Aus dem Charité Comprehensive Cancer Center
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Liquid Biopsy – Blood-based biomarkers for the molecular analysis of cancer

Liquid Biopsy – Blut-basierte Biomarker zur molekularen Charakterisierung von Tumoren

zur Erlangung des akademischen Grades
Doctor rerum medicinalium (Dr. rer. medic.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Sandra Liebs
aus Berlin (Deutschland)

Datum der Promotion: 30.11.2023

Table of contents

List of tables iii

List of figures iv

List of abbreviations.. v

Abstract 1

1 Introduction..4

1.1 Liquid biopsy: The analysis of CTCs and cfDNA 4

1.2 Research questions 5

2 Methods..7

2.1 Patients 7

2.2 Isolation of blood-based biomarkers including cfDNA and CTCs 7

2.3 Nucleic acid preparation from CTCs, whole blood and tissue specimens 8

2.4 Mutational analysis using Droplet Digital™ PCR and Next Generation Sequencing 9

2.5 Data analysis 10

2.6 Statistical analysis 10

3. Results 11

3.1 Previous work: The pre-clinical OncoTrack platform 11

3.2 *KRAS* and *BRAF* mutation profiling in cfDNA from CRC patients in relation to disease stage 11

3.3 Identification of the predominant tumor in a patient with synchronous primary cancers using cfDNA analysis..14

3.4 Comparative analysis of mutation profiles of tumor tissue, CTCs and cfDNA in CRC, HNSCC and MEL patients..15

3.5 Liquid biopsy assessment of tumor heterogeneity...19

3.6 Detection of subclonal resistance in cfDNA..20

4. Discussion 22

4.1 Short summary of results 22

4.2 Interpretation and embedding of results into the current state of research 22

4.3 Implications for practice and future research 25

5. Conclusions 27

Reference list 28

Statutory Declaration 32

Declaration of your own contribution to the publications 33

Excerpt from Journal Summary List “Oncology” 2017 35

Printing copy of the publication: Liebs S, Keilholz U, Kehler I, Schweiger C, Haybäck J, Nonnenmacher A. Detection of mutations in circulating cell-free DNA in relation to disease stage in colorectal cancer. Cancer Med. 2019 40

Excerpt from Journal Summary List “Oncology” 2019 49

Printing copy of the publication: Liebs S, Eder T, Klauschen F, Schütte M, Yaspo ML, Keilholz U, Tinhofer I, Kidess-Sigal E, Braunholz D. Applicability of liquid biopsies to represent the mutational profile of tumor tissue from different cancer entities. Oncogene. 2021 51

Curriculum Vitae 60

Publication list 61

Acknowledgments 63

List of tables

Table 1: Patient characteristics....12

Table 2: Concordance of the *BRAF* and *KRAS* gene status between tumor tissue and cfDNA....13

List of figures

Figure 1: Quantitative analysis of cfDNA concentrations in relation to disease stage ...13

Figure 2: Comparative analysis of the representation of tissue mutations in corresponding plasma samples with levels of detectable cfDNA and CTCs 14

Figure 3: Total CTC counts and cfDNA concentrations possibly influenced by clinical parameters 15

Figure 4: Representation of tissue-derived mutations in LB samples 17

Figure 5: Represenation of cfDNA mutations in corresponding tissue samples 18

Figure 6: Shared and private mutations of tumor tissue and CTCs likely correlate with the requirements for tumor growth and dissemination to distant organs 19

Figure 7: Schematic illustration of the clinical course of a melanoma patient refractory to immunotherapy and BRAF-MEK-inhibition 21

List of abbreviations

AF	Allele frequency
cfDNA	Cell-free DNA
CRC	Colorectal cancer
CTCs	Circulating tumor cells
ctDNA	Circulating tumor DNA
ddPCR	Droplet Digital™ PCR
EDTA	Ethylenediaminetetraacetic acid
EGFR	Endothelial growth factor receptor
EMT	Epithelial-mesenchymal-transition
EpCAM	Epithelial cell adhesion molecule
FDA	Food and Drug Administration
FFPE	Formalin-fixed and paraffin-embedded
HNSCC	Head and neck squamous cell carcinoma
LB	Liquid biopsy
MCSP	Melanoma-associated chondroitin sulfate proteoglycan antigen
MEL	Melanoma
NGS	Next generation sequencing
PBMC	Peripheral blood mononuclear cells
QC-PCR	Quality control polymerase chain reaction
WES	Whole exome sequencing
WGA	Whole genome amplification

Abstract

Tissue analysis is the current gold standard for cancer diagnosis and characterization, although it may neither fully represent spatial tumor heterogeneity nor clonal evolution under treatment pressure. The analysis of circulating tumor cells (CTCs) and cell-free DNA (cfDNA) holds great potential to partially overcome this limitation. One major uncertainty is, however, whether both constituents (CTC vs. cfDNA) provide clinical informative value in a competitive or complementary way. Therefore, reflection of the mutational profile of tumor tissue in CTCs and cfDNA was investigated.

Applicability of cfDNA-based mutation analysis in colorectal cancer (CRC) patients in relation to disease stage was systematically investigated using Droplet Digital™ PCR. From 65 patients, the *KRAS* and *BRAF* gene status was assessed in plasma and compared to tumor tissue. In 17 of 25 (68%) patients with stage IV tumors, the tissue status was displayed in plasma. In patients with stage I-III tumors, concordance was only 43% (12 of 28 patients). Interestingly, in one stage II patient, cfDNA analysis revealed a different mutation compared to the respective colon cancer. Instead, the *KRAS* mutation of the synchronous stage IV tumor of the pancreas was detected in plasma. This case indicated the ability of liquid biopsy (LB) to identify the predominant cancer in patients with simultaneous malignancies.

In contrast to cfDNA levels, CTC detection rate in the CRC cohort was independent of tumor stage, indicating complementarity of cfDNA and CTCs. To investigate this hypothesis in neoplasms with different metastatic organotropisms, possibly affecting the clinical value of LB, panel sequencing of cfDNA and CTCs from patients with advanced CRC, head and neck squamous cell carcinoma (HNSCC) and melanoma (MEL) was performed. Only one of seven CTC samples isolated from four of 18 patients reflected the status of the solid tumor. In contrast, 78% of tissue mutations were displayed in high input cfDNA samples (30-100 ng, N=8). Highest concordance was observed in MEL and CRC with 100% and 92%, respectively, compared to only 50% in HNSCC.

These results emphasized that, when analyzing cancer patients in the advanced setting, cfDNA is superior to CTCs with respect to sample handling and mutation concordance. CTCs implicated clinical use in earlier cancer stages and for the analysis of tumor heterogeneity. Overall, clinical value of LB analysis was demonstrated in special patient cases

by detecting tumor heterogeneity and clonal dynamics under selective pressure, which represent the main drivers of acquired resistance and subsequent treatment failure.

Zusammenfassung

Gewebeanalysen stellen den gegenwärtigen Goldstandard für die Krebsdiagnose und -charakterisierung dar, obwohl dadurch weder die räumliche Tumorheterogenität noch die klonale Evolution unter selektivem Druck einer Therapie vollständig widergespiegelt werden. Die Analyse zirkulierender Tumorzellen (CTCs) und zellfreier DNA (cfDNA) besitzt ein hohes Potential diese Limitation partiell zu überwinden. Allerdings besteht Ungewissheit darüber, ob beide Bestandteile der Flüssigbiopsie (LB) Informationen von klinischer Relevanz übermitteln und ob diese von kompetitiver oder komplementärer Natur sind. Folglich wurde untersucht, inwiefern CTCs und cfDNA das Mutationsprofil des soliden Tumorgewebes repräsentieren.

Die Eignung der cfDNA-basierten Mutationsanalyse in Relation zum Krankheitsstatus von Patienten mit Kolorektalkarzinom (CRC) wurde mittels Droplet Digital™ PCR systematisch untersucht. Der *KRAS* und *BRAF* Genstatus wurde in Plasmaproben von 65 Patienten ermittelt und mit dem im Gewebe verglichen. In 17 von 25 (68%) Patienten mit Tumoren im Stadium IV, hat das Plasma den bekannten Gewebestatus wiedergegeben. In Patienten mit Krebsstadium I-III lag die Konkordanz bei nur 43% (12 von 28 Patienten). Interessanterweise, wurde in einem Patienten ein zum Tumorgewebe (Stadium II) widersprüchliches Ergebnis in der cfDNA offenbart. Stattdessen wurde im Plasma der KRAS-Status des synchronen Pankreastumors (Stadium IV) widergespiegelt. Dieser Fall implizierte den Anwendungsbereich, anhand der LB den vorherrschenden Tumor in Patienten mit multiplen Krebserkrankungen zu identifizieren.

Im Gegensatz zum cfDNA-Level war die CTC-Detektionsrate in der CRC-Kohorte unabhängig vom Tumorstadium, was auf eine Komplementarität der Komponenten hindeutete. Zur Untersuchung dieser Hypothese in Neoplasien mit unterschiedlichem Organotropismus wurde eine Panel-Sequenzierung von cfDNA und CTCs von Patienten mit fortgeschrittenem CRC, Kopf-Hals-Karzinom und Melanom durchgeführt. Nur eine von sieben CTC-Proben, die von vier der 18 Patienten isoliert wurde, spiegelte das Mutationsprofil

des soliden Tumors wider. Im Gegensatz dazu, wurden 78% der Gewebemutationen in hoch konzentrierten cfDNA-Proben detektiert (30-100 ng, N=8). Die höchste Übereinstimmung lag bei 100% und 92% in Patienten mit Melanom und CRC, verglichen zu 50% bei den Kopf-Hals-Karzinomen.

Diese Ergebnisse zeigten, dass cfDNA-Analysen in Patienten mit fortgeschrittenem Tumorstadium bezüglich der Probenhandhabung und Repräsentation des Tumormutationsprofils den CTC-Analysen überlegen sind. CTCs schienen hingegen zur Untersuchung der Tumorheterogenität als auch in früheren Krebsstadien einen klinischen Nutzen zu versprechen. Insgesamt wurde in einigen speziellen Patientenfällen der klinische Stellenwert der LB-Analyse demonstriert, indem Tumorheterogenität und klonale Dynamiken unter selektiven Therapiedruck detektiert wurden, welche die Haupttreiber erworbener Resistenz und folgendem Therapieversagen repräsentieren.

1 Introduction

Precision oncology implies different strategies to identify and target cancer-related alterations. This includes the routine analysis of predictive tissue markers to indicate sensitivity to targeted treatment, such as wild type status of *KRAS* and *BRAF* in colorectal cancer (CRC) with regard to cetuximab treatment and the presence of *BRAF* mutations in melanoma (MEL) as a prerequisite for BRAF-MEK-inhibition. Frequently, however, initial clinical response to small molecules and monoclonal antibodies is transitory. At present, initial cancer diagnosis and characterization is based on the analysis of a section of diagnostic tumor tissue biopsy, with the inherent limitation to fully represent the entire spatial and temporal tumor portrait. In particular, heterogeneous tumor genetics play a pivotal role in the development of acquired resistance to targeted treatments, since certain subclones, present at low frequencies and often not recognized in a single tissue biopsy, might gain competitive advantages and expand during the selective pressure of treatment. In accordance with this, Khan et al. reported a significant fraction of *RAS* wild type tumors to remain refractory to cetuximab treatment, due to expanding clones with resistance mutations already present initially with allele frequencies below the detection threshold (1). To anticipate emerging resistance precociously, collection of consecutive tissue samples from all coexisting lesions would be necessary, but impractical due to limitations in access to tumor sites.

1.1 Liquid biopsy: The analysis of CTCs and cfDNA

Liquid biopsy is suggested as a minimal invasive alternative for serial monitoring of cancer, by analyzing tumor-derived biomarkers in body fluids, including blood, urine and cerebrospinal fluid. Especially blood-based analysis of CTCs and cfDNA received considerable interest for the potential use as a surrogate of inter- and intratumoral heterogeneity. However, the informative value and clinical relevance of liquid biopsies and their different constituents remains elusive, requiring further investigation prior to clinical application.

CTCs represent cancer cells shedding from the solid tumor into circulation, with only 0.01% of tumor cells estimated to result in metastases (2). The multi-step nature of metastasis to distant organs requires tumor cell survival despite detachment from the extracellular matrix (anoikis-resistance), evasion of the immune system, arrest within blood vessels and subsequent invasion of surrounding tissue (diapedesis). A prognostic value

of CTCs has been shown in multiple studies of CRC, breast and prostate cancer, correlating the presence of ≥ 3 and ≥ 5 tumor cells in 7.5 ml blood with reduced progression-free survival and overall survival (3-5). Due to the low abundance of CTCs in blood (1-10 CTCs and 5-10 x 10^6 white blood cells per milliliter of whole blood) (6) and various possible phenotypes, including epithelial, mesenchymal, EMT-like (epithelial-mesenchymal-transition) and cancer stem cell characteristics, manifold approaches were established to enrich CTCs prior to detection and further characterization. Methodologies can be divided in two groups: biological and physical property-dependent technologies. The former utilizes marker expression for negative and positive cell selection (depletion of CD45-positive leucocytes vs. capture of tumor marker-expressing CTCs), whereas the latter uses size exclusion based on larger cell dimensions of CTCs compared to normal blood cells. To allow genomic analysis of a single cell (6 pg total DNA) (7), whole genome amplification is required, which is susceptible to errors based on amplification bias as well as complete failure due to insufficient DNA integrity. Besides those methodological constraints, analysis of viable CTCs will have a prominent role in enlightening mechanisms of cancer progression and reflecting tumor heterogeneity.

In comparison, cfDNA is released by healthy and diseased cells, undergoing apoptosis or necrosis (8). Therefore, circulating tumor DNA (ctDNA) can only be identified by the detection of genomic aberrations, such as single nucleotide variations and copy number alterations. Cancer-related mutations are present in frequencies as low as 0.01% (9), necessitating highly sensitive and specific detection methods. At present, five cfDNA assays have approval, covering *EGFR* testing in lung cancer and detection of *RAS* mutations in colorectal cancer (10). In contrast, only the CellSearch® system for CTC detection and isolation was FDA-cleared (U.S. Food and Drug Administration), allowing clinical use in specific countries.

1.2 Research questions

Shedding of CTCs and ctDNA into the bloodstream might be affected by tumor size, its anatomic location and vascularization, potentially limiting the clinical value of LB for various tumor entities and early cancer stages. Moreover, the tendency of a certain tumor type to spread to specific organs (metastatic organotropism) might also affect the utility of LB. To elucidate those issues, cfDNA and CTCs were quantified and characterized in

two patient cohorts and the genetic compositions were compared between LB and corresponding tissues. Within the first study as part of the OncoTrack research project, suitability of cfDNA analysis for mutation detection in *KRAS* and *BRAF* with respect to disease stage was investigated in CRC patients (stage I-IV). To analyze differences in tumor entities with distinct metastatic patterns, patients with HNSCC (predominant locoregional disease progression), CRC (primarily metastatic spread to the liver through the portal vein) and MEL (systemic hematogenous dissemination) were enrolled in the second study, in which 327 cancer-related genes were profiled via panel sequencing.

2 Methods

2.1 Patients

For all studies, informed consent was obtained prior to tissue and blood collection. The OncoTrack research project enrolled patients with early and advanced CRC (stage I-IV), whereas the following project focused on CRC, HNSCC and MEL patients presented with distant metastasis and therefore an elevated risk of high CTC numbers and increased levels of ctDNA in blood. The ethics committee of the Charité University Medicine approved all studies (EA 1/069/11 and EA 4/087/15). The Medical University of Graz and the St John of God Hospital Graz approved patient recruitment for the OncoTrack study in Graz (23-015 ex 10/11).

2.2 Isolation of blood-based biomarkers including cfDNA and CTCs

Peripheral blood was collected prior to surgery and processed within two hours. With increasing experience on the preservation of blood-based biomarkers, including type of collection tubes and protocols for sample processing, methods differed between publications. Between 2010 and 2016, BD Vacutainer® PST™ II heparin tubes were used for blood collection of the OncoTrack patient cohort. When working with cfDNA nowadays, one preferred anti-coagulant is ethylenediaminetetraacetic acid (EDTA) (11, 12), which is why blood was collected in BD Vacutainer™ K2-EDTA tubes for the comparative study of CTCs and cfDNA (recruitment from 2016-2017).

For cfDNA analysis, initial blood processing included two centrifugation steps to isolate and purify plasma prior to storage at -80°C (see respective publications for details (13, 14)). Cell-free DNA was isolated from plasma with two different kits, depending on the coagulant present in vacutainers employed. Plasma samples from heparin tubes were processed with the QIAamp DNA Blood Midi kit (Qiagen, Hilden, Germany), whereas EDTA samples were compatible with the QIAamp Circulating Nucleic Acid kit (Qiagen), which was reported to increase the yield of smaller, tumor-derived ctDNA fragments (15, 16). Independently of the cfDNA isolation kit, samples were concentrated following the specifications of Zymo`s DNA Clean & Concentrator®-5 kit (Irvine, USA).

In contrast to the isolation of plasma-derived biomarkers, enrichment and isolation of living CTCs was performed immediately after blood sampling. Previously, the EasySep™

Human CD45 Depletion kit (Stemcell Technologies) was used to enrich CTCs, applying a tetrameric antibody complex and magnetic particles to deplete CD45-positive cells (OncoTrack study). However, internal comparisons demonstrated superior performance of the RosetteSep™ Human CD45 Depletion Cocktail with respect to enhanced depletion efficiency of blood cells and higher CTC detection levels (data not published). Therefore, the RosetteSep™ kit was used for the sequencing project. Here, the depletion cocktail clusters erythrocytes, granulocytes and peripheral blood mononuclear cells (PBMCs), which pellet, when centrifuged over a density gradient centrifugation medium, whereas unbound cells (including CTCs) are present at the interface between plasma and density medium. In both projects, enriched cells were stained with fluorescence-labelled antibodies to differentiate between remaining PBMCs and CTCs (see respective publications for details (13, 14)). Additionally, the LIVE/DEAD™ Fixable Blue Dead Cell Stain for UV excitation (Life Technologies) was used to identify only viable tumor cells, specifically stained for the corresponding tumor markers. Viable CTCs were identified based on their CD45-negativity and the detection of at least one expressed tumor marker (MEL: melanoma-associated chondroitin sulfate proteoglycan antigen (MCSP); CRC/HNSCC: Epithelial cell adhesion molecule (EpCAM), endothelial growth factor receptor (EGFR) and CD73, a regulatory molecule of tumor growth, metastasis and immune evasion (17)). The DMI3000B fluorescence microscope (Leica, Wetzlar, Germany) was utilized for microscopic analysis, including micromanipulator-assisted single cell isolation using the Microinjector IM-9B (Narishige Group, Tokyo, Japan).

2.3 Nucleic acid preparation from CTCs, whole blood and tissue specimens

The sequencing project included genotyping of tissue, whole blood, cfDNA and CTCs. Isolated CTCs were subjected to an overnight whole genome amplification (WGA) using the REPLI-g Single Cell kit (Qiagen). An insufficient DNA integrity of single cells might lead to unsuccessful amplification during WGA. Therefore, a quality control PCR (QC-PCR) was performed with 1 µl of the WGA product, amplifying up to four DNA regions of various length and chromosomal location to predict successful downstream application (Ampli1™ QC kit from Menarini Silicon Biosystems, Castel Maggiore, Italy).

