger coverage drops in all region sets in samples collected from CRC patients compared to healthy controls. In 5% (3/55) of healthy controls significantly stronger coverage drops in one of the analyzed region sets were detected when comparing the coverage to all 54 other healthy control samples. Therefore, to ensure a specificity of ≥ 95% for ctDNA detection based on the coverage in CRC-specific active chromatin regions, significantly stronger coverage drops need to be identified in at least two of the analyzed region sets rather than one. Overall, we detected ctDNA based on differential coverage in 33% (44/134) of samples collected from CRC patients with clinically diagnosed tumor burden (Additional file 2: Table S6). However, we obtained similar values [32% (12/37)] for remission patients and [33% (3/9)] for remission patients more than six weeks post-treatment. Taken together, coverage-based chromatin site analysis for ctDNA detection is suitable at diagnosis, but not for recurrence (also not > 6 weeks).

Quantification of the ctDNA fraction in CRC patients

To quantify the ctDNA fraction as a complement to fragmentation and coverage-based chromatin site analysis, we used the ichorCNA tool [17], which led to correct prediction of ctDNA in only 35% (47/134) of samples with clinically diagnosed tumor burden, even when



C

	diagnosis	therapy	partial remission	stable disease	progressive disease	remission	remission >6 weeks
global fragment size distribution	26% (11/43)	35% (20/57)	28% (5/18)	50% (18/36)	20% (2/10)	24% (9/37)	22% (2/9)
regional fragmentation 5 Mb	77% (33/43)	79% (45/57)	100% (18/18)	100% (36/36)	100% (10/10)	68% (25/37)	22% (2/9)
meta-learner	77% (33/43)	79% (45/57)	100% (18/18)	97% (35/36)	100% (10/10)	68% (25/37)	22% (2/9)

Fig. 3 Performance of ML classifiers based on global and regional fragmentation as well as a meta-learner. Performance was assessed over 100 bootstrapping iterations with fivefold cross validation **A** using the best performing model out of four classifiers for each iteration and **B** only a support vector machine over all iterations. **C** The three final classifiers detect ctDNA in CRC patients with high sensitivity

selectively enriching for ctDNA-associated 90–150 bp fragments (Additional file 1: Figure S3) [18, 20].

Detection of genome-wide and focal SCNAs in CRC patients

To identify genome-wide and focal SCNAs we applied a combination of the Illumina DRAGEN CNV workflow and Plasma-Seq [15, 22], considering ctDNA-associated 90–150 bp fragments (Additional file 1: Methods, Figures S4 and S5) [18, 20]. We analyzed paired tumor tissue and plasma samples collected at diagnosis to validate the SCNA pipeline. To correct for germline CNVs, constitutional DNA from saliva was additionally analyzed. In 44% (12/27) of patients with localized- and in 94% (15/16) of patients with metastatic CRC genome-wide SCNA profiles were highly concordant to the corresponding tissue. SCNAs unique to plasma were identified in 78% (21/27) of patients with localized- and 82% (13/16) of patients with metastatic CRC (Fig. 4A). In addition, we identified focal SCNAs in plasma matching tumor tissue in 4% (1/27) of patients with localized-, and in 63% (10/16) of patients with metastatic CRC, and focal SCNAs only in plasma in 15% (4/27) of patients with localized-, and in 63% (10/16) of patients with metastatic CRC (Fig. 4B). Certain genetic events found in plasma may not be present in tumor tissue because of the representation of only one site of the entire tumor mass rather than the complete tumor heterogeneity including metastatic sites. It is likely that low amplitude SCNAs may not be detected in plasma since ctDNA represents only a fraction of total cfDNA. Overall, although some SCNAs might be missed in plasma, with our approach we are able to detect genome-wide SCNAs in plasma from CRC patients over all stages, including subclonal events not identified in tumor tissue.