Formalin-fixed and paraffin-embedded (FFPE) tissue slides from primary and metastatic tumor tissue were deparaffinized and processed accordingly to manufacturer`s protocol of the High Pure FFPET DNA Isolation kit (Roche, Basel, Switzerland). To differentiate

between germline variants and tumor-specific somatic mutations, DNA from 1-2 ml whole blood was isolated using the QIAamp DNA Blood Midi kit (Qiagen). Accordingly to expected concentrations, DNA was quantified using the Implen NanoPhotometer® P-Class P 330 (Implen, Munich, Germany) or the highly sensitive Qubit® dsDNA HS Assay kit (Thermo Fisher Scientific, Waltham, USA). Additionally, Agilent`s High Sensitivity DNA Kit was used with the 2100 Bioanalyzer (Agilent, Eugen, USA) to analyze cfDNA fragment length.

2.4 Mutational analysis using Droplet Digital™ PCR and Next Generation Sequencing

Two highly sensitive methods for the mutation detection in cancer-related genes were applied. The analysis of cfDNA of CRC patients was based on the knowledge of reported mutations in the two oncogenes *KRAS* and *BRAF* from tumor tissue. Therefore, the Droplet Digital™ PCR (ddPCR) platform was used to investigate to which extend cfDNA reflected the gene status of the solid tumor. Here, a water-oil emulsion droplet technology fractionated each PCR sample into 20,000 droplets, resulting in individual PCR reactions with approximately one amplicon per droplet. The two detection channels were used to differentiate the wild type sequence from the point mutation (HEX and FAM-labelled probes, respectively). Multiple assays were designed, detecting the V600E variant of the *BRAF* gene as well as the *KRAS* mutations G12A/C/D/V and G13D with a limit of detection of 0.01% and a false positive rate of one event (see respective publications for details (13, 14)). Analysis of patient-derived material was performed in duplicates, furthermore including corresponding controls, harboring the mutation of interest with a frequency of 1% or a non-template control (positive and negative controls, respectively).

To compare informative potential of cfDNA and CTCs in different types of neoplastic disease, next generation sequencing (NGS) was used for a broader detection range. The Haloplex™ HS target enrichment system (Agilent, Santa Clara, CA) was used for library preparation, enriching the exonic sequence (1.47 Mb) of our in-house panel of 327 frequently mutated genes (18). The High Output v2 sequencing kit (300 cycles, Illumina, San Diego, CA) was used to perform paired-end sequencing on the Illumina NextSeq500 platform executed by *amedes genetics* (amedes Medizinische Dienstleitungen GmbH, Berlin, Germany). Only sequencing of tumor tissue from the MEL subcohort was con-

ducted differently as part of the precision oncology program Treat20 Plus (19). Collaborators at the Max Planck Institute performed DNA isolation from the metastatic tissue and whole exome sequencing (WES) on the HiSeq™ system using the Nextera Rapid Capture Exome and Expanded Exome kit (Illumina, 62 Mb).

2.5 Data analysis

Final evaluation of ddPCR results considered only samples with ≥10,000 analyzed droplets to guarantee reliable statistical analysis of the QuantaSoft™ software. Wildtype and mutation events were differentiated based on their various fluorescence amplitudes, resulting from different probe concentrations used respectively. Outliers were excluded prior to quantification of positive events. Furthermore, only those samples with mutation event counts above the false positive rate were considered as positive.

Regarding the NGS analysis, raw fastq files were trimmed and aligned to the hg19 reference genome using Agilent`s SureCall software (version 3.5.1.46), furthermore, removing duplicates and identifying preliminary variants for subsequent analysis. Personal alterations were excluded when detected in the patient-specific germline sample. Common polymorphisms (minor allele frequency >2%) and artifacts were filtered out and relevant missense or nonsense mutations were sieved based on the predicted damaging effects annotated by the two data bases COSMIC (20) and Cancer Genome Interpreter (21). Alterations from FFPE and cfDNA with an allele frequency below 5% were excluded. Variant calling in CTC pools was not limited by an allele frequency cut-off due to different cell counts in each sample and an inestimable amplification bias during WGA. CTC and cfDNA-derived variants with a sequencing depth >30 and ≥ 5 for total and altered reads were integrated into final analysis. Discordant results between tissue and liquid biopsy samples from the same individual underwent manual inspection, partly identifying subthreshold mutations. WES results of metastatic tissue from MEL patients were provided by *Alacris Theranostics GmbH* (Berlin, Germany).

2.6 Statistical analysis

Continuous variables were summarized by median and range, and categorical variables by frequency. Due to the small sample size, only exploratory analyses but no formal comparisons were made.

3. Results

3.1 Previous work: The pre-clinical OncoTrack platform (published in Schütte et al., Nature Communications (22))

Our laboratory was part of the OncoTrack consortium (2011-2016), aiming to identify novel markers associated with treatment effects in stage I-IV CRC. I joined the laboratory in July 2014 and was involved in the methodological work for CTC detection since then. Extensive omics data from patient-derived xenograft (N=59) and organoid models (N=35) as well as 116 matched tumor samples highlighted the contribution of intra-tumor heterogeneity and clonal dynamics to therapy resistance (22). From this patient cohort, CTCs were quantified from blood samples in our laboratory (details in section 3.4), however, molecular analysis failed at that time due to the low input material originating from single cells. Subsequently, corresponding CTC protocols were improved and additional methods for the isolation and analysis of cfDNA were established for the following liquid biopsy projects.

3.2 *KRAS* and *BRAF* mutation profiling in cfDNA from CRC patients in relation to disease stage (published in Liebs et al., Cancer Medicine (13))

Plasma samples from 65 patients with early and advanced CRC (OncoTrack cohort) were analyzed to detect common point mutations in *KRAS* and *BRAF* in cfDNA and compared to solid tissue. Tumors from 10 patients (15%) harbored the *BRAF* V600E mutation, a *KRAS* mutation in codon 12 or 13 was reported in 25 patients (38%), and tumors from 18 patients (28%) were wild-type for both oncogenes (Table 1). For 12 patients, the *KRAS* and *BRAF* tissue status was unknown.

Table 1: Patient characteristics

Characteristics Number of patients	Total N = 65	Stage I N = 9	Stage II N =12	Stage III N =15	Stage IV N = 29
Age at enrollment, years					
Median	67	67	69	70	63
Range	36-92	49-79	46-79	39-83	36-92
Sex, n (%)					
Male	39 (60%)	6 (67%)	7 (58%)	10 (67%)	16 (55%)
Female	26 (40%)	3 (33%)	5 (42%)	5 (33%)	13 (45%)
Tissue gene status, n (%)					
KRAS-MUT	25 (38%)	2 (22%)	2 (17%)	6 (40%)	15 (52%)
BRAF-MUT	10 (15%)	2 (22%)	4 (33%)	1 (7%)	3 (10%)
WT	18 (28%)	2 (22%)	4 (33%)	5 (33%)	7 (24%)
Unknown	12 (18%)	3 (33%)	2 (17%)	3 (20%)	4 (14%)
CTC detection rate, n (%)					
Performed CTC analysis	54 (83%)	7 (78%)	12 (100%)	13 (87%)	22 (76%)
Patients with CTCs	29 (54%)	4 (57%)	7 (58%)	8 (62%)	10 (45%)
Patients without CTCs	25 (46%)	3 (43%)	5 (42%)	5 (38%)	12 (55%)
CTC numbers					
Median	1	1	2	1	0
Range	0-8	0-4	0-8	0-6	0-5
Not available	11	2	0	2	7

KRAS-MUT comprises the amino acid substitutions G12D, G12V, G12C and G13D, the V600E mutation is listed as *BRAF*-MUT. From Liebs et al. (13) Copyright © 2019 The Authors (Reproduced with permission from Springer Nature)

Independent of plasma volume or DNA concentration, cfDNA was detected in 100% of patient samples. CfDNA concentrations increased with higher tumor burden, ranging between 59 ng/ml in healthy donors to 156 ng/ml in patients with distant metastasis (Figure 1). Across all tumor stages, ddPCR assays demonstrated 100% specificity, verifying all wild type statuses from tumors in the corresponding cfDNA sample (Table 2). Sensitivity was limited with only 11 of 35 (31%) retrieved tissue mutations in plasma, including 2 of 10 (20%) *BRAF* and 9 of 25 (36%) *KRAS* mutations. Mutant copies were detected with 2 to 227 ddPCR events, resulting in allele frequencies (AF) of 0.01 to 0.52.

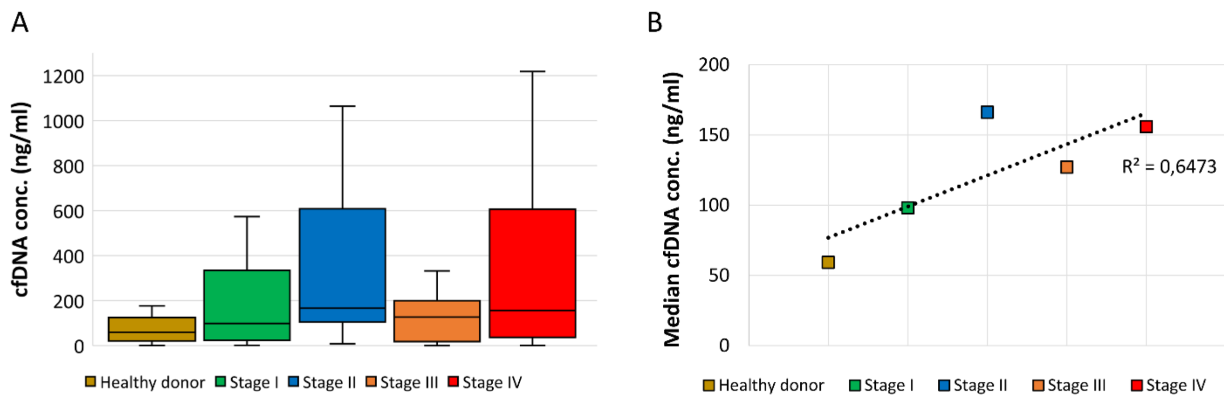


Figure 1: Quantitative analysis of cfDNA concentrations in relation to disease stage: (A) CfDNA levels in stage I-IV CRC patients in comparison to healthy individuals. Box plot showing median, first and third quartiles with whiskers from minimum to maximum. (B) Median cfDNA concentrations increase with higher tumor burden. From Liebs et al. (13) Copyright © 2019 The Authors (Reproduced with permission from Springer Nature)

Table 2: Concordance of the *BRAF* and *KRAS* gene status between tumor tissue and cfDNA

		cfDNA analysis									
		Total (N=53)		**Stage I**		**Stage II**		**Stage III**		**Stage IV**	
		MUT	WT	MUT	WT	MUT	WT	MUT	WT	MUT	WT
Tissue analysis	MUT	11	24	0	4	1	5	0	7	10	8
	WT	0	18	0	2	0	4	0	5	0	7
Sensitivity		31%		0%		17%		0%		56%	
Specificity		100%		100%		100%		100%		100%	
Accuracy		55%		33%		50%		42%		68%	

From Liebs et al. (13) Copyright © 2019 The Authors (Reproduced with permission from Springer Nature)

Despite the detection of increasing ddPCR event counts when analyzing higher cfDNA concentrations, no correlation of high event counts with successful tissue mutation detection in plasma was observed (Figure 2). However, ten of the eleven verified mutations were detected in the stage IV cohort, resulting in a total concordance rate of 68% for CRC patients with distant metastasis (all wild-type samples (N=7) and 10 of 18 (56%) mutations were recovered). In contrast, only one of 17 (6%) mutations in all stage I-III tumors was also detected in the periphery, resulting in an overall concordance rate of 43%.

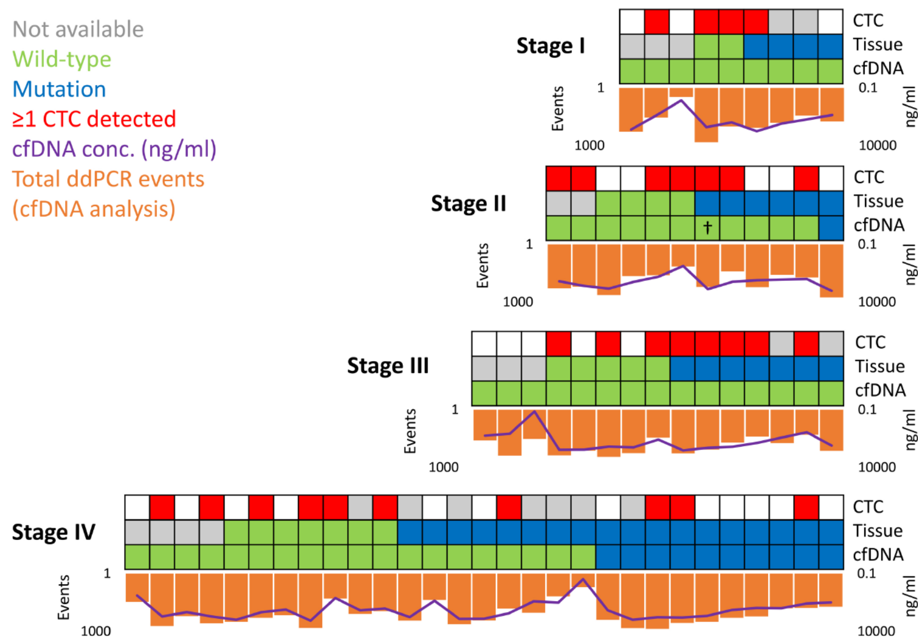


Figure 2: Comparative analysis of the concordance of tissue and plasma mutations with levels of detectable cfDNA and CTCs: Total ddPCR events increased in higher concentrated cfDNA samples, but did not correlate with the detection of tissue-reported variants in plasma. CTCs were detected independently of cancer stage, suggesting complementarity of both LB components. † In patient 374-CB-M, the *KRAS* G12C mutation from the CRC was not detected in plasma, but the G12D variant from the synchronous stage IV cancer of the pancreas. From Liebs et al. (13) Copyright © 2019 The Authors (Reproduced with permission from Springer Nature)

3.3 Identification of the predominant tumor in a patient with synchronous primary cancers using cfDNA analysis (published in Liebs et al., ESMO Open (23))

One OncoTrack patient with colon cancer and liver metastases was of particular interest, since only in this case a discrepancy in the *KRAS* mutation status of cfDNA and tissue was identified (Figure 2). Whereas a *KRAS* G12D mutation was detected in plasma with an AF of 0.10, the stage II colon cancer harbored a *KRAS* G12C mutation (AF: 0.41). However, cfDNA results were in concordance with the detected alteration in both the liver metastasis (AF: 0.47) and the synchronous adenocarcinoma of the pancreas (AF: 0.23). Those results suggested the pancreas tumor as the origin of ctDNA and the metastatic lesion, which was verified by further histologic studies.

3.4 Comparative analysis of mutation profiles of tumor tissue, CTCs and cfDNA in CRC, HNSCC and MEL patients (published in Liebs et al., Oncogene (14))

In contrast to high cfDNA levels associated with advanced disease, CTC counts were independent of tumor stage in the OncoTrack cohort, varying between 1-8 CTCs in 29 of 54 CRC patients (54%). This observation emphasized the differences between cfDNA and CTCs, furthermore, indicating complementarity of both LB constituents. To investigate this hypothesis more closely and specifically in relation to different metastatic organotropisms, archival tissue and liquid biopsy samples from 18 patients with advanced CRC, HNSCC and MEL were sequenced using a 327 cancer gene panel. Median cfDNA concentrations were 139.7 ng/ml, 4.7 ng/ml and 7.1ng/ml in CRC, HNSCC and MM patients, respectively (Figure 3). In 13 of 18 (72%) patients, 1-33 CTCs were detected. However, from only 12 patients, CTC samples were isolated and whole genome amplified, resulting in sufficient DNA concentrations for NGS analysis from seven of 16 samples (44%).

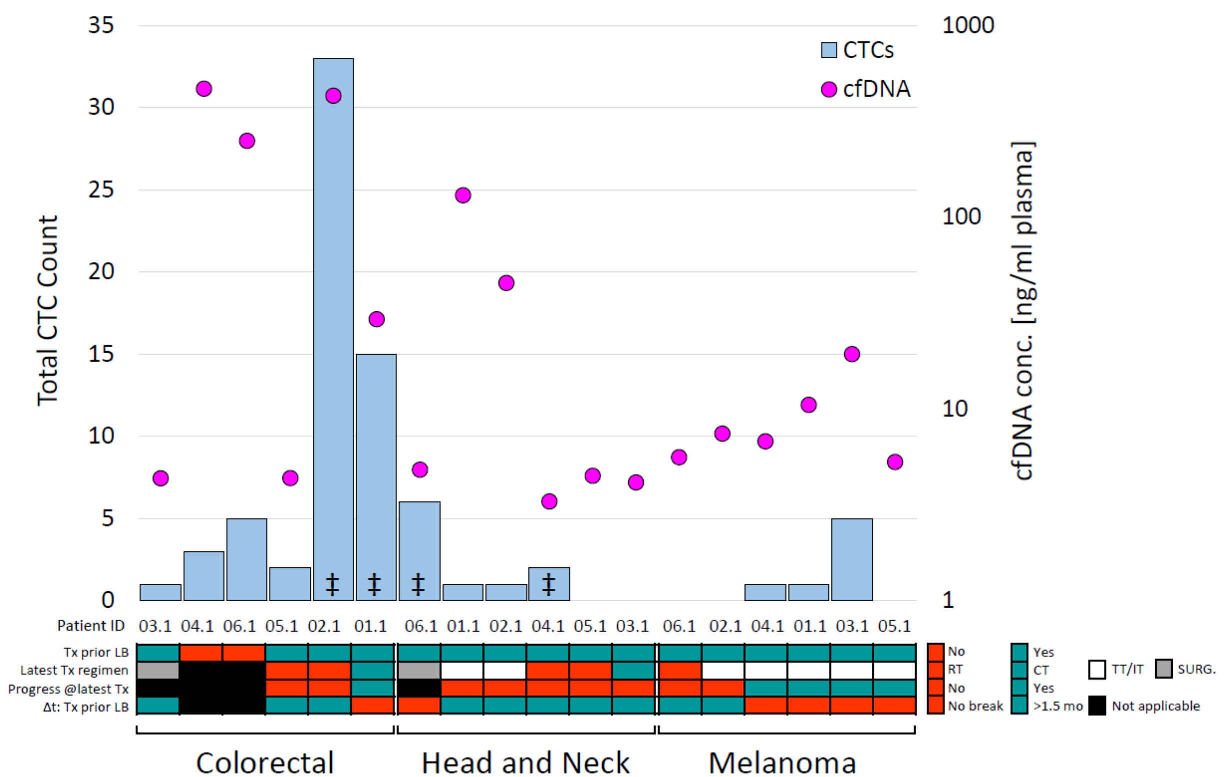


Figure 3: Total CTC counts and cfDNA concentrations possibly influenced by clinical parameters. CT: chemotherapy, IT: immunotherapy, LB: liquid biopsy collection, mo: months, RT: radiotherapy, SURG: surgery, TT: targeted therapy, Tx: treatment, Δt: time span, ‡ available NGS data

from whole genome amplified CTCs. From Liebs et al. (14) Copyright © 2021 The Authors (Reproduced with permission from Springer Nature)

In primary and metastatic tumor tissue specimens (N=30), 92 tissue mutations were identified and assessed for reflection in 18 cfDNA and seven CTC samples (Figure 4). Whereas highly concentrated cfDNA samples demonstrated a total concordance rate of 78% with tumor tissue, low input samples displayed only 8% of tissue mutations (44% of patients with 30-100 ng cfDNA input for NGS and 56% with <30 ng, respectively). Comparative mutation analysis of tissue with CTC samples was performed for only three patients, harboring one, two and 20 tumor mutations (after applying the filter algorithm described in method section 4.5, no tissue mutations were detected in the forth patient with sequenced CTCs). Only a pool of 13 CTCs mirrored the molecular profile of the colon tumor tissue from patient CRC002.1, although two additional CTC samples from that patient with comparable genome integrity indices were sequenced as well (eight and five CTCs harbored respective wild type sequences only). The *TP53* p.Q100* variant from patient CRC001.1 was not detected in either of the two CTC samples (one and five tumor cells), whereas the corresponding cfDNA reflected the mutation status. Only one of 20 rather sporadic tissue mutations from patient HNSCC004.1 was displayed subthreshold in the respective CTC sample (single tumor cell).

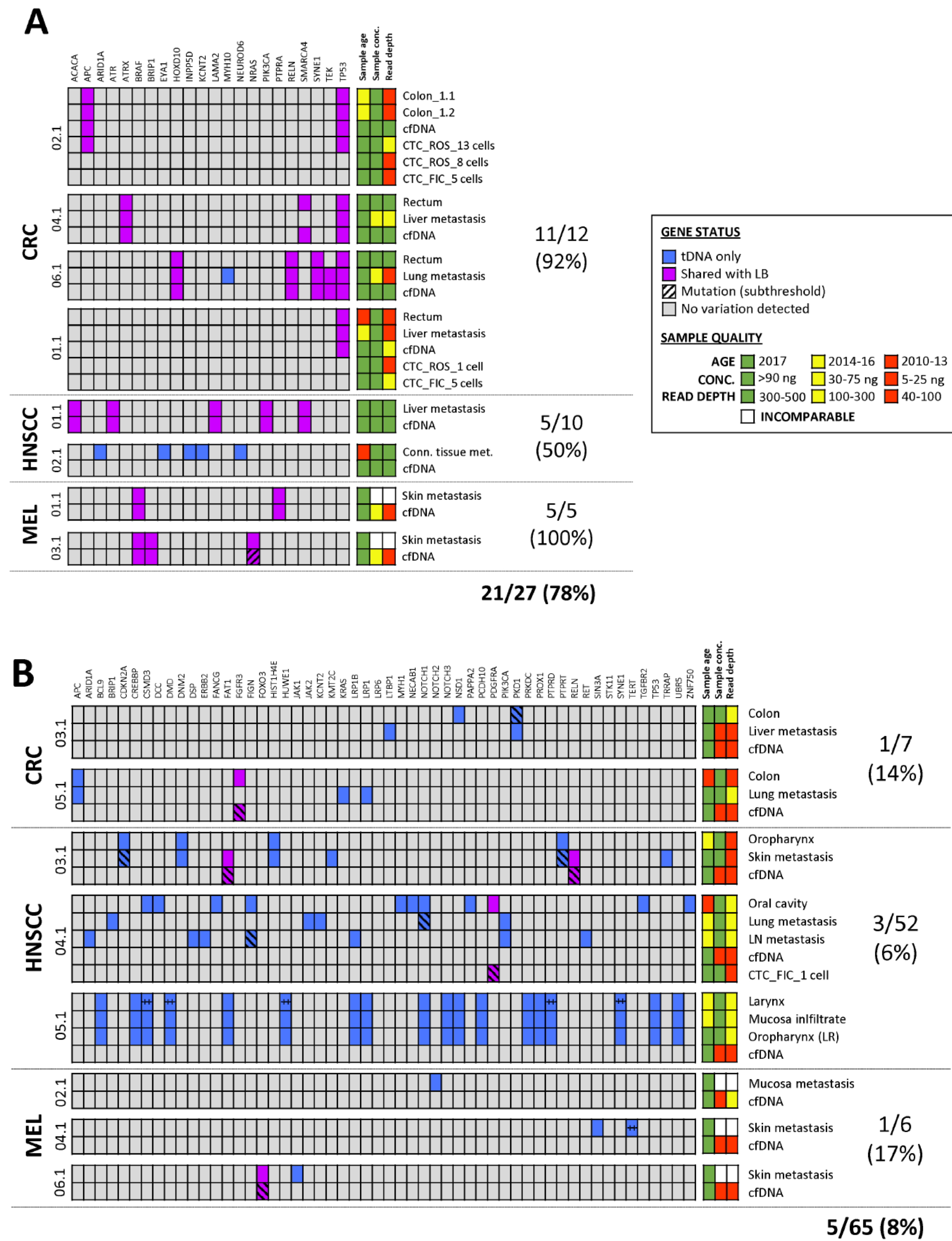


Figure 4: Representation of tissue-derived mutations in LB samples: Based on the total cfDNA input used for NGS analysis, samples were categorized as (A) high-input and (B) low-input material (≥30 ng and <30 ng, respectively). ‡ Multiple mutations were detected in the same gene. FIC: density gradient centrifugation-enriched CTCs, LB: Liquid biopsy, LR: local recurrence, NGS: next generation sequencing, ROS: RosetteSep™-enriched CTCs, tDNA: tumor-derived DNA. From Liebs et al. (14) Copyright © 2021 The Authors (Reproduced with permission from Springer Nature)

Subsequently, liquid biopsies were evaluated for additional mutations, which were originally not detected in tissue samples possibly due to tumor heterogeneity or the presence of rare subclones. In cfDNA and CTCs, 43 and 204 mutations were identified, respectively. After manual inspection, 15 of 43 (35%) and 18 of 204 (9%) mutations were also detected at subthreshold levels in tissue (Figure 5).

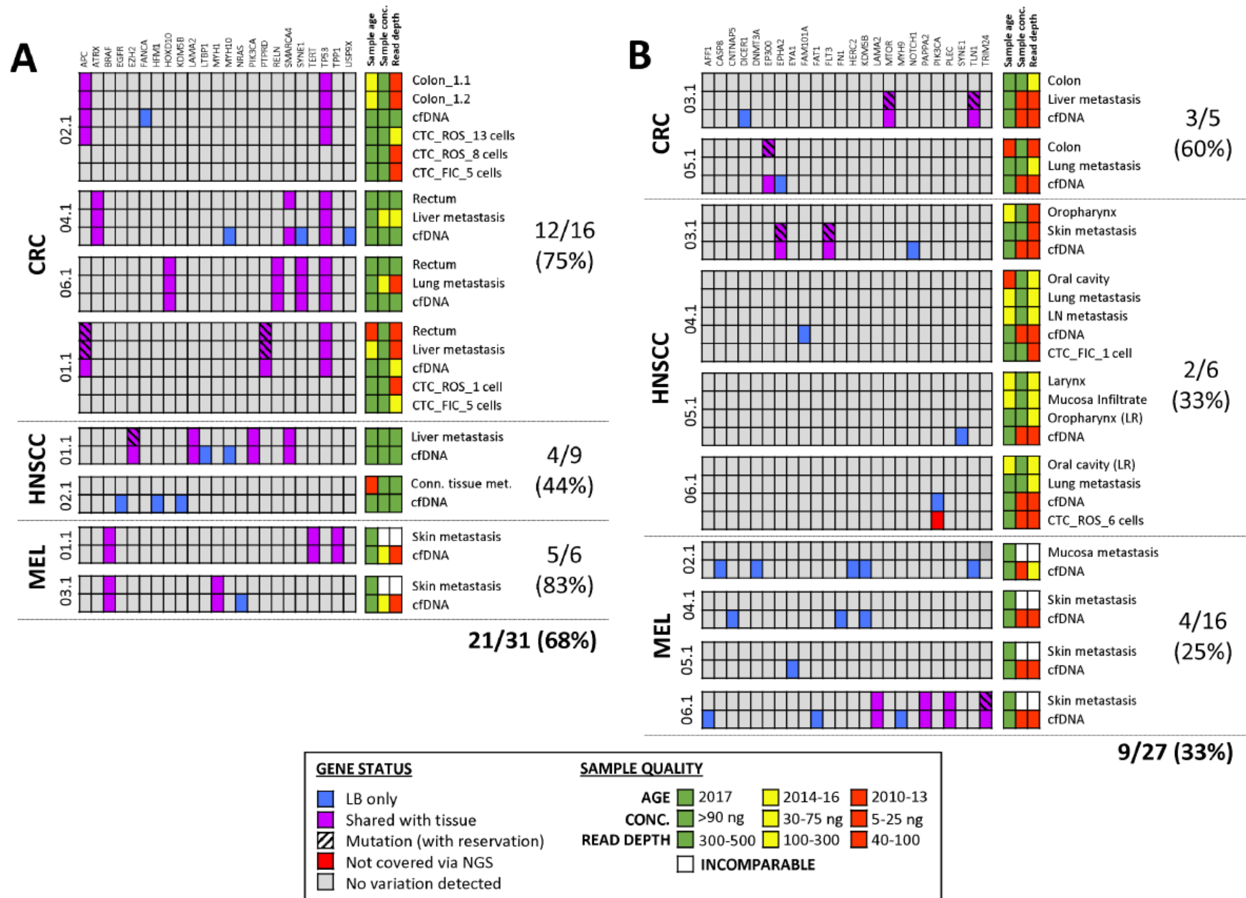


Figure 5: Representation of cfDNA mutations in corresponding tissue samples: Based on the total cfDNA input used for NGS analysis, samples were categorized as (A) high-input and (B) low-input material (≥30 ng and <30 ng, respectively). FIC: Ficoll-enriched CTCs, LB: Liquid biopsy, LR: local recurrence, NGS: next generation sequencing, ROS: RosetteSep™-enriched CTCs, tDNA: tumor-derived DNA. From Liebs et al. (14) Copyright © 2021 The Authors (Reproduced with permission from Springer Nature)

3.5 Liquid biopsy assessment of tumor heterogeneity (published in Liebs et al., Oncogene (14))

In patient CRC002.1, tumor heterogeneity was investigated to a broader extent by sequencing of cfDNA, three CTC pools and two spatial regions of the colon tumor tissue (Figure 6A). Two mutations in the tumor suppressor genes *APC* and *TP53* were detected in both tumor samples and reflected in one CTC and the cfDNA sample. The other two CTC samples only displayed the respective wild type sequence. In total, 121 variants were identified in all three CTC pools, of which only 34 (28%) were also found in at least one other specimen.

Mutated genes were assigned to corresponding pathways and cancer hallmarks using the databases KEGG (Kyoto Encyclopedia of Genes and Genomes) and COSMIC. Genes associated with genome instability, immune escape and tumor invasion were more frequently mutated in and private to CTC samples (Figure 6B/C). In contrast, mutations shared by tumor tissue and CTCs were rather related to induction of angiogenesis, proliferative signaling, inflammation and resistance to cell death.

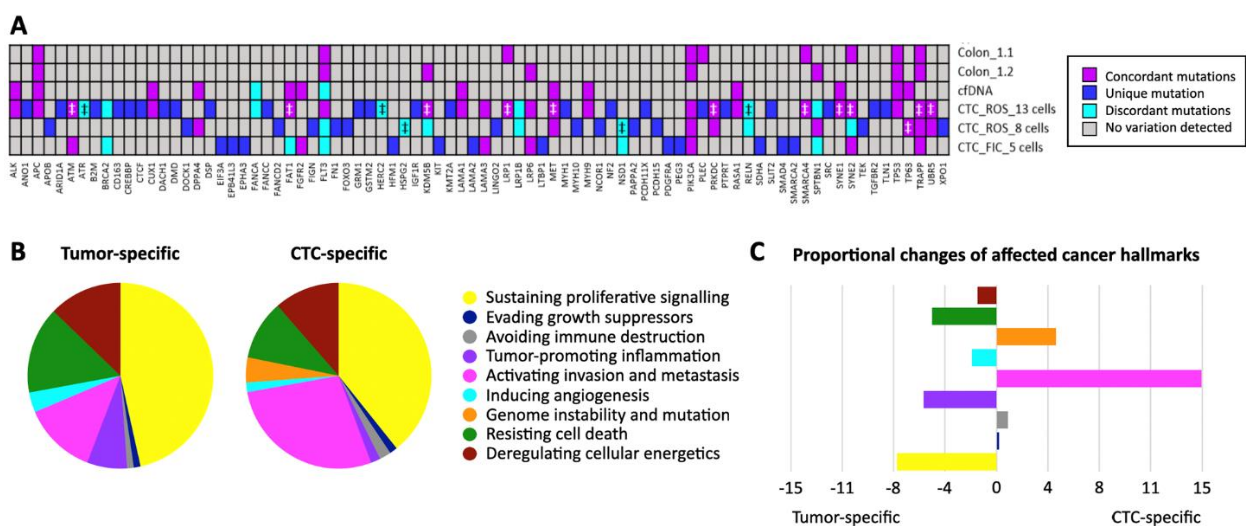


Figure 6: Shared and private mutations of tumor tissue and CTCs might be associated with the requirements for tumor growth and dissemination to distant organs: (A) Distinct mutation profiles of corresponding cfDNA, tissue and multiple CTC samples from patient CRC002.1 were assigned to cancer hallmarks, (B/C) demonstrating proportional changes in affected cancer-related pathways. Whereas mutations private to CTCs were more frequently associated with invasion and avoidance of immune destruction, shared mutations with tumor tissue were more frequently in-

volved in pathways of proliferative signalling, tumor-promoting inflammation and induction of angiogenesis. FIC: Ficoll-enriched CTCs, ROS: RosetteSep™-enriched CTCs. From Liebs et al. (14) Copyright © 2021 The Authors (Reproduced with permission from Springer Nature)

3.6 Detection of subclonal resistance in cfDNA (published in Liebs et al., Oncogene (14))

In a melanoma patient refractory to immunotherapy and BRAF-MEK-inhibition (MEL003.1), subclonal resistance was indicated when analyzing multiple metastatic lesions and cfDNA (Figure 7). Whereas initial analysis of a cutaneous metastasis revealed a *BRAF* V600E mutation, presence of a secondary *NRAS* G13R mutation was detected in a second skin metastasis when the patient progressed under BRAF-MEK-inhibition. Liquid biopsy was collected when the patient presented with progressive disease during subsequent immunotherapy. CTCs were detected, however, DNA integrity was insufficient for further analysis. In contrast, NGS of cfDNA did not only display both tissue mutations, but also the emergence of an additional *NRAS* Q61R mutation. Using ddPCR, four metastatic lesions (resected between 2015 and 2017) and cfDNA (isolated in 2017) were analyzed for the presence of *BRAF* V600E, *NRAS* G13R and Q61R. All three mutations were verified in plasma. *BRAF* V600E was detected in all four metastases, whereas, consistent with WES results, *NRAS* G13R was only found in one subcutaneous lesion. In contrast, *NRAS* Q61R was detected in none of the tissue samples.

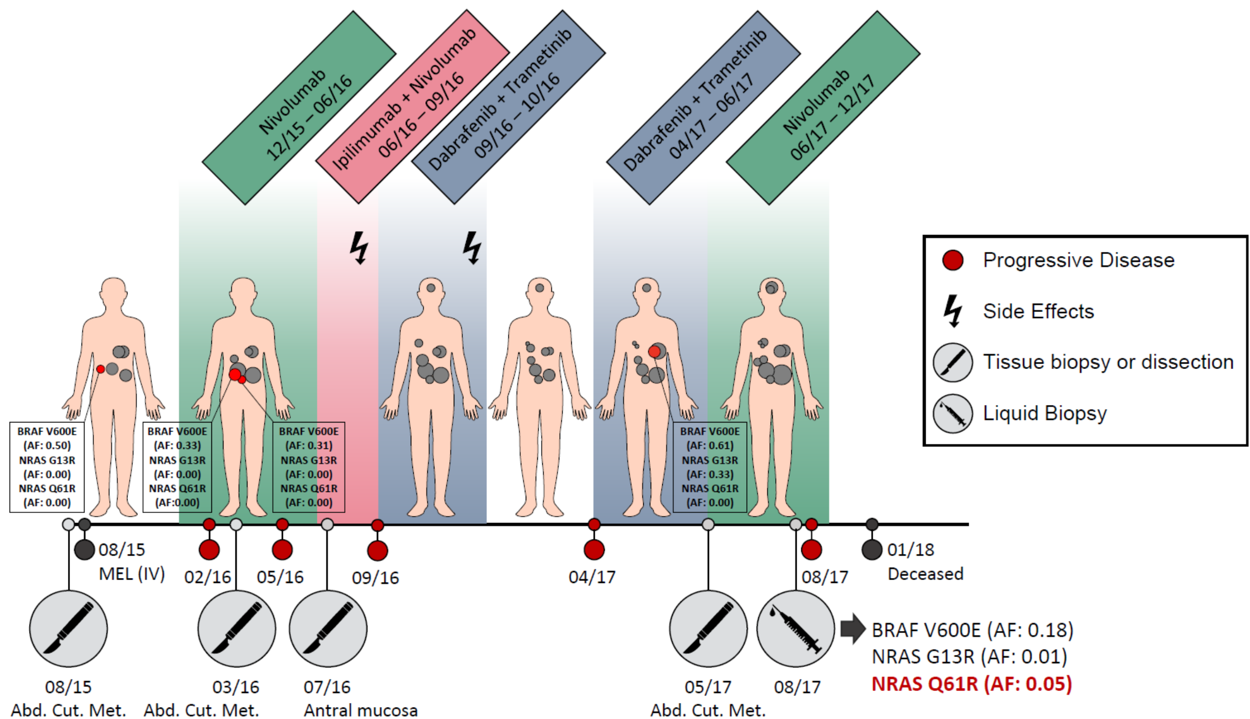


Figure 7: Schematic illustration of the clinical course of a melanoma patient refractory to immunotherapy and BRAF-MEK-inhibition: Tissue and plasma samples were collected (indicated by scalpel and syringe, respectively) and analysed for the presence of *BRAF* and *NRAS* mutations using NGS and ddPCR, demonstrating the emergence of potentially resistance-associated *NRAS* mutations (G13R and Q61R) at different time points during treatment. AF: Allele frequency. From Liebs et al. (14) Copyright © 2021 The Authors (Reproduced with permission from Springer Nature)

4. Discussion

4.1 Short summary of results

The presented studies clearly emphasized what a promising disease surveillance tool liquid biopsy may be to guide cancer management. Analysis of cfDNA from the Onco-Track cohort highlighted the limited utility of cfDNA analysis for early cancer detection, but its great potential for cancer monitoring in CRC patients with metastatic disease. The case report of a patient with synchronous primary cancers displayed the ability of liquid biopsy to identify the predominant tumor burden. Comparative analysis of CTCs and cfDNA proved superiority of cfDNA-based tissue mutation detection compared to CTCs in CRC, MEL and HNSCC, but also underlined the advantages of CTC analysis for the investigation of tumor heterogeneity. In one MEL patient, emergence of a mutation possibly mediating resistance to the given treatment was identified in cfDNA but not in available tissue samples.

All of these examples indicated various advantages of LB, a real-time tool that is suggested to provide a more comprehensive cross-section of the complex clonal divergence of single and coexisting lesions compared to individual tissue profiling. Nevertheless, the potential and possible pitfalls of this methodology have to be critically evaluated and validated before LB will be implemented into clinical routine. Thus, based on the findings of our publications, advantages and disadvantages of LB will be discussed in the following sections, furthermore, including additional aspects from the current literature.