Complementary ctDNA detection by combining cfDNA features

Based on our results showing that global and regional fragmentation as well as chromatin signatures, and SCNAs are capable to independently detect ctDNA, we compared the sensitivity of all features in CRC patients in general and across stages considering the time point of sample collection in the course of disease (Fig. 5A, B).

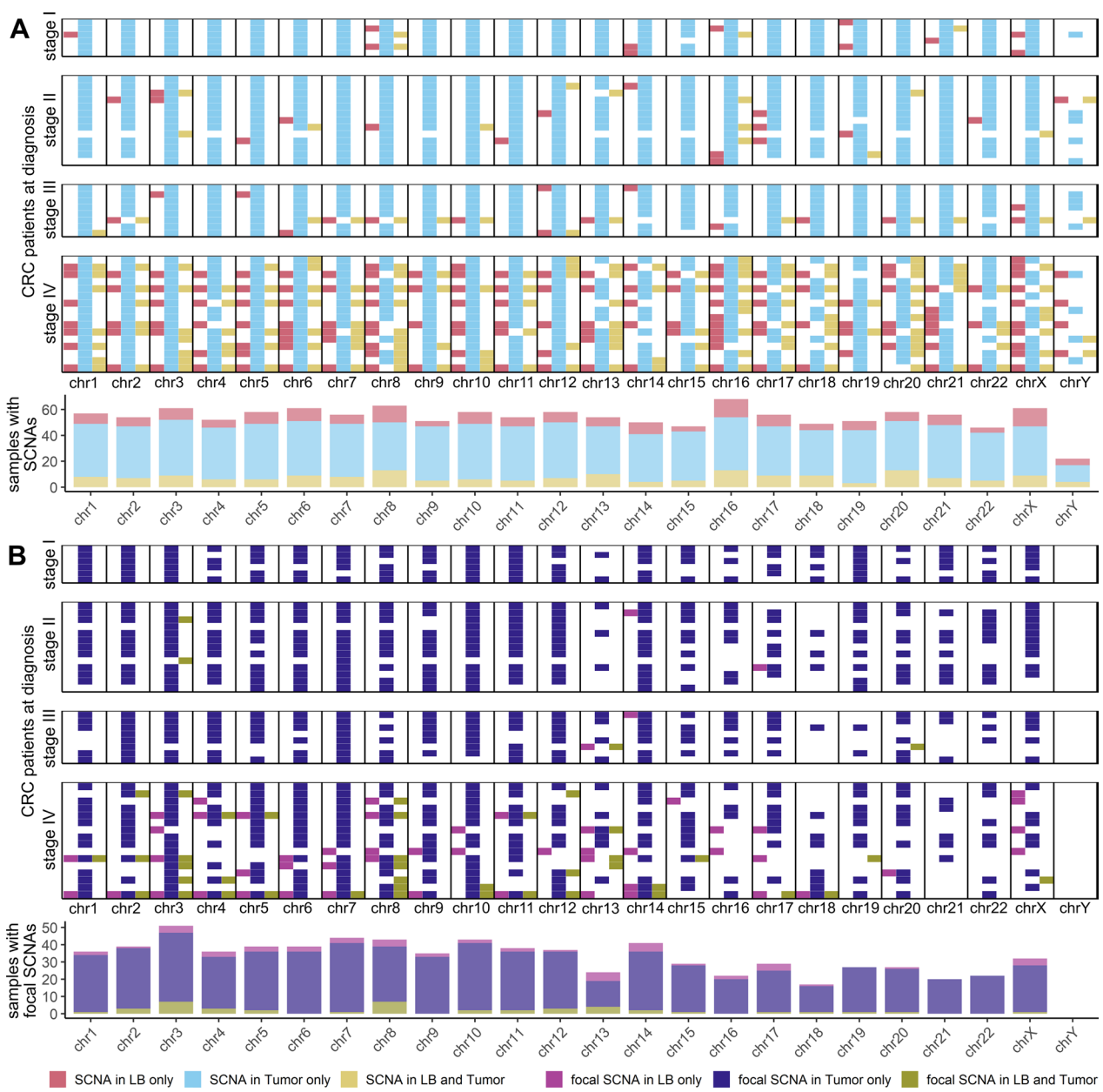


Fig. 4 Matched plasma and tumor analysis. To validate the SCNA analysis integrated in LIFE-CNA we performed a matched analysis of plasma samples collected at diagnosis with tumor tissue. **A** Total SCNAs present in plasma (red) or tumor (blue) only or in both plasma and tumor (yellow) and **B** focal SCNAs present in plasma (pink) or tumor (violet) only or in both plasma and tumor (green) present on each chromosome for individual patients and summarized over all patients below. Since more than one SCNA can be present per chromosome, it is possible that on the same chromosome different SCNAs are detected in plasma only, tissue only or in both plasma and tumor tissue

(See figure on next page.)

Fig. 5 LIFE-CNA enables accurate disease monitoring in CRC patients. SCNAs, focal SCNAs (foc. SCNA), tumor fraction in all (tum. frac.) and filtered fragments (tum. frac. short), enrichment in fragments from 90 to 150 bp (glob. frag.), regional fragmentation (reg. frag.), and significantly stronger coverage drops (low cov.) were analyzed with LIFE-CNA. In addition ctDNA was predicted with machine learning classifiers based on global (ML glob. frag.) and regional fragmentation (ML reg. frag.), and a meta-learner (ML Meta.) integrated into LIFE-CNA. To assess performance of LIFE-CNA, hotspot variants (SNVs) cfDNA concentration (cfDNA) and CEA were analyzed **A** in samples from CRC patients collected at different time points during disease summarized over all samples and **B** stratified by disease stage. **C** LB-CRC-32 was used as one example to show response and resistance to treatment throughout the course of disease