4.2 Interpretation and embedding of results into the current state of research

In Liebs et al. (Cancer Medicine (13)), 11 of 35 (31%) tissue mutations were retrieved in plasma samples, including only one of 17 (6%) mutations in stage I-III and 10 of 18 (56%) mutations in stage IV CRC patients. Low concordance between tumor tissue and cfDNA might not only be affected by biological factors such as cancer stage and tumor burden, but also by pre-analytical and analytical factors as reported by Guo et al (24). In our study, sample age as well as the utilized cfDNA isolation kit might have limited assay performance (kit compatibility with anticoagulant in blood collection tube had to be taken into account, although a different kit was reported to result in superior cfDNA quantities (25)).

Additionally, Beije et al reported that different analysis methods might vary in their sensitivity and specificity of mutation detection (26)). Taken all of this into account, the detection of cancer-related aberrations (up to 10 - 1000 mutant copies per 5 ml plasma (27)) in front of the background of wild-type signals originating from non-cancerous cells requires highly sensitive and specific detection methods as well as standard operating protocols for plasma handling to guarantee successful detection of circulating tumor DNA even at low allele frequencies. Thus, in the last decade and still ongoing, many research groups tested different techniques for the exploration of cfDNA to identify the optimal setting for reliable mutation detection in concordance with the underlying disease.

In our study, the analysis of plasma samples from 53 CRC patients with known *BRAF* and *KRAS* gene status resulted in only one discordant finding between tissue and plasma. A patient with a *KRAS* G12C mutation in the stage II CRC tumor displayed a *KRAS* G12D mutation in plasma, which instead displayed the status of the synchronous stage IV pancreatic cancer. Although no serial LB sampling was performed since the patient died before systemic treatment was started, our case report demonstrated the utility of cfDNA to identify and track the predominant cancer over time (23), which was consistent with observations from other groups. Lakis et al reported different cancer driver mutations of different allele frequencies in *EGFR* and *KRAS* in synchronous pulmonary lesions, of which the *KRAS*-mutated lesion did not response to targeted therapy resulting in cancer progression and subsequent treatment failure (28). The identification of the unresponsive cancer histology via the detection of an increase of circulating melanoma cells, whereas epithelial CTCs originating from the synchronous CRC decreased under chemotherapy, was published by Fusi et al (29). However, in this context, it is also important to emphasize that prerequisites for disease monitoring via LB include the existence and stability of disease markers over time and a sufficient release of CTCs and/or cfDNA into the periphery to detect those traces in blood samples.

Another crucial variable to be investigated with regard to applicability of LB is if the diagnostic value of CTCs and cfDNA is affected by different tumor characteristics of certain cancer histologies. Clinical manifestation of metastasis in a secondary organ follows a cascade of stochastic events, which are affected by extrinsic and intrinsic factors, including anatomical site and circulation patterns, vascularization, the ability to cross physical barriers as well as the interaction with and modification of the host organ microenviron-

ment (27, 30). Consequently, different organ-specific patterns of metastasis were observed in multiple tumor entities, including predominant locoregional progress of HNSCC, sequential organ-specific metastasis of CRC in liver and then lung, and the ability of MEL to colonize many different distant organ sites. In our study, no significant difference in the informative value of CTCs and cfDNA between those three entities was demonstrated (Liebs et al, Oncogene (14)). However, this observation requires further validation in a bigger patient cohort to prove LB as a biomarker independent of the underlying tumor entity.

Superiority of cfDNA over CTC analysis was already suggested in previous studies (31, 32), however, the biology of CTC and cfDNA release into the bloodstream and therefore the potential informative value might be different. Whereas cfDNA is released by apoptotic cells rather reflecting the overall profile of cancer cells, a subpopulation of CTCs depicts cells which escape treatment and patient`s immune response, eventually forming new tumor lesions. Thus, the applicability of cfDNA and CTCs to represent the mutation tissue profile was compared (Liebs et al, Oncogene (14)), demonstrating that cfDNA outperformed CTC analysis in terms of concordance with corresponding tissue. Besides the already outlined limitations of cfDNA analysis, also CTC detection and characterization are still restricted in multiple manners. Different CTC phenotypes (mesenchymal, epithelial, hybrids) express distinct subsets of proteins on their surfaces, which have to be considered when establishing an antibody panel for the detection of the entire set of existing CTCs per blood sample. This is especially necessary due to the low frequency and quantity of CTCs (33, 34), which is a major limitation for their implementation into clinical practice. CTCs have to be enriched before isolation and whole genome amplification prior to mutation detection is prone for technical defects including allelic imbalance and/or dropout caused by insufficient DNA integrity in isolated single cells. Furthermore, only 0.01% of CTCs have the capacity to form metastases at distant sites. Nevertheless, CTC analysis yields multiple benefits including the possible identification of new therapeutically targetable signatures and the investigation of tumor heterogeneity to assess possible resistance mechanisms. The latter was also demonstrated in our study, showing that consistent with other publications (35-37) the majority of CTC mutations (79%) were private. Interestingly, unique CTC mutations were associated with the cancer hallmarks „activating invasion and metastasis" as well as „avoiding immune destruction“ (38). In contrast,

shared mutations by tumor tissue and CTCs were assigned to pathways such as „induction of angiogenesis", „deregulation of cellular energetics" and „sustainability of proliferative signaling" being in line with basic conditions of progressing tumor lesions. Besides the other outstanding case in our cohort, in which a possible resistance-associated mutation was detected in cfDNA but not in available tissue, did multiple other studies even predict therapy resistance by the emergence of mutations in blood before relapse clinically demonstrated (32, 39-41).

4.3 Implications for practice and future research

All three studies as well as findings from other research groups proposed manifold capabilities of disease surveillance via liquid biopsies. Despite the high need for informative biomarkers in precision oncology, validity and utility of liquid biopsy for clinical routine are still subject to debate. One explanatory variable for this is that at current state, multiplicity of liquid biopsy approaches for the isolation and analysis of cfDNA and CTCs hampered agreement of the clinical and scientific community on the clinical value of LB, due to the absence of robust and consistent results verified in large comparative studies. Furthermore, in many cases, abundance of gene alterations in liquid biopsy can jeopardize clinical benefit, when targets of therapeutic relevance are lacking and knowledge about interpretation and translation into clinic is low. In order to resolve those issues, multiple aspects should be considered in the future application of liquid biopsies: Procedures of sample processing and profiling have to be standardized and verified in bigger patient cohorts than is presently the case to allow highest possible sample quality for the detection of mutations even of low allele frequencies. Associated with that, assay performance including sensitivity and specificity have to be further optimized. Especially for clinical use, cancer panels targeting mutations of clinical relevance have to be implemented and applied to longitudinal liquid biopsy samples to allow early detection of therapy-induced emergence of resistant subclones or changes in mutation allele frequencies as an indicator of treatment response. With regard to unknown variants and their involvement in cancer progression, more detailed research has to be performed in close cooperation between laboratories analyzing LB samples and those performing more basic research on aberrated cancer pathways. For this, a database, such as COSMIC, collecting presumably irrelevant alterations from LB samples might expose more abundant variants correlated with cancer progression. Thus, further research on the identification of new

targetable signaling pathways of tumor cells has to be performed followed by the establishment of novel biomarkers and inhibitors. Additionally, simultaneous analysis of CTCs, cfDNA and even further blood components such as exosomes should be taken into consideration to increase the sensitivity of tissue profiling via LB by covering the diverse footprints of those complementary disease markers.

5. Conclusions

At current state and apart from a few clinical applications (42, 43), LB is of experimental value only and not accepted as an alternative to standard tissue profiling. However, LB should not only be an option for patients from which serial invasive sampling would be contraindicated or no sufficient DNA quantities are available for mutation profiling. Instead, LB should rather be recognized as a relevant complementary biomarker to tumor tissue analysis, since from a clinical perspective, assistance to track changes in the highly dynamic clonal composition of cancers is urgently needed to predict treatment outcome and identify patients who are at risk for relapse. Especially, serial liquid biopsies are most promising to predict treatment failure before clinical recurrence, allowing dynamic patient monitoring that will potentially precede image-based detection of clinical progress and identify potential targets for medical intervention.

Reference list

1. Khan KH, Cunningham D, Werner B, Vlachogiannis G, Spiteri I, Heide T, Mateos JF, Vatsiou A, Lampis A, Damavandi MD, Lote H, Huntingford IS, Hedayat S, Chau I, Tunariu N, Mentrasti G, Trevisani F, Rao S, Anandappa G, Watkins D, Starling N, Thomas J, Peckitt C, Khan N, Rugge M, Begum R, Hezelova B, Bryant A, Jones T, Proszek P, Fassan M, Hahne JC, Hubank M, Braconi C, Sottoriva A, Valeri N. Longitudinal Liquid Biopsy and Mathematical Modeling of Clonal Evolution Forecast Time to Treatment Failure in the PROSPECT-C Phase II Colorectal Cancer Clinical Trial. Cancer discovery. 2018;8(10):1270-85.
2. Tremblay PL, Huot J, Auger FA. Mechanisms by which E-selectin regulates diapedesis of colon cancer cells under flow conditions. Cancer Res. 2008;68(13):5167-76.
3. Cohen SJ, Punt CJ, Iannotti N, Saidman BH, Sabbath KD, Gabrail NY, Picus J, Morse M, Mitchell E, Miller MC, Doyle GV, Tissing H, Terstappen LW, Meropol NJ. Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2008;26(19):3213-21.
4. Cristofanilli M, Budd G, Ellis M, Stopeck A, Matera J, Miller M, Reuben J, Doyle G, Allard W, Terstappen L, Hayes D. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med. 2004;351:781 - 91.
5. de Bono JS, Scher HI, Montgomery RB, Parker C, Miller MC, Tissing H, Doyle GV, Terstappen LW, Pienta KJ, Raghavan D. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008;14(19):6302-9.
6. Alvarez Cubero MJ, Lorente JA, Robles-Fernandez I, Rodriguez-Martinez A, Puche JL, Serrano MJ. Circulating Tumor Cells: Markers and Methodologies for Enrichment and Detection. Methods in molecular biology (Clifton, NJ). 2017;1634:283-303.
7. Bäumer C, Fisch E, Wedler H, Reinecke F, Korfhage C. Exploring DNA quality of single cells for genome analysis with simultaneous whole-genome amplification. Scientific reports. 2018;8(1):7476.
8. Diaz LA, Jr., Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2014;32(6):579-86.
9. Elazezy M, Joosse SA. Techniques of using circulating tumor DNA as a liquid biopsy component in cancer management. Comput Struct Biotechnol J. 2018;16:370-8.
10. Ou SI, Nagasaka M, Zhu VW. Liquid Biopsy to Identify Actionable Genomic Alterations. Am Soc Clin Oncol Educ Book. 2018;38:978-97.
11. Kang Q, Henry NL, Paoletti C, Jiang H, Vats P, Chinnaiyan AM, Hayes DF, Merajver SD, Rae JM, Tewari M. Comparative analysis of circulating tumor DNA stability In K(3)EDTA, Streck, and CellSave blood collection tubes. Clinical biochemistry. 2016;49(18):1354-60.
12. Alidousty C, Brandes D, Heydt C, Wagener S, Wittersheim M, Schäfer SC, Holz B, Merkelbach-Bruse S, Büttner R, Fassunke J, Schultheis AM. Comparison of Blood Collection Tubes from Three Different Manufacturers for the Collection of Cell-Free DNA for Liquid Biopsy Mutation Testing. J Mol Diagn. 2017;19(5):801-4.

13. Liebs S, Keilholz U, Kehler I, Schweiger C, Haybäck J, Nonnenmacher A. Detection of mutations in circulating cell-free DNA in relation to disease stage in colorectal cancer. Cancer Med. 2019;8(8):3761-9.
14. Liebs S, Eder T, Klauschen F, Schütte M, Yaspo M-L, Keilholz U, Tinhofer I, Kidess-Sigal E, Braunholz D. Applicability of liquid biopsies to represent the mutational profile of tumor tissue from different cancer entities. Oncogene. 2021.
15. Warton K, Graham LJ, Yuwono N, Samimi G. Comparison of 4 commercial kits for the extraction of circulating DNA from plasma. Cancer Genet. 2018;228-229:143-50.
16. Underhill HR, Kitzman JO, Hellwig S, Welker NC, Daza R, Baker DN, Gligorich KM, Rostomily RC, Bronner MP, Shendure J. Fragment Length of Circulating Tumor DNA. PLoS Genet. 2016;12(7):e1006162.
17. Zhang B. CD73 promotes tumor growth and metastasis. Oncoimmunology. 2012;1(1):67-70.
18. Eder T, Hess AK, Konschak R, Stromberger C, Jöhrens K, Fleischer V, Hummel M, Balermpas P, von der Grün J, Linge A, Lohaus F, Krause M, Baumann M, Stuschke M, Zips D, Grosu AL, Abdollahi A, Debus J, Belka C, Pigorsch S, Combs SE, Budach V, Tinhofer I. Interference of tumour mutational burden with outcome of patients with head and neck cancer treated with definitive chemoradiation: a multicentre retrospective study of the German Cancer Consortium Radiation Oncology Group. Eur J Cancer. 2019;116:67-76.
19. Leyvraz S, Konietschke F, Peuker C, Schütte M, Kessler T, Ochsenreither S, Ditzhaus M, Sprünken ED, Dörpholz G, Lamping M, Rieke DT, Klinghammer K, Burock S, Ulrich C, Poch G, Schäfer R, Klauschen F, Joussen A, Yaspo ML, Keilholz U. Biomarker-driven therapies for metastatic uveal melanoma: A prospective precision oncology feasibility study. Eur J Cancer. 2022;169:146-55.
20. Tate JG, Bamford S, Jubb HC, Sondka Z, Beare DM, Bindal N, Boutselakis H, Cole CG, Creatore C, Dawson E, Fish P, Harsha B, Hathaway C, Jupe SC, Kok CY, Noble K, Ponting L, Ramshaw CC, Rye CE, Speedy HE, Stefancsik R, Thompson SL, Wang S, Ward S, Campbell PJ, Forbes SA. COSMIC: the Catalogue Of Somatic Mutations In Cancer. Nucleic acids research. 2019;47(D1):D941-d7.
21. Tamborero D, Rubio-Perez C, Deu-Pons J, Schroeder MP, Vivancos A, Rovira A, Tusquets I, Albanell J, Rodon J, Tabernero J, de Torres C, Dienstmann R, Gonzalez-Perez A, Lopez-Bigas N. Cancer Genome Interpreter annotates the biological and clinical relevance of tumor alterations. Genome medicine. 2018;10(1):25.
22. Schütte M, Risch T, Abdavi-Azar N, Boehnke K, Schumacher D, Keil M, Yildiriman R, Jandrasits C, Borodina T, Amstislavskiy V, Worth CL, Schweiger C, Liebs S, Lange M, Warnatz HJ, Butcher LM, Barrett JE, Sultan M, Wierling C, Golob-Schwarzl N, Lax S, Uranitsch S, Becker M, Welte Y, Regan JL, Silvestrov M, Kehler I, Fusi A, Kessler T, Herwig R, Landegren U, Wienke D, Nilsson M, Velasco JA, Garin-Chesa P, Reinhard C, Beck S, Schäfer R, Regenbrecht CR, Henderson D, Lange B, Haybaeck J, Keilholz U, Hoffmann J, Lehrach H, Yaspo ML. Molecular dissection of colorectal cancer in pre-clinical models identifies biomarkers predicting sensitivity to EGFR inhibitors. Nat Commun. 2017;8:14262.
23. Liebs S, Nonnenmacher A, Klauschen F, Keilholz U, Vecchione L. Liquid biopsy assessment of synchronous malignancies: a case report and review of the literature. ESMO Open. 2019;4(4):e000528.
24. Guo Q, Wang J, Xiao J, Wang L, Hu X, Yu W, Song G, Lou J, Chen J. Heterogeneous mutation pattern in tumor tissue and circulating tumor DNA warrants parallel NGS panel testing. Mol Cancer. 2018;17(1):131.

25. Devonshire AS, Whale AS, Gutteridge A, Jones G, Cowen S, Foy CA, Huggett JF. Towards standardisation of cell-free DNA measurement in plasma: controls for extraction efficiency, fragment size bias and quantification. Anal Bioanal Chem. 2014;406(26):6499-512.
26. Beije N, Helmijr JC, Weerts MJA, Beaufort CM, Wiggin M, Marziali A, Verhoef C, Sleijfer S, Jansen M, Martens JWM. Somatic mutation detection using various targeted detection assays in paired samples of circulating tumor DNA, primary tumor and metastases from patients undergoing resection of colorectal liver metastases. Molecular oncology. 2016;10(10):1575-84.
27. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, Bartlett BR, Wang H, Luber B, Alani RM, Antonarakis ES, Azad NS, Bardelli A, Brem H, Cameron JL, Lee CC, Fecher LA, Gallia GL, Gibbs P, Le D, Giuntoli RL, Goggins M, Hogarty MD, Holdhoff M, Hong SM, Jiao Y, Juhl HH, Kim JJ, Siravegna G, Laheru DA, Lauricella C, Lim M, Lipson EJ, Marie SK, Netto GJ, Oliner KS, Olivi A, Olsson L, Riggins GJ, Sartore-Bianchi A, Schmidt K, Shih I M, Oba-Shinjo SM, Siena S, Theodorescu D, Tie J, Harkins TT, Veronese S, Wang TL, Weingart JD, Wolfgang CL, Wood LD, Xing D, Hruban RH, Wu J, Allen PJ, Schmidt CM, Choti MA, Velculescu VE, Kinzler KW, Vogelstein B, Papadopoulos N, Diaz LA, Jr. Detection of circulating tumor DNA in early- and late-stage human malignancies. Science translational medicine. 2014;6(224):224ra24.
28. Lakis S, Heukamp LC, Griesinger F. Detection of Discrepant Driver Mutations in a Patient with Two Synchronous Primary Non-Small Cell Lung Cancers (NSCLCs) with Liquid Biopsy. J Thorac Oncol. 2017;12(11):e186-e8.
29. Fusi A, Liu Z, Schmittel A, Tinhofer I, Trefzer U, Keilholz U. Monitoring of circulating tumor cells in a patient with synchronous metastatic melanoma and colon carcinoma. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2010;21(8):1734-5.
30. Haber DA, Velculescu VE. Blood-based analyses of cancer: circulating tumor cells and circulating tumor DNA. Cancer discovery. 2014;4(6):650-61.
31. Freidin MB, Freydina DV, Leung M, Montero Fernandez A, Nicholson AG, Lim E. Circulating tumor DNA outperforms circulating tumor cells for KRAS mutation detection in thoracic malignancies. Clinical chemistry. 2015;61(10):1299-304.
32. Dawson SJ, Tsui DW, Murtaza M, Biggs H, Rueda OM, Chin SF, Dunning MJ, Gale D, Forshew T, Mahler-Araujo B, Rajan S, Humphray S, Becq J, Halsall D, Wallis M, Bentley D, Caldas C, Rosenfeld N. Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med. 2013;368(13):1199-209.
33. Alama A, Truini A, Coco S, Genova C, Grossi F. Prognostic and predictive relevance of circulating tumor cells in patients with non-small-cell lung cancer. Drug Discov Today. 2014;19(10):1671-6.
34. Miller MC, Doyle GV, Terstappen LW. Significance of Circulating Tumor Cells Detected by the CellSearch System in Patients with Metastatic Breast Colorectal and Prostate Cancer. J Oncol. 2010;2010:617421.
35. Pestrin M, Salvianti F, Galardi F, De Luca F, Turner N, Malorni L, Pazzagli M, Di Leo A, Pinzani P. Heterogeneity of PIK3CA mutational status at the single cell level in circulating tumor cells from metastatic breast cancer patients. Molecular oncology. 2015;9(4):749-57.
36. Pailler E, Auger N, Lindsay CR, Vielh P, Islas-Morris-Hernandez A, Borget I, Ngo-Camus M, Planchard D, Soria JC, Besse B, Farace F. High level of chromosomal instability in circulating tumor cells of ROS1-rearranged non-small-cell lung cancer.

Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2015;26(7):1408-15.
37. De Luca F, Rotunno G, Salvianti F, Galardi F, Pestrin M, Gabellini S, Simi L, Mancini I, Vannucchi AM, Pazzagli M, Di Leo A, Pinzani P. Mutational analysis of single circulating tumor cells by next generation sequencing in metastatic breast cancer. Oncotarget. 2016;7(18):26107-19.
38. Hanahan D, Weinberg Robert A. Hallmarks of Cancer: The Next Generation. Cell. 2011;144(5):646-74.
39. Yamada T, Matsuda A, Takahashi G, Iwai T, Takeda K, Ueda K, Kuriyama S, Koizumi M, Shinji S, Yokoyama Y, Ohta R, Yoshida H. Emerging RAS, BRAF, and EGFR mutations in cell-free DNA of metastatic colorectal patients are associated with both primary and secondary resistance to first-line anti-EGFR therapy. Int J Clin Oncol. 2020;25(8):1523-32.
40. Almodovar K, Iams WT, Meador CB, Zhao Z, York S, Horn L, Yan Y, Hernandez J, Chen H, Shyr Y, Lim LP, Raymond CK, Lovly CM. Longitudinal Cell-Free DNA Analysis in Patients with Small Cell Lung Cancer Reveals Dynamic Insights into Treatment Efficacy and Disease Relapse. J Thorac Oncol. 2018;13(1):112-23.
41. Taus Á, Camacho L, Rocha P, Hernández A, Longarón R, Clavé S, Fernández-Ibarrondo L, Salido M, Hardy-Werbin M, Fernández-Rodríguez C, Albanell J, Bellosillo B, Arriola E. Plasmatic KRAS Kinetics for the Prediction of Treatment Response and Progression in Patients With KRAS-mutant Lung Adenocarcinoma. Arch Bronconeumol (Engl Ed). 2021;57(5):323-9.
42. Brown P. The Cobas® EGFR Mutation Test v2 assay. Future Oncol. 2016;12(4):451-2.
43. Warren JD, Xiong W, Bunker AM, Vaughn CP, Furtado LV, Roberts WL, Fang JC, Samowitz WS, Heichman KA. Septin 9 methylated DNA is a sensitive and specific blood test for colorectal cancer. BMC Med. 2011;9:133.

Statutory Declaration

"I, Sandra Liebs, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic "Liquid Biopsy – Blood-based biomarkers for the molecular analysis of cancer" / "Liquid Biopsy – Blut-basierte Biomarker zur molekularen Charakterisierung von Tumoren", independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; http://www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date Signature

Declaration of your own contribution to the publications

Sandra Liebs contributed the following to the below listed publications:

Publication 1: Liebs S, Keilholz U, Kehler I, Schweiger C, Haybäck J, Nonnenmacher A, Detection of mutations in circulating cell-free DNA in relation to disease stage in colorectal cancer. Cancer Med. 2019

Contribution:

- Study design and conception
- Contribution to the experimental work (DNA isolation from FFPE tissue)
- Supervision of findings, provision of scientific advice and analysis of pre-processed re-sults to draft the manuscript
- Design of Figures 1B and 2 as well as Table 1 and 2
- Editing of Figure 1A, Supplementary Figure 1 and Supplementary Table 1, 2 and 3
- Manuscript drafting, finalization and submission (S. Liebs is the corresponding author)

Publication 2: Liebs S, Eder T, Klauschen F, Schütte M, Yaspo ML, Keilholz U, Tinhofer I, Kidess-Sigal E, Braunholz D. Applicability of liquid biopsies to represent the mutational profile of tumor tissue from different cancer entities. Oncogene. 2021

Contribution:

- Sample collection including the plasma isolation from 7/18 patients, collection of germline samples from 9/18 patients, CTC enrichment from 18/18 patients
- Performance of the experimental work including CTC isolation and whole genome ampli-fication of 15/16 samples, quality control PCR of 16/16 CTC samples, isolation of cfDNA from 18/18 plasma samples, DNA isolation from 7/18 whole blood and 30/30 FFPE spec-imens and library preparation of 59/67 samples
- Analysis of the entire data set of sequencing results
- As follows, figures and tables were created based on
    - my performance of the QC-PCR and ddPCR (shown in Supplementary Figure 1 and Figure 6, respectively)
    - my analysis of CTC counts, DNA concentrations and the summary of the clinical data of enrolled patients (as summarized in Figure 1 and 6 and Supplementary ta-ble 1 and 2)
    - my analysis of the sequencing results resulting in the comparative analysis of tu-mor mutations displayed in Figure 2, 3, 4 and 5A
- Design of Figures 1, 2, 3, 4, 5A and 6, Supplementary Figure 1 and 2 as well as Supple-mentary Table 1, 2 and 3
- Editing of Figure 5B and 5C, which were designed based on my identification of muta-tion-affected pathways
- Manuscript drafting, finalization and submission (S. Liebs is the corresponding author)

Signature, date and stamp of first supervising university professor / lecturer

Signature of doctoral candidate

Excerpt from Journal Summary List "Oncology" 2017

The excerpt from the "Oncology" Journal Summary List of 2017 will be listed here, including the journal *Cancer Medicine* (position 110 of 222), to which the manuscript "Detection of mutations in circulating cell-free DNA in relation to disease stage in colorectal cancer" was submitted in March 2019.

Journal Data Filtered By: **Selected JCR Year: 2017** Selected Editions: SCIE,SSCI

Selected Categories: **"ONCOLOGY"** Selected Category Scheme: WoS

Gesamtanzahl: 222 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	CA-A CANCER JOURNAL FOR CLINICIANS	28,839	244.585	0.066030
2	NATURE REVIEWS CANCER	50,407	42.784	0.079730
3	LANCET ONCOLOGY	44,961	36.418	0.136440
4	JOURNAL OF CLINICAL ONCOLOGY	156,474	26.303	0.285130
5	Nature Reviews Clinical Oncology	8,354	24.653	0.026110
6	Cancer Discovery	11,896	24.373	0.065350
7	CANCER CELL	35,217	22.844	0.096910
8	JAMA Oncology	5,707	20.871	0.027770
9	ANNALS OF ONCOLOGY	38,738	13.926	0.095780
10	JNCI-Journal of the National Cancer Institute	37,933	11.238	0.052550
11	Journal of Thoracic Oncology	15,010	10.336	0.033280
12	CLINICAL CANCER RESEARCH	81,859	10.199	0.132210
13	SEMINARS IN CANCER BIOLOGY	6,330	10.198	0.010740
14	LEUKEMIA	25,265	10.023	0.059580
15	NEURO-ONCOLOGY	10,930	9.384	0.030350
16	Cancer Immunology Research	4,361	9.188	0.021180
17	CANCER RESEARCH	139,291	9.130	0.130190

18	Journal for ImmunoTherapy of Cancer	1,675	8.374	0.007130
19	BIOCHIMICA ET BIOPHYSICA ACTA-REVIEWS ON CANCER	5,276	8.220	0.009300
20	Blood Cancer Journal	1,804	8.125	0.007660
21	CANCER TREATMENT REVIEWS	7,870	8.122	0.015820
22	Molecular Cancer	10,301	7.776	0.017280
23	INTERNATIONAL JOURNAL OF CANCER	51,800	7.360	0.071870
24	Journal of Hematology & Oncology	4,098	7.333	0.009750
25	EUROPEAN JOURNAL OF CANCER	29,883	7.191	0.050170
26	ONCOGENE	66,411	6.854	0.075960
27	CANCER	68,221	6.537	0.074740
28	CANCER LETTERS	29,311	6.491	0.042280
29	Journal of the National Comprehensive Cancer Network	5,143	6.471	0.017530
30	Advances in Cancer Research	2,343	6.422	0.003690
31	JOURNAL OF PATHOLOGY	16,156	6.253	0.024060
32	Therapeutic Advances in Medical Oncology	1,020	6.238	0.002650
33	JOURNAL OF EXPERIMENTAL & CLINICAL CANCER RESEARCH	5,661	6.217	0.008740
34	BREAST CANCER RESEARCH	11,022	6.142	0.020000
35	Pigment Cell & Melanoma Research	4,430	6.115	0.007840
36	Clinical Epigenetics	2,172	6.091	0.007720
37	CANCER AND METASTASIS REVIEWS	6,106	6.081	0.006870
38	BRITISH JOURNAL OF CANCER	46,723	5.922	0.065130
39	STEM CELLS	21,694	5.587	0.035680
40	INTERNATIONAL JOURNAL OF RADIATION ONCOLOGY BIOLOGY PHYSICS	46,595	5.554	0.055060
41	OncoImmunology	5,963	5.503	0.020500
42	MOLECULAR CANCER THERAPEUTICS	19,211	5.365	0.031690
43	ENDOCRINE-RELATED CANCER	7,114	5.331	0.012410

44	Cancers	3,897	5.326	0.008990
45	ONCOLOGIST	11,433	5.306	0.020480
46	Molecular Oncology	4,529	5.264	0.013160
47	CARCINOGENESIS	21,776	5.072	0.021960
48	Gastric Cancer	4,290	5.045	0.006460
49	NEOPLASIA	6,801	4.994	0.008860
50	SEMINARS IN ONCOLOGY	5,409	4.942	0.007270
52	CELLULAR ONCOLOGY	1,322	4.761	0.002020
53	Oncogenesis	1,348	4.722	0.004480
54	ORAL ONCOLOGY	8,949	4.636	0.013760
55	Cancer Biology & Medicine	816	4.607	0.002330
56	MOLECULAR CANCER RESEARCH	7,834	4.597	0.013490
57	JOURNAL OF ENVIRONMENTAL SCIENCE AND HEALTH PART C-ENVIRONMENTAL CARCINOGENESIS & ECOTOXICOLOGY REVIEWS	895	4.586	0.000810
58	CANCER EPIDEMIOLOGY BIOMARKERS & PREVENTION	19,976	4.554	0.029440
59	GYNECOLOGIC ONCOLOGY	23,652	4.540	0.034310
60	Journal of Oncology	1,573	4.528	0.002410
61	BONE MARROW TRANSPLANTATION	12,506	4.497	0.020810
62	CRITICAL REVIEWS IN ONCOLOGY HEMATOLOGY	6,956	4.495	0.012190
63	LUNG CANCER	11,340	4.486	0.019070
64	Frontiers in Oncology	6,599	4.416	0.024250
65	CANCER SCIENCE	11,994	4.372	0.016230
66	CANCER IMMUNOLOGY IMMUNOTHERAPY	7,509	4.225	0.012830
67	Clinical Lung Cancer	2,360	4.204	0.005450
68	PROSTATE CANCER AND PROSTATIC DISEASES	2,022	4.099	0.004890
69	CANCER GENE THERAPY	2,928	4.044	0.003610
70	SEMINARS IN RADIATION ONCOLOGY	2,480	4.027	0.003620

71	Cancer Prevention Research	5,348	4.021	0.011930
72	American Journal of Cancer Research	3,246	3.998	0.008250
73	Cancer Cell International	2,393	3.960	0.004960
74	Targeted Oncology	1,008	3.877	0.002560
75	CANCER CYTOPATHOLOGY	2,544	3.866	0.004380
76	Clinical Colorectal Cancer	1,264	3.861	0.002620
77	ANNALS OF SURGICAL ONCOLOGY	26,592	3.857	0.053440
78	MOLECULAR CARCINOGENESIS	5,244	3.851	0.007630
79	JOURNAL OF IMMUNOTHERAPY	3,093	3.826	0.004590
80	BIODRUGS	1,435	3.825	0.002460
81	Chinese Journal of Cancer	2,161	3.822	0.003960
82	Journal of Cancer Survivorship	2,225	3.713	0.007530
83	Cancer Management and Research	739	3.702	0.001970
84	Molecular Therapy-Oncolytics	254	3.690	0.000830
85	Chinese Journal of Cancer Research	1,128	3.689	0.002420
86	EJSO	7,996	3.688	0.014750
87	CURRENT OPINION IN ONCOLOGY	2,962	3.653	0.005630
88	BREAST CANCER RESEARCH AND TREATMENT	19,709	3.605	0.037840
89	CURRENT TREATMENT OPTIONS IN ON-COLOGY	1,242	3.562	0.002670
90	CANCER JOURNAL	2,899	3.519	0.005390
91	INVESTIGATIONAL NEW DRUGS	4,450	3.502	0.009350
92	Journal of Bone Oncology	280	3.500	0.000860
93	ACTA ONCOLOGICA	7,207	3.473	0.013060
94	CLINICAL & EXPERIMENTAL METASTASIS	3,506	3.455	0.004330
94	PSYCHO-ONCOLOGY	10,201	3.455	0.019830
96	INTERNATIONAL JOURNAL OF HYPERTHERMIA	3,350	3.440	0.004040
97	AMERICAN JOURNAL OF CLINICAL ONCOLO-GYCANCER CLINICAL TRIALS	4,247	3.424	0.005470
98	ONCOLOGY-NEW YORK	2,317	3.398	0.003800

99	UROLOGIC ONCOLOGY- SEMINARS AND ORIGINAL INVESTIGATIONS	4,787	3.397	0.013310
100	CANCER BIOLOGY & THERAPY	7,577	3.373	0.008280
101	GENES CHROMOSOMES & CANCER	5,116	3.362	0.006970
102	Journal of Geriatric Oncology	895	3.359	0.003320
103	Journal of Gynecologic Oncology	957	3.340	0.002260
104	INTERNATIONAL JOURNAL OF ONCOLOGY	15,493	3.333	0.022360
105	EXPERIMENTAL CELL RESEARCH	19,420	3.309	0.019610
106	BMC CANCER	24,272	3.288	0.053080
107	JOURNAL OF CANCER RESEARCH AND CLINICAL ONCOLOGY	7,401	3.282	0.010800
108	Journal of Cancer	2,710	3.249	0.006580
109	Cancer Research and Treatment	1,873	3.230	0.004340
110	Cancer Medicine	3,123	3.202	0.011220
111	HEMATOLOGICAL ONCOLOGY	1,007	3.193	0.002060
112	Surgical Oncology Clinics of North America	1,139	3.178	0.002150
113	ONCOLOGY RESEARCH	1,573	3.143	0.001570
114	World Journal of Gastrointestinal Oncology	1,069	3.140	0.002520
115	MELANOMA RESEARCH	2,356	3.135	0.004620
116	Current Oncology Reports	1,650	3.122	0.003720
117	HEMATOLOGY-ONCOLOGY CLINICS OF NORTH AMERICA	2,277	3.098	0.004500
118	Translational Oncology	1,791	3.071	0.004510
119	American Journal of Translational Research	3,677	3.061	0.008470
120	JOURNAL OF NEURO-ONCOLOGY	10,858	3.060	0.017330
121	CLINICAL ONCOLOGY	3,372	3.055	0.005910
122	CANCER IMAGING	1,150	3.016	0.002250

Printing copy of the publication

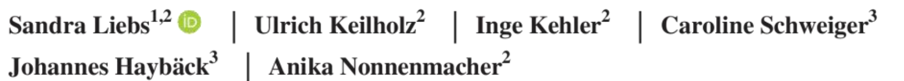
Received: 28 March 2019 | Revised: 18 April 2019 | Accepted: 20 April 2019

DOI: 10.1002/cam4.2219

ORIGINAL RESEARCH

Cancer Medicine Open Access WILEY

Detection of mutations in circulating cell-free DNA in relation to disease stage in colorectal cancer



Sandra Liebs[1,2] | Ulrich Keilholz[2] | Inge Kehler[2] | Caroline Schweiger[3] | Johannes Haybäck[3] | Anika Nonnenmacher[2]

[1]German Cancer Consortium (DKTK), German Cancer Research Center (DKFZ), Heidelberg, Germany

[2]Charité - Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Charité Comprehensive Cancer Center, Berlin, Germany

[3]Institute of Pathology, Medical University Graz, Graz, Austria

Correspondence
Sandra Liebs, Charité University Hospital Berlin, Charité Comprehensive Cancer Center, Charitéplatz 1/Hufelandweg 9, Berlin 10117, Germany.
Email: sandra.liebs@charite.de

Funding information
OncoTrack received research funding from the Innovative Medicines Initiative Joint Undertaking under grant agreement n° 115234 resources of which are composed of financial contribution from the European Union's Seventh Framework Programme (FP7/2007-2013) and EFPIA companies' in kind contribution. The funding period for the project ended on 31 December 2016. Our analysis was financed by the German Cancer Consortium (DKTK), which is funded as one of the National German Health Centers by the Federal German Ministry of Education and Research. Purchase of the DeNovix DS-11 FX+ (Biozym Scientific GmbH) used for concentration measurement of DNA isolated from tissue and plasma specimens was funded by the Sonnenfeld-Stiftung.

Abstract

Enthusiasm has emerged for the potential of liquid biopsies to provide easily accessible genetic biomarkers for early diagnosis and mutational cancer characterization. We here systematically investigated the suitability of circulating cell-free DNA (cfDNA) analysis for mutation detection in colorectal cancer (CRC) patients with respect to clinicopathological disease stage. Droplet Digital PCR (ddPCR) was performed to detect common point mutations in the *KRAS* and *BRAF* oncogenes in cfDNA from 65 patients and compared to mutations in tumor tissue. Stage of disease was classified according to UICC (Union for International Cancer Control) criteria. In tumor tissue, *KRAS* or *BRAF* mutations were present in 35 of 65 cases (44% UICC stage I, 50% stage II, 47% stage III, and 62% stage IV). Although cfDNA was detected in 100% of patients, ddPCR displayed the tumor tissue mutation in only 1 of 6 (17%) stage II patients, whereas 10 of 18 (56%) reported variants were verified in cfDNA samples of the stage IV cohort. No *BRAF* or *KRAS* mutation was detected in cfDNA from patients with wild-type tumor tissue. In one case of mutant stage II colon cancer (*KRAS*-G12C), the G12D variant was detected in cfDNA instead. Further workup revealed that circulating tumor-derived DNA and liver metastases originated from a synchronous *KRAS*-mutated cancer of the pancreas. Our results demonstrate that ddPCR-based analysis is highly specific and useful for mutation monitoring, but the sensitivity limits its usefulness for early cancer detection.

KEYWORDS
BRAF, circulating cell-free DNA, colorectal cancer, *KRAS*

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2019 The Authors. *Cancer Medicine* published by John Wiley & Sons Ltd.