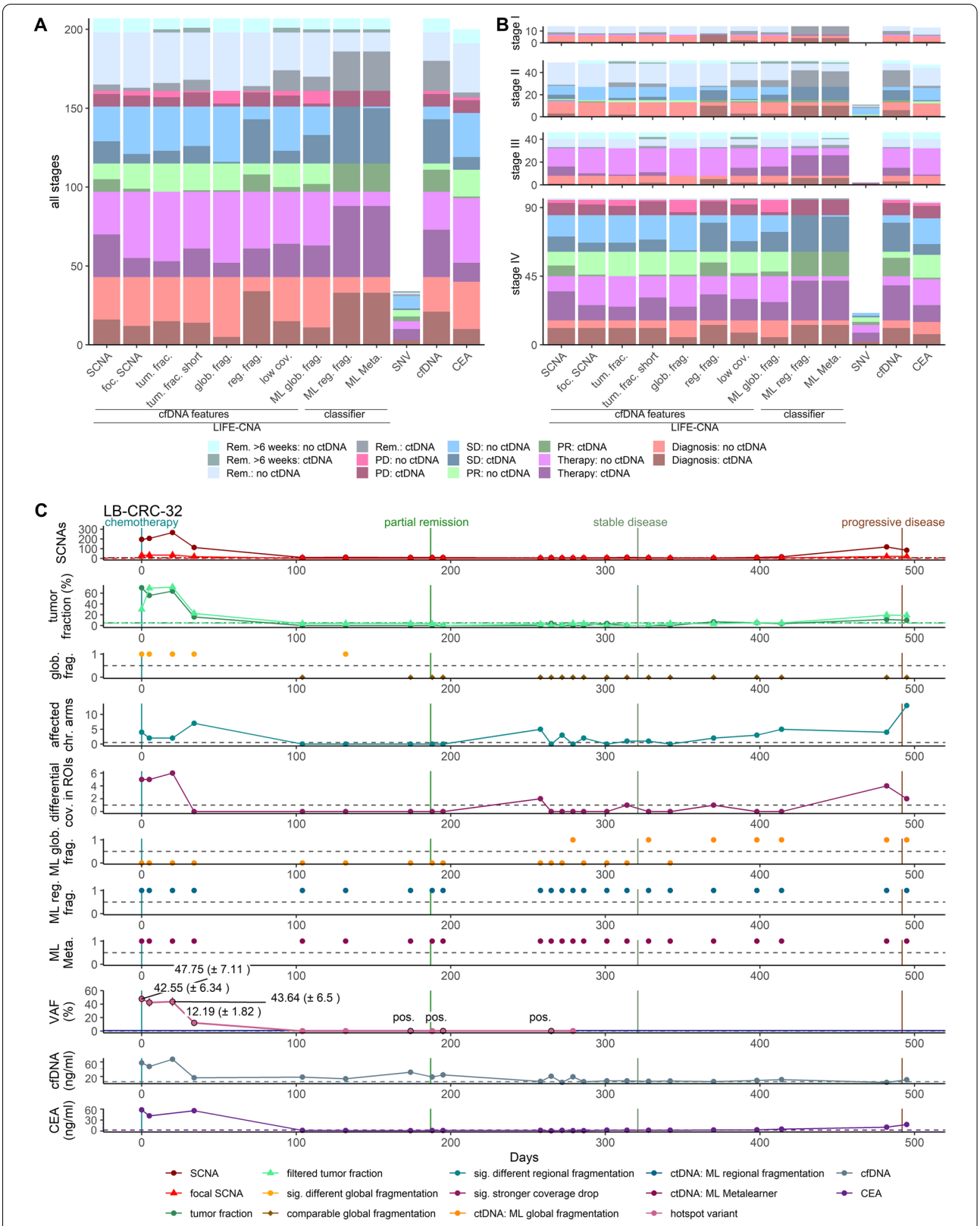


Fig. 5 (See legend on previous page.)

Regional fragmentation and coverage in active chromatin enabled ctDNA detection in 77% (33/43) and 23% (10/43) of patients with localized- and in 74% (67/91) and 37% (34/91) of patients with metastatic CRC with clinically diagnosed tumor burden, respectively. As expected, increased numbers of called SCNAs as well as elevated tumor fractions (Additional file 1: Data) were mainly observed in patients with metastatic CRC (57%, 52/91 vs. 26%, 11/43 and 45%, 41/91 vs. 14%, 6/43, respectively). Enriched short cfDNA fragments enabled ctDNA detection only in a small number of patients with metastatic CRC (19%, 17/91). Considering the three ML classifiers integrated in our LIFE-CNA workflow, we observed that the classifiers based on regional fragmentation and the meta-learner have a higher sensitivity for ctDNA detection (90%, 121/134 and 120/134, respectively), compared to the classifier based on global fragmentation (36%, 48/134). However, when focusing on samples collected within the first six weeks post-surgery, we observed ctDNA predictions with all non-genetic cfDNA features besides the global fragmentation, with the highest numbers of 68% (25/37) being with the ML classifiers based on regional fragmentation and the meta-learner. When focusing on only those samples collected from patients in remission more than six weeks post-treatment ctDNA detection rates decreased.