1 | INTRODUCTION

Colorectal cancer (CRC) is the second most common cause of cancer death in Europe.[1] The 5-year survival rate of 92% in stage I cancer patients decreases to 12% in those present with distant metastasis, demonstrating the crucial need for early detection and treatment.[2] Up to 40% of CRC patients are unlikely to benefit from EGFR-targeted therapies, such as cetuximab and panitumumab, due to mutations in the *KRAS* oncogene.[3] Even early responders with *RAS* wild-type tumors develop secondary resistance under pressure of EGFR-directed treatments due to emerging tumor subclones.[4] Furthermore, 8%-15% of CRC patients with *KRAS* wild-type tumors harbor *BRAF* mutations, which have been proven to be an additional negative predictor of response to anti-EGFR treatment. Given that, patient management requires mutational monitoring of the disease as a basis for personalized medicine. In clinical practice, tissue biopsies are obtained for molecular profiling although a fragment of a single lesion might be inadequate to reflect intratumoral heterogeneity presented at low frequencies. Therefore, blood-based mutational profiling is suggested as a promising approach to provide a more comprehensive molecular profile of the disease in a minimally invasive manner. Liquid biopsy includes the analysis of tumor-derived biomarkers in any body fluid, such as plasma, urine, and cerebrospinal fluid. In particular, serial blood testing is proposed as a convenient real-time tool to identify spatial and temporal heterogeneity predicting response or resistance to targeted agents.[5]

Circulating cfDNA is composed of small nucleic acid fragments liberated from cells by rupture, necrosis or apoptosis originating from normal and deceased cells. Thus, circulating tumor-derived DNA (ctDNA) is only identified via the detection of cancer-related mutations. In correlation with tumor burden, mutant allele frequencies were reported to range between less than 10 and up to 1000 mutant copies per 5 mL plasma in stage I-IV cancer patients,[6] suggesting limitations in early stage cancer. We here systematically investigated the sensitivity and specificity of the analysis of somatic mutations in plasma samples from CRC patients in relation to disease stage. Since circulating tumor cells (CTC) can provide an alternative source of genetic information in liquid biopsies, the mutation detection in cfDNA was compared with the presence of CTCs.

2 | MATERIAL AND METHODS

2.1 | Patients

Patients with early and advanced CRC were included in the OncoTrack research project at the Charité and the Medical University Graz between 2010 and 2016.[7] Informed consent was obtained prior to blood and tissue specimen collection. The study was approved by the ethics committee of the Charité University Medicine (Charitéplatz 1, 10117 Berlin, Germany; EA 1/069/11). It was also approved and confirmed by the ethics commission of the Medical University of Graz (Auenbruggerplatz 2, 8036 Graz, Austria) and the ethics committee of the St John of God Hospital Graz (23-015 ex 10/11), respectively. Disease stage was classified according to the criteria of the Union for International Cancer Control (7th edition).[8]

2.2 | Cell lines

DNA isolated from human-derived cell lines with reported wild-type or mutation status in the oncogenes *KRAS* and *BRAF* was used to establish Droplet Digital PCR (ddPCR) assays (Table S1). All cell lines were cultured in media supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Waltham, USA) and 1% penicillin/streptomycin (Biochrom GmbH, Berlin, Germany) at 37°C and 5% CO_2. Prior to DNA isolation, cell lines were tested negative for mycoplasma using the Promokine PCR Mycoplasma Test KIT I/C following manufacturer`s specifications (PromoCell GmbH, Heidelberg, Germany). Cell line authenticity was validated by single nucleotide polymorphism profiling with Multiplexion GmbH (Friedrichshafen, Germany). Following manufacturer's instructions, the GeneJET Genomic DNA Purification kit (Thermo Scientific, Waltham, USA) was used to isolate DNA eluted in 100 µL double distilled water.

2.3 | Nucleic acid preparation from blood and tissue specimens

Prior to tumor resection, peripheral blood samples were collected in BD Vacutainer® PST™ II heparin tubes (BD, Franklin Lakes, USA) and directly processed by centrifugation for initial plasma storage at −80°C (1500 *g* for 10 minutes or 10 minutes at 800 *g* followed by 1600 *g* for 10 minutes). Furthermore, heparin blood from six healthy donors was centrifuged at 1811 *g* for 7 minutes followed by 3061 *g* for 10 minutes. In 2010, when patient recruitment started for the OncoTrack research project, the knowledge about stabilizing ctDNA in plasma samples was not as advanced as it is today. Most publications regarding the superior effect of EDTA and other blood collection tubes on preserving cfDNA and CTCs, while preventing hematopoietic cells from lysis, were published since 2016.[9,10] In 2004, Lam et al reported that EDTA is a superior anticoagulant compared to heparin, but only when blood processing was delayed, whereas comparable results regarding DNA concentrations were obtained when plasma was isolated within 6 hours after blood draw.[11] In our study, plasma was directly isolated after blood collection. Furthermore, at the

time of patient recruitment, internal analysis in our group demonstrated comparable DNA concentrations when using EDTA and heparin collection tubes, which, however, was not published. Based on this knowledge, we decided to use the stored plasma samples from the OncoTrack project for the analysis of cfDNA. A cfDNA assay system developed for heparin blood samples was employed. All plasma samples were centrifuged at 2000 *g* for 15 minutes prior to cfDNA isolation using the QIAamp DNA Blood Midi kit (Qiagen, Hilden, Germany). Briefly, 0.4-3.0 mL plasma was incubated with protease and Buffer AL for 10 minutes at 70°C, transferred to the QIAamp Midi column and washed with Buffer AW1 and AW2 at 4258 g for 1 minute and 15 minutes, respectively. Nucleic acid was eluted in 250 µL Ultra Pure water and further concentrated to 55 µL using Zymo`s DNA Clean & Concentrator®-5 kit according to the protocol specifications (Irvine, USA).

Within the large scale deep sequencing program of OncoTrack, whole genome and whole exome sequencing of tumor tissue specimens was performed, resulting in an accessible database of omics data.[7] In tissue samples not sequenced within the OncoTrack program, variant detection was performed using the same ddPCR assay as for cfDNA samples. Ten micrometer thick formalin-fixed paraffin-embedded (FFPE) tissue slides were deparaffinized and processed following the specifications of the High Pure FFPET DNA Isolation kit (Roche, Basel, Switzerland). The GeneJET Genomic DNA Purification kit (Thermo Scientific, Waltham, USA) was used to isolate DNA from fresh frozen tissue following the manufacturer`s instructions using the double amount of enzymatic solutions. Digestion of tumor tissue was performed for 2 hours at 56°C each. After purification, DNA from fresh frozen tissue was eluted in 150 µL double distilled water, whereas FFPE-derived DNA was eluted in 30 µL.

2.4 | DNA quantification and fragment analysis

DNA concentrations were quantified using the DeNovix DS-11 FX+ (Biozym Scientific GmbH, Hessisch Oldendorf, Germany). DNA isolated from fresh frozen tissue and cell lines was quantified via UV-Vis absorbance, whereas concentrations of FFPE-derived and circulating cell-free DNA (cfDNA) were determined using the Qubit® dsDNA HS Assay kit (Thermo Fisher Scientific). Additionally, fragment length of cfDNA was analyzed on the Agilent 2100 Bioanalyzer using the High Sensitivity DNA Kit (Agilent, Eugen, USA). To exclude cfDNA from normal cells of higher fragment size from tumor-derived DNA fragments, the correlation area under the curve in the region from 50-260 bp was determined to compare the resulting cfDNA concentrations (ng/mL) between patients of different tumor stages.

2.5 | Variant detection via ddPCR

Expecting low allele frequencies of mutant variants in cfDNA, the highly sensitive Droplet Digital™ PCR platform was used for mutation detection (Bio-Rad Laboratories GmbH, Munich, Germany). Our study design consisted of two parts: a first evaluation of 2-3 somatic mutations or the wild-type of the *KRAS* oncogene detected via a multiplex assay and a verification duplex PCR only detecting the wild-type or one of the mutations. Two *KRAS* multiplex assays were designed, one detected variants G12D/A or G13D (*KRAS* I multiplex) whereas the second assay detected G12V/C (*KRAS* II multiplex). Differentiation between mutations in multiplex assays was enabled by using different concentrations of FAM-labeled probes whereas the wild-type was detected with a HEX-labeled probe. Due to our main focus on the V600E variant of the *BRAF* gene, only a duplex PCR was used here for sample testing without further verification. Primers and probes were designed and tested for specificity using the Primer3, Primer-BLAST, and UCSC In-Silico PCR software.[12-14]

Each ddPCR reaction mixture was prepared using 3 µL DNA and 17 µL mastermix containing 2X ddPCR Supermix for Probes with no dUTP (Bio-Rad Laboratories GmbH, Munich, Germany), each primer at final concentrations of 900 nM and probe concentrations as listed in Table S2. Analyzing cell line-derived gDNA as control samples, EcoRI-HF (New England Biolabs) was further added to the reaction mix resulting in a final enzyme concentration of 0,5 units/µL. Droplets were generated using the QX200 Droplet generator, manually transferred to a 96-well PCR plate (Eppendorf, Hamburg, Germany) and heat-sealed with the PX1 Plate Sealer (Bio-Rad). PCR reactions were performed in the T-100 thermal cycler (Bio-Rad) with the following program: 1 cycle at 95°C for 10 minutes, 40 cycles at 94°C for 30 seconds and at 56°C or 59°C for 1 minute (*BRAF* or *KRAS* assays, respectively), and 1 cycle at 98°C for 10 minutes. Droplets were read in the QX200 Droplet Reader (Bio-Rad) and analyzed using the QuantaSoft software (version 1.7.4, Bio-Rad). Patient-derived samples were analyzed in duplicates. Each run included nontemplate controls to exclude the presence of contaminations. Cell line-derived gDNA harboring the mutations of interest were diluted in wild-type gDNA with a frequency of 1% to demonstrate successful target amplification within each run.

2.6 | Determination of assay performance and evaluation strategy of ddPCR results

False-positive rate (FPR) and limit of detection (LOD) were determined for multiplex and duplex assays. FPR was evaluated by determining the number of unspecific events in the mutation channel when analyzing nontemplate

controls and only wild-type cell line-derived DNA samples with many and few copies per microliter adjusted to expectant cfDNA levels (500 and 100 cpm, respectively). All assays demonstrated a FPR of 0 to 0.8 events, resulting in a defined cutoff value of one event. Mutant gDNA was diluted in constant wild-type gDNA (ranging from 10% to 0.001%), identifying a LOD of 0.01% for all established assays.

The evaluation strategy is depicted in Figure S1A. Briefly, only samples with ≥10.000 generated droplets were included into the final analysis. Two dimensional plots of gDNA samples derived from cell lines harboring the mutation of interest were used for first threshold setting, which was corrected if necessary, using the 1D plot. Outliers regarding high-fluorescence signals were excluded during quantification of positive events. Events in the wild-type and mutation channel were quantified and evaluated by being dispersed or overlapping with the positive controls in the 2D plot. Despite an FPR of one event in the multiplex set up, when analyzing the complete data set, three or more events in the multiplex PCR were proven to be positive in the validation duplex as well.

2.7 | Circulating tumor cell enrichment and quantification

Up to 50 mL of whole blood was collected in BD Vacutainer® heparin tubes for the enrichment and detection of circulating tumor cells. Between 8 and10 mL of whole blood was added to 40 mL of 1X Red blood cell lysis buffer (Stemcell Technologies, Vancouver, Canada) and incubated at room temperature for a maximum of 15 minutes. Remaining cells were washed with PBS (290 *g*, 5 minutes, 4°C) and resuspended in PBS containing 2% FCS and 2 mM EDTA to a concentration of $\leq 5 \times 10^7$ cells per milliliter for subsequent CD45 depletion using the EasySep™ Human CD45 Depletion kit (Stemcell Technologies). Incubation with the CD45-recognizing tetrameric antibody complex as well as the incubation with the magnetic particles was performed at 4°C for 15 minutes each. Labeled cells were separated using the EasySep™ magnet for 5 minutes at room temperature. The depleted cell fraction was washed and resuspended in 100 µL PBS prior to incubation with 10 µL FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) for 10 minutes at 4°C. To discriminate remaining leukocytes from tumor cells, an antibody against IgG1-AF555 (1 µL Life Technologies, Carlsbad, USA) recognizing the CD45 depletion cocktail as well as anti-EpCAM-AF488 (2 µL Biolegend, San Diego, USA) and anti-CEA-AF488 (2 µL Biolegend) were incubated for 20 minutes at 4°C. Additionally, 2 µL LIVE/DEAD™ Fixable Blue Dead Cell Stain for UV excitation (Life Technologies) was incubated for 10 minutes at 4°C to identify dead cells. Tumor cell quantification was performed using the DMI3000B fluorescence microscope (Leica, Wetzlar, Germany), whereby only living cells positive for EpCAM and/or CEA but negative for CD45 were identified as CTCs.

2.8 | Statistical analysis

Categorical variables were summarized by frequency and continuous variables by median and range. Assay performance was evaluated by the detection of reported *KRAS* and *BRAF* tissue mutations in cfDNA samples (sensitivity) and by confirming plasma samples determined as wild-type from the tissue analysis (specificity).

3 | RESULTS

3.1 | Characteristics of the patient cohort

From the OncoTrack cohort, 65 plasma samples collected prior to treatment and resection of tissue specimens were accessible for cfDNA isolation. Patients' median age was 67 years (range 36-92 years). Thirty-nine patients were male (60%) and 26 were female (40%). Ten patients (15%) had tumors with a *BRAF* V600E mutation and 25 patients (38%) had tumors with *KRAS* mutations in codon 12 or 13 (G12D/V/C or G13D). Patients with a reported *BRAF* mutation were presumed to harbor *KRAS* wild-type and vice versa, since coexistence of mutations in both oncogenes occurs with a probability of only 0.0001%.[15] A detailed overview of patients' clinicopathological characteristics was presented in Table 1.

3.2 | Quantitative analysis of cfDNA

Quantitative analysis of cfDNA samples demonstrated an increase in cfDNA concentrations with higher tumor burden varying from 59 ng/mL in healthy individuals to 156 ng/mL in patients with metastasized colon cancer (Figure 1A, 1). Correlating with increasing cfDNA level, ddPCR analysis detecting the *BRAF* and *KRAS* oncogenes resulted in higher events in the wild-type and mutation channel, which, however, did not correlate with successful tissue mutation retrieval in cfDNA samples (Figure 2). Highly concentrated cfDNA samples did not necessarily present circulating tumor DNA.

3.3 | Mutation status analysis from tumor tissue and plasma

Within our study cohort, *KRAS* or *BRAF* mutations were present in 35 of 65 (54%) tumor specimens. cfDNA was detected in 100% of patients independently of plasma volume or DNA concentration. No correlation between plasma volume and successful ctDNA detection was observed. Comparably low plasma volumes (≤0.5 mL) were available from only three patients harboring a tissue mutation; however, the cfDNA concentration

TABLE 1 Demographic and clinical characteristics of study participants

Characteristics	Total	Stage I	Stage II	Stage III	Stage IV
Number of patients	**N = 65**	**N = 9**	**N = 12**	**N = 15**	**N = 29**
Age at enrollment, years					
Median	67	67	69	70	63
Range	36-92	49-79	46-79	39-83	36-92
Sex, n (%)					
Male	39 (60%)	6 (67%)	7 (58%)	10 (67%)	16 (55%)
Female	26 (40%)	3 (33%)	5 (42%)	5 (33%)	13 (45%)
Tissue gene status, n (%)					
KRAS-MUT	25 (38%)	2 (22%)	2 (17%)	6 (40%)	15 (52%)
BRAF-MUT	10 (15%)	2 (22%)	4 (33%)	1 (7%)	3 (10%)
WT	18 (28%)	2 (22%)	4 (33%)	5 (33%)	7 (24%)
Unknown	12 (18%)	3 (33%)	2 (17%)	3 (20%)	4 (14%)
CTC detection rate, n (%)					
Performed CTC analysis	54 (83%)	7 (78%)	12 (100%)	13 (87%)	22 (76%)
Patients with CTCs	29 (54%)	4 (57%)	7 (58%)	8 (62%)	10 (45%)
Patients without CTCs	25 (46%)	3 (43%)	5 (42%)	5 (38%)	12 (55%)
CTC numbers					
Median	1	1	2	1	0
Range	0-8	0-4	0-8	0-6	0-5
Not available	11	2	0	2	7

KRAS-MUT includes the G12D, G12V, G12C and G13D variants, whereas *BRAF*-MUT refers to the V600E mutation.

Abbreviation: CTC, circulating tumor cells.

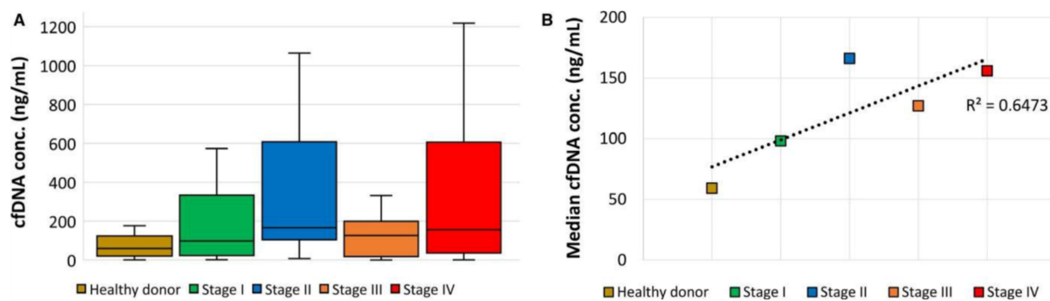


FIGURE 1 Cell-free DNA (cfDNA) concentrations und mutation detection in relation to disease stage. A, Quantitative analysis of cfDNA isolated from stage I-IV colon cancer patients in comparison to healthy individuals. Box plot showing median, first and third quartiles with whiskers from minimum to maximum. B, Median cfDNA levels demonstrate an increase with higher tumor burden

from only one of them was very limited (22.86 ng/mL) possibly explaining the absence of the tumor-derived mutation in plasma (Table S3). Mutational profiling of cfDNA verified CRC-related mutations in 11 of 35 (31%) corresponding plasma samples (Figure 2), including 2 of 10 (20%) *BRAF* and 9 of 25 (36%) *KRAS* mutations. Independently of tumor stage, mutant allele frequencies ranged between 0.01 and 0.52 (more than 50-fold) with mutations detected with 2 to 227 ddPCR

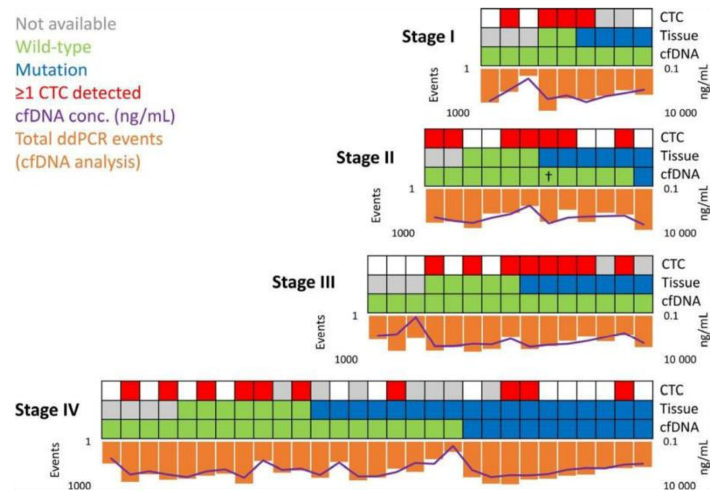


FIGURE 2 Retrieval of tissue-reported mutations in plasma in comparison to cell-free DNA (cfDNA) concentration and the detection of circulating tumor cells (CTCs). Corresponding to higher cfDNA levels, total Droplet Digital PCR detection events in the wild-type and mutation channel increased, which, however, did not correlate with successful retrieval of tissue-reported variants in plasma. CTCs were detected in blood samples from patients of all cancer stages, highlighting that the analysis of tumor-derived cells in the periphery will possibly complement the limited information received by cfDNA analysis. †The CRC-derived *KRAS* mutation (G12C) was not verified in plasma from patient 374-CB-M; however, the G12D variant originating from the synchronous stage IV cancer of the pancreas was detected

events (more than 100-fold). Individual data for each mutant cfDNA sample are shown in Figure S1B. No *BRAF* or *KRAS* mutation was detected in cfDNA from patients with wild-type tumor tissue. Thus, ddPCR assays showed 100% specificity throughout all stages with increasing accuracy in patients with higher tumor burden (Table 2). However, sensitivity was very limited with a maximum of 56% in stage IV patients. Only 1 of 17 (6%) CRC-derived gene variants was verified in all stage I-III patients. In stage II patient 249-CB-P, the *BRAF* mutation in the tumor was detected in the corresponding plasma sample with an allele frequency of 0.05 (27 mutation events). In comparison to cfDNA levels of the remaining stage II patients (median: 143.6 ng/mL), 249-CB-P demonstrated a strikingly higher concentration (1064.25 ng/mL), increasing the possibility of successful ctDNA detection.