LIFE-CNA for accurate treatment monitoring in CRC patients

The analysis of multiple ctDNA features improves the sensitivity of untargeted ctDNA detection. To assess the clinical validity of LIFE-CNA for disease monitoring, we assessed changes of our measures over a median follow-up time of 7.5 months (range 1–35.5 months) in 15 patients and correlated these changes with treatment outcome as a proof-of-concept (Additional file 2: Table S6). In addition to LIFE-CNA, we analyzed the commonly used serum protein marker CEA, plasma cfDNA concentration, and SNVs for patients with available hotspot variant data ($n=5$). We were able to predict response to treatment in 77% (10/13) of patients (7/7 metastatic, 3/5 localized) by decreasing numbers of SCNAs, normalizing regional or global fragmentation, and/or normalizing coverage in regions of interest. CEA was informative in only 25% (3/12) of patients in two of those patients ~ 2 months later than LIFE-CNA, and decreasing plasma cfDNA concentrations could be correlated to treatment response in only 46% (6/13) of patients in one of those patients ~ 1 month later than LIFE-CNA. Further, LIFE-CNA correctly predicted progressive disease in 100% (5/5) of patients up to four months before clinical evidence with increasing differences to healthy controls of all analyzed cfDNA features. CEA was informative in only 80% (4/5) of patients in one of those patients ~ 3.5 months later than LIFE-CNA and cfDNA concentration was informative in only 20% (1/5) of patients ~ 9 months later than LIFE-CNA, respectively (Additional file 1: Figures S6–S20). For example, response and resistance to treatment could be detected with LIFE-CNA in patient LB-CRC-32 up to five and three months before clinical evidence, respectively (Fig. 5C). CEA identified response to treatment > 2 months later and resistance to treatment in parallel to LIFE-CNA. Although, decreasing cfDNA concentration was associated with response to treatment, at the time of progression no increase could be observed which is in line with previous reports showing low sensitivity and specificity of cfDNA concentration for treatment monitoring [34]. For SNVs, response to treatment could be identified in 3/4 samples, whereas no data were available to evaluate changing SNV levels for progression detection.

LIFE-CNA for cancer screening but not for MRD

To analyze whether LIFE-CNA could be applied for the detection of MRD post-surgery, plasma samples of 33 CRC patients collected up to 8 days pre-surgery and follow-up samples collected between 1 and 42 days post-surgery were analyzed (Additional file 1: Figure S21). Pre-surgery, we detected ctDNA in 92% (22/24) of patients with localized- and in 89% (8/9) of patients with metastatic CRC. Post-surgery, ctDNA was identified in 96% (23/24) of patients with localized- and in 100% (9/9) of patients with metastatic CRC, in particular due to the classifiers based on regional fragmentation and the meta-learner. Further, significant differences in coverage were observed in a large number of post-surgery samples (Additional file 1: Figures S21–S54). Decreasing ctDNA predictions more than six weeks post-treatment might enable the application of LIFE-CNA for recurrence monitoring (Fig. 5A&B, turquoise: remission more than six weeks post-treatment). In addition, the high sensitivity of ctDNA detection at diagnosis of patients with localized CRC (92%) suggests the great potential of LIFE-CNA for cancer screening.

Proof-of-principle of LIFE-CNA using six healthy controls and in silico dilutions

We evaluated the specificity of all cfDNA features by analyzing six additional healthy controls not included in the reference set. Of all analyzed cfDNA features only differential regional fragmentation was detected in 1/6 healthy controls while the remaining cfDNA features did not indicate ctDNA (Fig. 6). The ML classifiers based on regional fragmentation and the meta-learner, predicted ctDNA in 2/6 healthy controls. These results indicate