3.4 | Discordance between colon tissue and cfDNA

There was one discrepancy in the *KRAS* gene status between the colon tumor tissue and cfDNA. Enrolled in the OncoTrack study with an adenocarcinoma of the colon and synchronous liver metastasis, the *KRAS* G12C variant detected in the primary tumor was not displayed in

TABLE 2 *BRAF* and *KRAS* gene status concordance between tumor tissue and cfDNA

	cfDNA analysis									
	Total (N = 53)		Stage I		Stage II		Stage III		Stage IV	
	MUT	WT	MUT	WT	MUT	WT	MUT	WT	MUT	WT
Tissue analysis										
MUT	11	24	0	4	1	5	0	7	10	8
WT	0	18	0	2	0	4	0	5	0	7
Sensitivity	31%		0%		17%		0%		56%	
Specificity	100%		100%		100%		100%		100%	
Accuracy	55%		33%		50%		42%		68%	

Abbreviation: cfDNA, cell-free DNA.

the corresponding cfDNA from patient 374-CB-M (Figure S1C, D). In contrast, the *KRAS* G12D mutation was found in plasma with an allele frequency of 0.1 (15 mutation events) as well as in the metastatic tissue. This indicated that both ctDNA and the metastasis were originated from the synchronous stage IV cancer of the pancreas, which was further verified pathologically.

3.5 | Circulating tumor cells

Blood samples for CTC quantification were available from 54 of 65 patients (83%), the tissue status of whom was known for 42 patients. CTCs were enriched from 50 mL whole blood and identified via fluorescence microscopy detecting EpCAM and/or CEA tumor marker expression. In 29 of 54 patients (54%), CTCs were successfully detected independently from tumor stage with a range of 1-8 CTCs per patient (Table 1). The detection of ctDNA was rather limited to patients with stage IV cancers, whereas circulating tumor-derived cells were detected even in patients with nonmetastasized CRC (Figure 2), emphasizing the differences between cfDNA and CTCs, making them not equivalent but complementary biomarkers for prognosis of the overall cancer disease for clinical management.

4 | DISCUSSION

One of the most desirable clinical applications of cfDNA analysis might be cancer diagnosis prior to metastatic spread, allowing early treatment to improve patients' survival. In recent years, different studies demonstrated the prognostic value of cfDNA in the breast, pancreatic, prostate, and CRC[16-19] further hypothesizing that its analysis might identify patients with localized tumors who are at risk of recurrence. Therefore, our study systematically investigated the utility of cfDNA to reflect molecular characteristics of the underlying disease with respect to tumor stage. Our assays have proven the highest precision with all variants detected in cfDNA being consistent with reported tissue status, except for one patient with stage II cancer of the right flexure of the colon. Here, cfDNA analysis revealed the *KRAS* mutation of the synchronous stage IV cancer of the pancreas. No *BRAF* or *KRAS* mutation was detected in cfDNA from patients with wild-type tumor tissue, resulting in 100% assay specificity among all four cancer stages. However, we observed a considerable difference in sensitivities regarding the retrieval of known mutations from tissue in cfDNA between patients of different tumor burden. No mutations were detected in cfDNA in stage I and stage III patients and only 1 of 6 mutations was verified in the stage II cohort. Highest accuracy (68%) was achieved in patients with distant metastases, demonstrating that cfDNA analysis in patients with noninvasive cancer is limited.

Beije et al concluded that performance of ctDNA detection assays varies, inter alia, according to the methods applied. When comparing various targeted detection assays in paired samples of cfDNA and tumor tissue from 12 mCRC patients, sensitivity was highest with digital PCR.[20] Here, 13 of 14 mutations (93%) observed in the primary tumor and/or the metastases were also detected in cfDNA. In contrast, next generation sequencing retrieved only a limited number of reported variants with a concordance between cfDNA and primary tumor and the metastasis of 39% and 55%, respectively. Guo et al used panel sequencing to detect tissue-matched mutations in cfDNA of 56 early-stage and advanced-stage patients with nonsmall cell lung cancer (NSCLC). They reported an overall concordance rate of 54.6% and 80%, respectively.[21] Of particular importance is their observation that the concordance rate can be strongly affected by multiple pre-analytical, analytical, and biological factors. Regarding that, we might explain the sporadic mutation detection in our patient cohort with limitations, such as sample age and inconsistent processing, storing, and delivery conditions at two different hospitals. Furthermore, due to blood being collected in heparin vacutainers, we used the QIAamp DNA Blood Midi kit for cfDNA isolation. In contrast to other isolation kits, such as the QIAamp Circulating Nucleic Acid kit, the QIAamp DNA Blood Midi kit is reported to be inferior regarding the isolation of short-fragmented ctDNA.[22] Considering that there is room for improvement in study design, different studies confirmed that ctDNA concentrations increase with tumor size and cancer stage.[23] This is consistent with the analysis of Bettegowda et al, who revealed a 47% sensitivity of *KRAS* mutation detection in cfDNA in stage I CRC patients, which increased to 87% in stage IV cancer.[6] Diehl et al reported that the number of mutant *APC* gene molecules in the circulation of CRC patients depends on tumor stage being as little as 0.01% in stage I patients.[24] Although the detection limit of our assays theoretically allowed for variant detection of an allelic frequency of 0.01%, the total amount of detected *KRAS* or *BRAF* molecules was so low in plasma samples of the stage I cohort that mutation detection would be below the FPR.

Taken together, we have confidence in the reliability when detecting a cancer-related mutation in plasma, however, the absence of detectable mutant molecules does not eliminate the occurrence of genomic alterations in blood possibly undiscovered due to low allelic frequency or technical limitations. Those challenges highlight the urgent need of standard operating protocols to guarantee optimal sample management regarding storage, processing and analysis of plasma samples. Furthermore, most studies complement their method of choice by enlarging their panel of cancer-related genes further including the detection of methylation patterns or circulating proteins, resulting in a more robust approach toward earlier cancer detection and disease monitoring.[25,26] In our case, we strongly recommend the use of CTCs and

cfDNA as complementary biomarkers as we successfully detected circulating tumor cells in 29 of 54 patients (54%) independently of tumor burden. Inter- and intratumoral heterogeneity remains a challenge in cancer treatment, emphasizing the importance of individualized therapy. Therefore, liquid biopsy comprising the analysis of CTCs and cfDNA as a complementary approach holds great potential for precision cancer medicine.

5 | CONCLUSION

In the last decade, administration of targeted therapies improved cancer patient management. Nevertheless, real-time detection of mechanisms of early and acquired resistance is still needed, requiring accurate biomarkers that can be applied in a minimally invasive manner. The analysis of cfDNA has proven to be convenient regarding sample preservation and processing. However, its analysis for early diagnosis and monitoring of patients with localized and advanced tumor is still of limited value, even though method sensitivities and specificities are constantly improving. Mutation detection in plasma was only sporadically successful in our stage I-III cohort, whereas only in patients with distant metastasis 68% concordance between tissue and cfDNA was demonstrated. Therefore, we hypothesize that a multi-marker approach, such as molecular profiling of cfDNA and CTCs, might be an alternative surrogate for tissue analysis to monitor an evolving genomic landscape of tumor cells and adapt treatment regimens accordingly.

ACKNOWLEDGMENTS

We thank the NGS technical team of the Department of Vertebrate Genomics/OWL Gene regulation and Systems Biology of Cancer and the Alacris Theranostics GmbH for the generation of the NGS data and Carl Steinbeisser for project management support.

DATA AVAILABILITY STATEMENT

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

ORCID

Sandra Liebs https://orcid.org/0000-0002-7099-0340

REFERENCES

1. Ferlay J, Colombet M, Soerjomataram I, et al. Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018. *Eur J Cancer*. 2018;103:356-387
2. American Cancer Society I.; Available from: https://www.cancer.org/cancer/colon-rectal-cancer/detection-diagnosis-staging/survival-rates.html.
3. My Cancer Genome. 2018. Available from: http://ww.mycancergenome.org.
4. Braig F, Marz M, Schieferdecker A, et al. Epidermal growth factor receptor mutation mediates cross-resistance to panitumumab and cetuximab in gastrointestinal cancer. *Oncotarget*. 2015;6(14):12035-12047.
5. Khan KH, Cunningham D, Werner B, et al. Longitudinal liquid biopsy and mathematical modeling of clonal evolution forecast time to treatment failure in the PROSPECT-C phase II colorectal cancer clinical trial. *Cancer Discov*. 2018;8(10):1270-1285.
6. Bettegowda C, Sausen M, Leary RJ, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med*. 2014;6(224): 224ra24.
7. Schutte M, Risch T, Abdavi-Azar N, et al. Molecular dissection of colorectal cancer in pre-clinical models identifies biomarkers predicting sensitivity to EGFR inhibitors. *Nat Commun*. 2017;8:14262.
8. Wittekind C, Meyer H-J. *TNM-Klassifikation maligner Tumoren. International Union against Cancer*. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA; 2010.
9. Kang Q, Henry NL, Paoletti C, et al. Comparative analysis of circulating tumor DNA stability In K3EDTA, Streck, and Cell Save blood collection tubes. *Clin Biochem*. 2016;49(18):1354-1360.
10. Alidousty C, Brandes D, Heydt C, et al. Comparison of blood collection tubes from three different manufacturers for the collection of cell-free DNA for liquid biopsy mutation testing. *J Mol Diagn*. 2017;19(5):801-804.
11. Lam NY, Rainer TH, Chiu RW, Lo YM. EDTA is a better anticoagulant than heparin or citrate for delayed blood processing for plasma DNA analysis. *Clin Chem*. 2004;50(1):256-257.
12. Information N.C.f.B. 2018. Available from: https://www.ncbi.nlm.nih.gov/tools/primer-blast/.
13. Untergasser A, Cutcutache I, Koressaar T, et al. Primer3–new capabilities and interfaces. *Nucleic Acids Res*. 2012;40(15):e115.
14. Kent J. 2018. Available from: http://rohsdb.cmb.usc.edu/GBshape/cgi-bin/hgPcr.
15. Larki P, Gharib E, Yaghoob Taleghani M, Khorshidi F, Nazemalhosseini-Mojarad E, Asadzadeh Aghdaei H. Coexistence of *KRAS* and *BRAF* mutations in colorectal cancer: a case report supporting the concept of tumoral heterogeneity. *Cell J*. 2017;19(Suppl 1):113-117.
16. Lecomte T, Berger A, Zinzindohoue F, et al. Detection of free-circulating tumor-associated DNA in plasma of colorectal cancer patients and its association with prognosis. *Int J Cancer*. 2002;100(5):542-548.
17. Tan G, Chu C, Gui X, Li J, Chen Q. The prognostic value of circulating cell-free DNA in breast cancer: A meta-analysis. *Medicine (Baltimore)*. 2018;97(13):e0197.
18. Chen L, Zhang Y, Cheng Y, Zhang D, Zhu S, Ma X. Prognostic value of circulating cell-free DNA in patients with pancreatic cancer: A systemic review and meta-analysis. *Gene*. 2018;679:328-334.
19. Kohli M, Li J, Du M, et al. Prognostic association of plasma cell-free DNA-based androgen receptor amplification and circu- lating tumor cells in pre-chemotherapy metastatic castration-re- sistant prostate cancer patients. *Prostate Cancer Prostatic Dis*. 2018;21(3):411-418.
20. Beije N, Helmijr JC, Weerts M, et al. Somatic mutation detection using various targeted detection assays in paired samples of

circulating tumor DNA, primary tumor and metastases from patients undergoing resection of colorectal liver metastases. *Mol Oncol*. 2016;10(10):1575-1584.
21. Guo Q, Wang J, Xiao J, et al. Heterogeneous mutation pattern in tumor tissue and circulating tumor DNA warrants parallel NGS panel testing. *Mol Cancer*. 2018;17(1):131.
22. Devonshire AS, Whale AS, Gutteridge A, et al. Towards standardisation of cell-free DNA measurement in plasma: controls for extraction efficiency, fragment size bias and quantification. *Anal Bioanal Chem*. 2014;406(26):6499-6512.
23. Yang YC, Wang D, Jin L, et al. Circulating tumor DNA detectable in early- and late-stage colorectal cancer patients. *Biosci Rep*. 2018;38(4):BSR20180322
24. Diehl F, Li M, Dressman D, et al. Detection and quantification of mutations in the plasma of patients with colorectal tumors. *Proc Natl Acad Sci U S A*. 2005;102(45):16368-16373.
25. Cohen JD, Li L, Wang Y, et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. *Science*. 2018;359(6378):926-930.
26. Liu L, Toung JM, Jassowicz AF, et al. Targeted methylation sequencing of plasma cell-free DNA for cancer detection and classification. *Ann Oncol*. 2018;29(6):1445-1453.

SUPPORTING INFORMATION

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

How to cite this article: Liebs S, Keilholz U, Kehler I, Schweiger C, Haybäck J, Nonnenmacher A. Detection of mutations in circulating cell-free DNA in relation to disease stage in colorectal cancer. *Cancer Med*. 2019;00:1–9. https://doi.org/10.1002/cam4.2219

Excerpt from Journal Summary List “Oncology” 2019

The excerpt from the “Oncology” Journal Summary List of 2019 will be listed here, including the journal Oncogene (position 26 of 244), to which the manuscript “Applicability of liquid biopsies to represent the mutational profile of tumor tissue from different cancer entities” was submitted in December 2020.

Journal Data Filtered By: **Selected JCR Year: 2019** Selected Editions: SCIE,SSCI

Selected Categories: **“ONCOLOGY”** Selected Category Scheme: WoS

Gesamtanzahl: 244 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	CA-A CANCER JOURNAL FOR CLINICIANS	39,917	292.278	0.093460
2	Nature Reviews Clinical Oncology	12,384	53.276	0.035980
3	NATURE REVIEWS CANCER	52,053	53.030	0.066030
4	LANCET ONCOLOGY	53,592	33.752	0.143420
5	JOURNAL OF CLINICAL ONCOLOGY	155,297	32.956	0.261940
6	Cancer Discovery	18,093	29.497	0.069280
7	CANCER CELL	41,064	26.602	0.095430
8	JAMA Oncology	13,794	24.799	0.064650
9	ANNALS OF ONCOLOGY	45,813	18.274	0.107060
10	Molecular Cancer	15,448	15.302	0.023990
11	Journal of Thoracic Oncology	18,136	13.357	0.038200
12	JNCI-Journal of the National Cancer Institute	36,018	11.577	0.045450
13	Trends in Cancer	2,351	11.093	0.010140
14	SEMINARS IN CANCER BIOLOGY	8,310	11.090	0.011730
15	Journal of Hematology & Oncology	6,732	11.059	0.015550
16	NEURO-ONCOLOGY	12,950	10.247	0.029050
17	CLINICAL CANCER RESEARCH	85,288	10.107	0.131520
18	Journal for ImmunoTherapy of Cancer	4,557	9.913	0.016030
19	CANCER RESEARCH	135,753	9.727	0.118680

20	Liver Cancer	1,131	9.720	0.002660
21	Journal of the National Comprehensive Cancer Network	6,912	9.316	0.020020
22	CANCER TREATMENT REVIEWS	9,427	8.885	0.017800
23	Cancer Immunology Research	6,969	8.728	0.026440
24	LEUKEMIA	25,819	8.665	0.048640
25	Blood Cancer Journal	2,800	8.023	0.010400
26	ONCOGENE	66,303	7.971	0.068320
27	Clinical and Translational Medicine	1,349	7.919	0.003280
28	npj Precision Oncology	500	7.717	0.001520
29	BIOCHIMICA ET BIOPHYSICA ACTARE-VIEWS ON CANCER	5,650	7.365	0.007800
30	CANCER LETTERS	34,162	7.360	0.044450
31	EUROPEAN JOURNAL OF CANCER	32,241	7.275	0.048170
32	Gastric Cancer	5,525	7.088	0.010730
33	JOURNAL OF EXPERIMENTAL & CLINICAL CANCER RESEARCH	9,316	7.068	0.014540
34	Therapeutic Advances in Medical On-cology	1,894	6.852	0.004260
35	Molecular Oncology	6,378	6.574	0.013820
36	CANCER AND METASTASIS REVIEWS	6,247	6.400	0.005940
37	Cancers	10,442	6.126	0.018740
38	Oncogenesis	2,775	6.119	0.007750
39	STEM CELLS	20,554	6.022	0.024110
40	npj Breast Cancer	814	6.000	0.003590
41	JOURNAL OF PATHOLOGY	16,307	5.979	0.017910

Printing copy of the publication

Oncogene www.nature.com/onc

ARTICLE OPEN

Applicability of liquid biopsies to represent the mutational profile of tumor tissue from different cancer entities

Sandra Liebs[1,2✉], Theresa Eder[1,3], Frederick Klauschen[1,4], Moritz Schütte[5], Marie-Laure Yaspo[6], Ulrich Keilholz[2], Ingeborg Tinhofer[3], Evelyn Kidess-Sigal[3,7,8,9] and Diana Braunholz[1,3,9]

© The Author(s) 2021

Genetic investigation of tumor heterogeneity and clonal evolution in solid cancers could be assisted by the analysis of liquid biopsies. However, tumors of various entities might release different quantities of circulating tumor cells (CTCs) and cell-free DNA (cfDNA) into the bloodstream, potentially limiting the diagnostic potential of liquid biopsy in distinct tumor histologies. Patients with advanced colorectal cancer (CRC), head and neck squamous cell carcinoma (HNSCC), and melanoma (MEL) were enrolled in the study, representing tumors with different metastatic patterns. Mutation profiles of cfDNA, CTCs, and tumor tissue were assessed by panel sequencing, targeting 327 cancer-related genes. In total, 30 tissue, 18 cfDNA, and 7 CTC samples from 18 patients were sequenced. Best concordance between the mutation profile of tissue and cfDNA was achieved in CRC and MEL, possibly due to the remarkable heterogeneity of HNSCC (63%, 55% and 11%, respectively). Concordance especially depended on the amount of cfDNA used for library preparation. While 21 of 27 (78%) tissue mutations were retrieved in high-input cfDNA samples (30–100 ng, $N = 8$), only 4 of 65 (6%) could be detected in low-input samples (<30 ng, $N = 10$). CTCs were detected in 13 of 18 patients (72%). However, downstream analysis was limited by poor DNA quality, allowing targeted sequencing of only seven CTC samples isolated from four patients. Only one CTC sample reflected the mutation profile of the respective tumor. Private mutations, which were detected in CTCs but not in tissue, suggested the presence of rare subclones. Our pilot study demonstrated superiority of cfDNA- compared to CTC-based mutation profiling. It was further shown that CTCs may serve as additional means to detect rare subclones possibly involved in treatment resistance. Both findings require validation in a larger patient cohort.