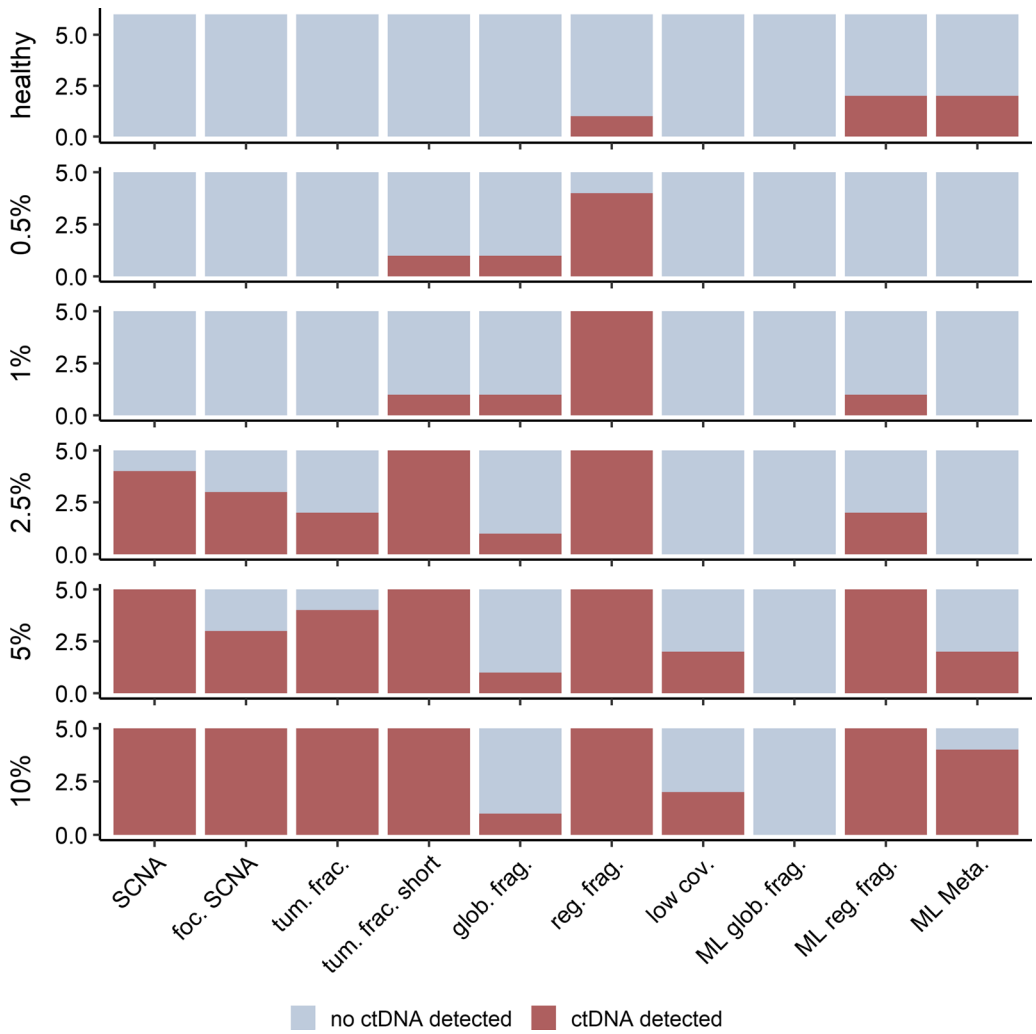


Fig. 6 Proof-of principle showing the high sensitivity of LIFE-CNA. Focal SCNAs (foc. SCNA), tumor fraction (tum. frac.), tumor fraction in 90 to 150 bp fragments(tum. frac. short), enrichment in fragments from 90 to 150 bp (glob. frag.), differential regional fragmentation (reg. frag.), significantly stronger coverage drop in at least to region sets (low cov.), classifier based on global fragmentation (ML glob. frag.), classifier based on regional fragmentation (ML reg. frag.), and classifier based on meta-learner (ML Meta.) were analyzed in six additional healthy controls not included in the panel of normals and in in silico dilutions with 0.5%, 1%, 2.5%, 5% and 10% tumor fraction as a proof-of-principle for ctDNA detection using LIFE-CNA

low specificity of the regional-fragmentation and meta-learner based classifiers for ctDNA detection.

In addition to specificity, we also assessed the sensitivity of LIFE-CNA for the detection of low ctDNA levels using in silico dilutions with tumor fractions of 0.5%, 1%, 2.5%, 5% and 10% (Additional file 2: Table S7). Analogous to disease monitoring, also for the in silico dilutions we observed the highest sensitivity for ctDNA detection based on regional fragmentation that correctly identified ctDNA in 4/5 samples with 0.5% tumor fraction and in all samples with 1% tumor fraction. At 0.5% tumor fraction, elevated tumor fractions based on ichorCNA and significant enrichment of short fragments could be predicted in one sample. Further, SCNAs could be detected in 4/5 samples with 2.5% tumor fraction. These results indicate that the sensitivity of our SCNA analysis could be increased compared to the previously described required tumor fractions above 5% to 10%. Focusing on the ML classifiers for ctDNA prediction, it was not possible to detect ctDNA based on global fragmentation in any of the in vitro dilutions. Using the classifier based on regional fragmentation, we detected ctDNA in 1/5 samples with 1% tumor fraction.

Discussion

Non-invasive and highly-sensitive ctDNA analyses allow real-time monitoring of patients throughout disease. The untargeted detection of ctDNA has the potential to extend the advantages of LB analysis to patients with cancer across all stages, and independently from knowledge about the presence of somatic hotspot variants. However, clinical validity of untargeted ctDNA analysis could so far mainly be shown for patients with metastatic cancer due to their high tumor fractions. Here, we developed LIFE-CNA for genome-wide ctDNA detection and disease monitoring based on multiple tumor-specific alterations across genetics, epigenetics and fragmentomics in patients with localized and metastasic CRC. We further provide analytical validation as well as a clinical proof-of-concept using a total of 259 plasma samples from 50 CRC patients and 61 healthy individuals. In contrast, a similar study conducted by Cristiano et al. [19] focused on one cfDNA feature (regional fragmentation analysis) for ctDNA detection only. Another study by Peneder et al. [20] also analyzed multiple cfDNA features in Ewing-sarcoma patients.

To facilitate clinical implementation of genome-wide ctDNA analysis suitable for all CRC patients, we defined distinct cutoffs or significance tests for each analyzed cfDNA feature. Establishing and validating definite criteria to report true ctDNA signals further are an important step towards the development of generic guidelines for the analytical validation of untargeted LB analyses, complementing the existing guidelines for targeted hotspot analyses [9, 35].

We evaluated performance of the various cfDNA features and of ML classifiers. CfDNA features achieved a higher sensitivity than ML classifiers for ctDNA detection at diagnosis of patients with localized and metastatic CRC, while false-positive predictions in external healthy controls were higher with the ML classifiers. Other applied ML classifiers reported in the literature achieved slightly better performance characteristics from training and testing procedures for early detection of ctDNA [20, 36]. One previous study performed external validation of a final ML classifier on a cohort of lung cancer patients and thereby achieved comparable sensitivity with slightly higher specificity compared to our ML classifier [37]. Although thorough external validation is required, considering an (albeit small) set of external samples indicates that our ML classifier might achieve a similar performance for CRC patients. Besides focusing solely on ML classifiers or the analysis of multiple cfDNA features, we also investigated whether a combination of ML classifiers with the analysis of multiple cfDNA features can improve the sensitivity of untargeted ctDNA detection. Concretely, combining the analysis of global and regional fragmentation, SCNAs and active chromatin coverage with the ML classifiers resulted in a slightly improved sensitivity for ctDNA detection at diagnosis of patients with localized and metastatic CRC and increased false-positive predictions in external healthy controls. We conclude that considering cfDNA features without ML classifiers may be favorable in cancer screening, as the number of false-positives is markedly reduced, with only a limited reduction in sensitivity, providing comparable performance to colonoscopies, the current gold standard in CRC screening [38]. However, before clinical implementation of LIFE-CNA, sensitivity and specificity needs to be externally validated in a larger cohort.

When evaluating the clinical sensitivity and specificity of LIFE-CNA for residual disease detection and treatment monitoring in a proof-of-concept study, we find a (too) high number of ctDNA positive predictions in R0-resected patients within the first six weeks post-treatment, showing that LIFE-CNA is probably not suited for residual disease detection. This may be explained by the fact that gene regulation and cfDNA fragment length, both factors being considered in the cfDNA features of LIFE-CNA, are perturbed after surgery. Multiple studies described altered gene regulation following surgery in response to cellular trauma [39–41] and the association of low-molecular weight heparin with increased levels of short cfDNA fragments [30, 31], which is given to patients directly after treatment.

There are some limitations that should be considered. Training the ML classifiers on a small a cohort of 134 CRC patient samples and 63 controls might cause overfitting. To overcome false-positive predictions caused by biological variability, larger control and positive cohorts to improve training and external validation for testing would be required before implementation of ML classifiers into clinical practice becomes feasible. Further, the median age of CRC patients (73) is much higher than the median age of healthy controls (32). With regard to the association between cfDNA fragmentation and nucleosome occupancy, which may change during life, future studies with age-matched healthy controls are highly important for validation of LIFE-CNA. Another limitation of this study is that a retrospective analysis of our rather small cohort enabled only the evaluation of a clinical proof-of-concept of LIFE-CNA but not the clinical validity. To establish the clinical utility a large prospective study would be required. If the clinical validity and utility of LIFE-CNA are demonstrated, simple blood sampling may allow rapid and non-invasive treatment monitoring, avoiding unnecessary colonoscopies and radiation introduced by imaging.

Conclusions

Taken together, we assume that considering multiple cfDNA features across different types of tumor-specific alterations in an untargeted genome-wide approach and evaluating them for various applications including screening and treatment monitoring, is an important step toward translating the high potential of liquid biopsy for future personalized medicine applications. Further, when analyzing active chromatin regions specific to other tumor entities we believe that LIFE-CNA can be easily transferred to all solid tumors.

Abbreviations

CEA: Carcinoembryonic antigen; cfDNA: Circulating free DNA; CNV: Copy number variation; CRC: Colorectal cancer; ctDNA: Circulating tumor DNA; ddPCR: Droplet digital PCR; DHS: DNase I hypersensitivity sites; LB: Liquid biopsy; LIFE-CNA: LIquid biopsy Fragmentation, Epigenetic signature and Copy Number Alteration analysis; ML: Machine learning; MRD: Molecular residual disease; S/L ratio: Short-to-long ratio; SCNA: Somatic copy number alteration; SNV: Single nucleotide variant; TSS: Transcriptional start site; WGS: Whole-genome sequencing.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13045-022-01342-z.

Additional file 1: Methods and Data including Table S1 and Figures S1–S54. **Methods** contain more detailed information about the study cohort and methods required to reproduce the experiments. **Data** contain detailed information on results not shown in the main document and results obtained for each individual patient analyzed for residual disease detection or treatment monitoring.

Additional file 2: Data required to reproduce the results shown in Tables S2–S8.

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Author contributions

AH and JP researched the literature to identify the features that could improve sensitivity of data analysis. AH and AB-P developed the experimental procedures. AH performed the experiments. EH provided the Plasma-Seq pipeline. AH and FS performed initial tests to determine the required coverage. AH and TW developed the bioinformatics analysis. AH analyzed and interpreted the data, and performed statistical analysis. UM provided input to statistical analysis and machine learning. JP and VS-L designed the study. VS-L was responsible for obtaining the positive ethics vote for patient recruitment. MR, HV, MdW, and CH contributed to patient recruitment. JP supervised the work. EH-F provided financial and technical resources to enable conduction of the study. AH and JP wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The sequence data have been deposited at the European Genome-phenome Archive (EGA) under accession number EGAS00001006490. These data are available under a controlled access regimen to ensure the protection of personally identifiable data; access can be obtained by contacting A.H. and J.P. Remaining data generated or analyzed during this study are included in this published article, and its supplementary information files or from the corresponding author on reasonable request. Code for sample analysis is modified from the publication by Heitzer et al. [15], Ulz et al. [22] and Peneder et al. [20] and is available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The study was approved by the ethics commission of the Bavarian Medical Association (No. 17059) and is registered with the German registry for clinical trials (trial registration ID: DRKS00012890). All participants provided informed written consent prior to blood and tissue specimen collection. The study was performed in accordance with the Declaration of Helsinki.

Consent for publication

All participants provided informed written consent to the publication of their data. This manuscript has been read and approved for publication by all named authors.

Competing interests

The authors declare no potential conflicts of interest.

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Publisher's Note

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