Oncogene; https://doi.org/10.1038/s41388-021-01928-w

INTRODUCTION

Tumor heterogeneity is a major driver of treatment failure in cancer management [1–3]. Genetic, epigenetic, and phenotypic differences between distinct subpopulations of cells within the same tumor lesion may foster a survival benefit for resistant subclones, resulting in primary or secondary resistance [4]. Serial analysis of spatial and temporal heterogeneity within a single lesion and between multiple tumor sites has been suggested to improve in-depth disease monitoring during systemic treatment [5, 6]. To circumvent the invasive procedure of tissue sampling and to overcome its limitations in depicting the highly dynamic genetic complexity of a tumor, the analysis of blood-based biomarkers (liquid biopsy, LB) might increase therapeutic precision. Uncertainty exists concerning the diagnostic information contained in different components of peripheral blood. Circulating tumor cells (CTCs) represent cells disseminating from the tumor tissue, which potentially initiate the formation of metastasis [7–9]. Cell-free DNA (cfDNA) is mainly released from apoptotic and necrotic cells [10]. Despite remaining technological limitations in detection and characterization of cfDNA and CTCs, there is emerging evidence that the analysis of both constituents might allow disease surveillance and therapy guidance [11–13]. Increased CTC numbers and cfDNA concentrations were demonstrated to be of prognostic and predictive value in various tumor entities [14, 15]. Diagnostic applications and longitudinal monitoring of treatment response were mainly based on mutation profiling of LB [13, 16, 17]. Several studies indicated complementarity of CTCs and cfDNA [18, 19], increasing the potential benefit of LB-based patient monitoring based on a single blood draw. The diagnostic potential of CTCs and cfDNA should depend on tumor features including its anatomic location, growth kinetics, invasiveness, and routes of metastatic spread [20, 21], and could thus differ between distinct tumor histologies. To elucidate the ability of liquid biopsies to depict mutations in solid cancers with

[1]German Cancer Consortium (DKTK), German Cancer Research Center (DKFZ), Heidelberg, Germany. [2]Charité Comprehensive Cancer Center, Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany. [3]Department of Radiooncology and Radiotherapy, Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany. [4]Institute of Pathology, Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany. [5]Alacris Theranostics GmbH, Berlin, Germany. [6]Department of Vertebrate Genomics/Otto Warburg Laboratory Gene Regulation and Systems Biology of Cancer, Max Planck Institute for Molecular Genetics, Berlin, Germany. [7]Department of Medicine, Division of Hepatology and Gastroenterology, Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany. [8]Berlin Institute of Health (BIH), Berlin, Germany. [9]These authors contributed equally: Evelyn Kidess-Sigal, Diana Braunholz. ✉email: sandra.liebs@charite.de

Received: 22 December 2020 Revised: 18 June 2021 Accepted: 24 June 2021
Published online: 06 July 2021

different metastatic routes, patients with head and neck squamous cell carcinoma (HNSCC), colorectal cancer (CRC), and melanoma (MEL) were enrolled in this study, and the mutation repertoire of primary and/or metastatic tumor tissue was compared with those of cfDNA and CTCs. The three tumor entities were selected based on their different metastatic patterns, as HNSCC is clinically characterized by a predominance of locoregional disease progression, CRC by primarily hepatic metastasis through the portal vein, and MEL by frequent systemic hematogenous spread.

RESULTS

Characteristics of the patient cohort

The study enrolled a total of 18 patients with metastatic HNSCC, CRC, or MEL (six each) at a time when high CTC counts and cfDNA levels were expected based on the underlying cancer progression or no previous or ongoing cancer therapy. Two patients had not received any treatment prior blood collection. Patients who had already undergone radio-, chemo-, immuno-, and/or targeted therapy had either a break from treatment (≥1.5 months) or were progressing under therapy at the time when LB was collected. Archival tissue samples were collected between 2010 and 2017, whereas blood samples were drawn in 2017. Tissue of distant metastasis or secondary cancer was available from 17 of 18 patients (94%). Except from four patients, metastatic tissue and LB collection was on the same day ($N = 5$) or after 3–16 weeks ($N = 9$). Sufficient material for paired analysis of primary and metastatic tumor tissue using next-generation sequencing (NGS) was available from eight patients (44%), allowing identification of persistent mutations in contrast to cancer plasticity under treatment pressure. A detailed summary of patient and sample characteristics is provided in Supplementary Tables 1 and 2.

Liquid biopsies

The median concentration of isolated cfDNA per milliliter plasma was 139.7 ng/ml (4.4–468 ng/ml), 4.7 ng/ml (3.3–130 ng/ml), and 7.1 ng/ml (5.3–19.3 ng/ml) in CRC, HNSCC, and MEL patients, respectively. In an exploratory analysis, high cfDNA concentrations were associated with shorter overall survival (OS) of CRC and MEL patients (≥29.4 and ≥10.5 ng/ml with OS ≤ 5 months, respectively), whereas the opposite was observed in the HNSCC cohort (Supplementary Table 1). No correlation between cfDNA concentrations and CTC counts was found. CTCs were detected in 13 of 18 patients (72%). Except for two CRC patients with 15 and 33 detectable CTCs (CRC01.1 and CRC002.1, respectively), total tumor cell counts ranged between zero and six cells within the entire patient cohort. The presence of three or more CTCs per 7.5 ml blood was associated with worse OS in CRC patients (≤ 5 months, Supplementary Table 1). No association was observed in MEL and HNSCC. CTC counts, cfDNA concentrations, and clinicopathological characteristics of each patient are presented in Fig. 1.

In total, 16 CTC samples were collected from 12 patients. For three patients, CTC enrichment was done in parallel using two different protocols, i.e., single CTCs were not only isolated after RosetteSep-based CD45 depletion but CTCs were also collected together with some remaining leucocytes after Ficoll density gradient centrifugation. PCR-based quality control (QC-PCR) results after whole genome amplification (WGA) suggested sufficient DNA integrity for NGS analysis in only 7 of 16 samples (44%) from four patients (Supplementary Fig. 1).

Representation of tissue mutations in cfDNA

In total, solid and LB samples from 18 patients were analyzed, originating from primary and metastatic tumor tissue ($n = 30$), cfDNA ($n = 18$), and CTCs ($n = 7$). Variant calling identified 92 somatic mutations in tissue samples (CRC: 19, HNSCC: 62, MEL: 11), which were examined with regard to their

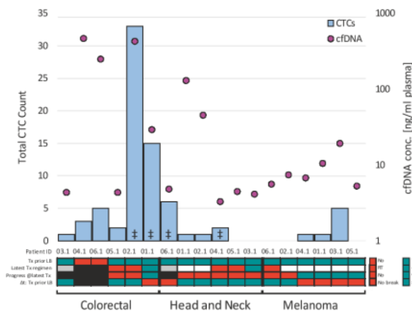


Fig. 1 Patient characteristics in comparison to the corresponding cfDNA concentrations and total CTC counts. For each patient, the detectable CTC count and cfDNA concentration were examined, possibly affected by the therapy status, including treatment prior to study enrollment and the time span (Δt) between the last therapy (Tx) and liquid biopsy collection (LB). CT chemotherapy, IT immunotherapy, RT radiotherapy, SURG surgery, TT targeted therapy, mo months. ‡available NGS data from whole genome amplified CTCs.

representation in cfDNA. Overall, tissue mutations were detected in 11 of 16 (69%) cfDNA samples (in the remaining two cases, no tissue mutation in the respective panel of genes was identified, leading to the exclusion of those two patients from the analysis of tissue mutation reflection in plasma). Successful retrieval of tissue mutations in plasma depended on the amount of cfDNA used for library preparation and not on the temporal distribution in sample collection. The analysis of high-input cfDNA samples (30–100 ng, $n = 8$) resulted in an overall concordance rate of 78% (CRC: 92%, HNSCC: 50%, MEL: 100%; Fig. 2A). Of the patients for whom the total yield of cfDNA was less than 30 ng ($n = 8$), only 4 of 65 (6%) tissue mutations were also found in plasma (Fig. 2B).

Representation of tissue mutations in CTCs

In addition to the retrieval of tissue alterations in plasma, CTC samples were analyzed to investigate concordance in mutation profiles with the solid cancer. Limited by the CTC detection rate (72%) and the fraction of samples with sufficient DNA integrity for WGA and sequencing (44%), NGS data were obtained from only seven CTC samples isolated from four patients. In patient HNSCC006.1, the analysis of tumor tissue from the local recurrence in the oral cavity and the lung metastasis did not reveal any mutations. Therefore, assessment of the concordance between tumor tissue and CTCs was limited to patients CRC001.1, CRC002.1, and HNSCC004.1, harboring 1, 2, and 20 tissue alterations, respectively.

For patient CRC002.1, mutations in the tumor suppressor genes *APC* and *TP53* identified in the colon tumor tissue were also found in a pooled sample of 13 CTCs (Fig. 2A). However, the analysis of two additional samples from five and eight CTCs revealed only the wildtype despite comparable DNA integrities (Supplementary Fig. 1). The *TP53* p.Q100* variant detected in the rectum and liver metastasis of patient CRC001.1 was not represented in either of the two available CTC samples (one and five tumor cells) but was detected in the respective cfDNA. Only 1 of 20 tissue mutations identified in patient HNSCC004.1 was verified at subthreshold allele frequency (AF) in the corresponding sample from a single CTC (confirmed to represent a high-quality sample per QC-PCR, Fig. 2B).

Indication of heterogeneity and clonal evolution in LB

Sequencing results from liquid biopsies were examined for additional alterations, which had not been identified in tissue.

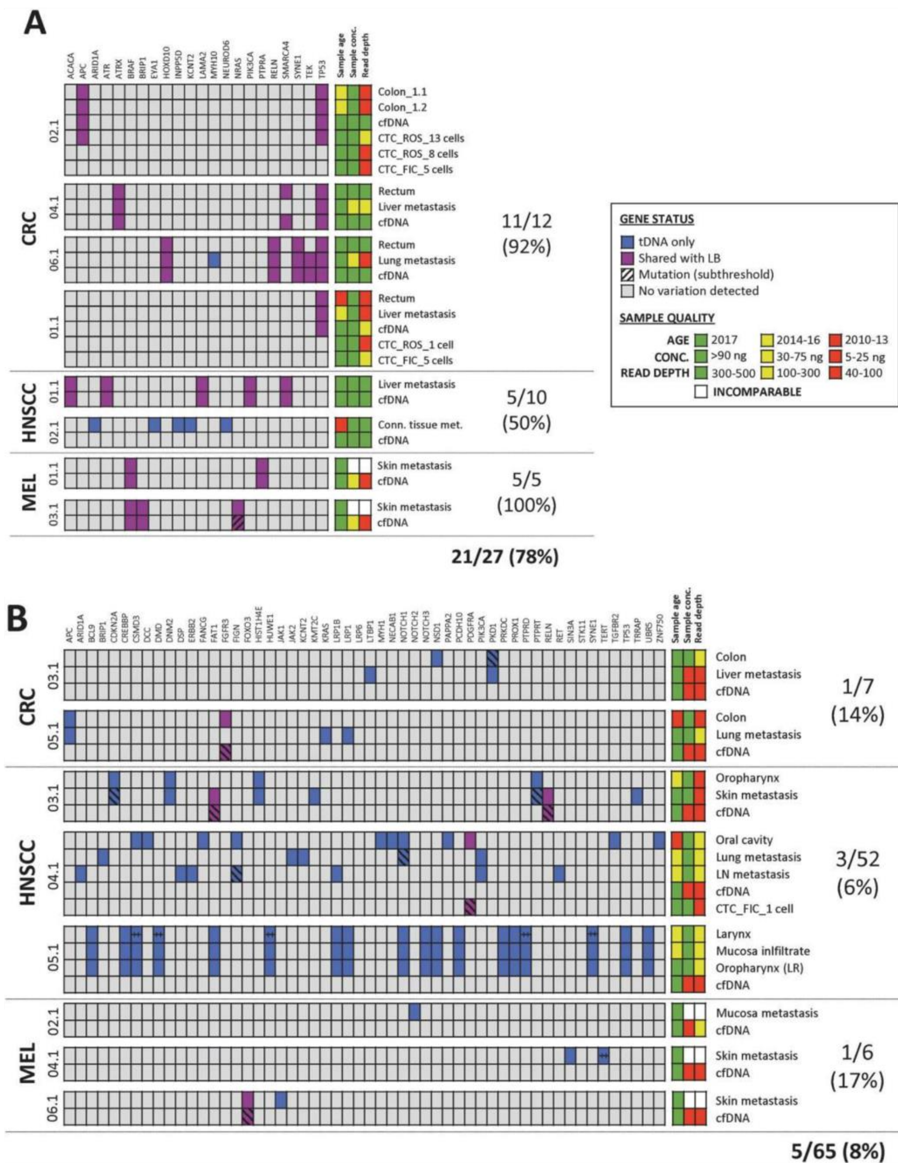


Fig. 2 Comparative analysis of tissue-derived mutations and their representation in LB samples. Patients were categorized into two subgroups referring to A high-input and B low-input cfDNA samples used for NGS analysis (≥30 and <30 ng, respectively). ‡Multiple mutations were detected in the same gene. FIC density gradient centrifugation-enriched CTCs, LB liquid biopsy, LR local recurrence, NGS next-generation sequencing, ROS RosetteSep™-enriched CTCs, tDNA tumor-derived DNA.

We hypothesized that these genetic variations could already be present in solid tumor tissue, though at very low frequencies, and might identify rare subclones that numerically expanded during disease course to the time point of LB collection. In cfDNA, variant calling determined 58 mutations in the entire patient cohort, including 15 tissue mutations (represented in high-input cfDNA samples only). After manual re-analysis (as described in the method section), 15 of 43 (35%) plasma-derived mutations were detected at subthreshold levels in tissue (Fig. 3). CTC samples harbored 206 variants in total, including two predominant tissue mutations (*APC* and *TP53* mutations in the tumor of CRC002.1). Forty-four (21%) CTC mutations were also found in at least one other specimen from the same patient, including another CTC, cfDNA, and/or formalin-fixed paraffin-embedded (FFPE) tissue (Fig. 4). However, only 18 (9%) CTC

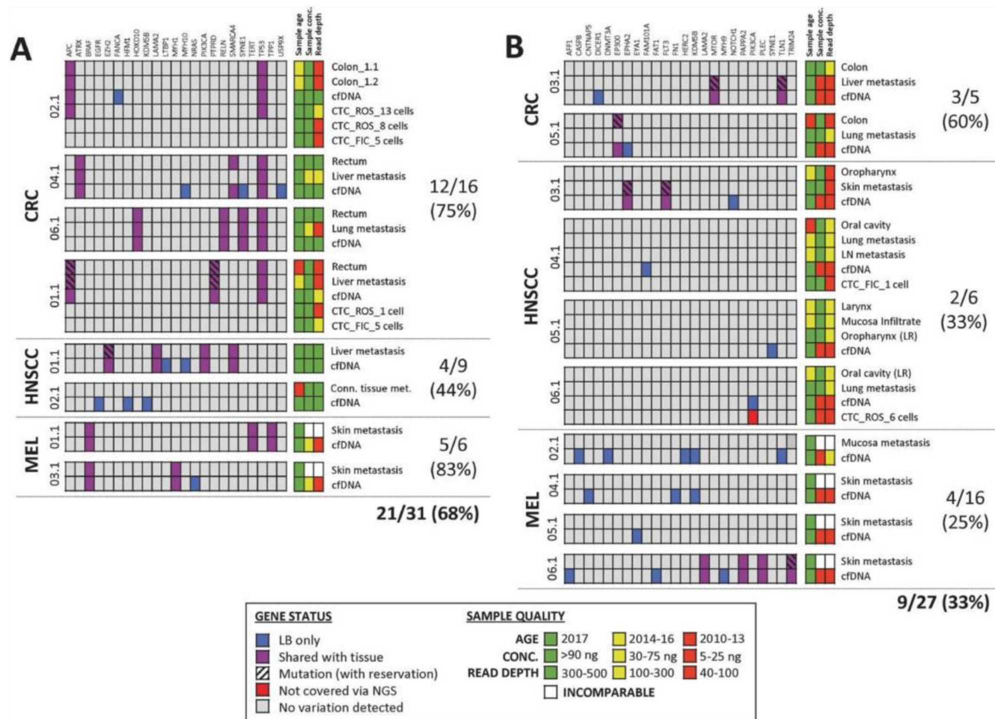


Fig. 3 Comparative analysis of cfDNA mutations and their concordance with corresponding tissue samples. Patients were assigned to a sub-group based on A high-input and B low-input cfDNA samples used for NGS analysis (≥30 and <30 ng, respectively). FIC Ficoll-enriched CTCs, LB liquid biopsy, LR local recurrence, NGS next-generation sequencing, ROS RosetteSep™-enriched CTCs, tDNA tumor-derived DNA.

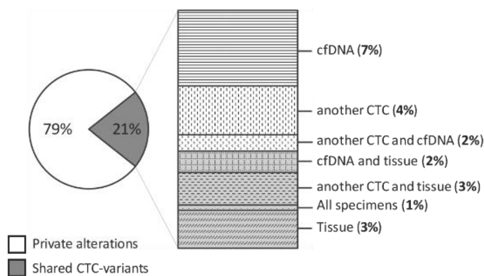


Fig. 4 Concordance of CTC-derived alterations with corresponding samples from the same individual. Of a total count of 206 CTC-derived mutations detected in four patients, 44 (21%) were also retrieved in another CTC, cfDNA, and/or tissue sample from the same patient, whereas 162 (79%) were unique for the analyzed circulating tumor cell.

alterations were also detected in corresponding tumor tissue, out of which 11 (61%) were furthermore retrieved in another LB sample.

Tumor heterogeneity in patient CRC002.1

Two cancer patients stood out from the entire cohort, highlighting the advantages of LB-based cancer profiling to analyze tumor heterogeneity (CRC002.1) and clonal evolution (MEL003.1). From patient CRC002.1, three CTC pools were sequenced, allowing broader assessment of tumor heterogeneity. Genome integrity indices were comparable between CTC samples with three to four bands in QC-PCR (Supplementary Fig. 1). CTC mutation burden increased with rising cell counts, ranging between 15 and 71 alterations (15 mutations in 5 cells, 36 mutations in 8 cells, and 71 mutations in 13 cells). In contrast, tumor tissue genotyping of two spatial areas of the colon revealed only two mutations in *APC* and *TP53*, which were also detected in the respective cfDNA sample (100 ng input). Only one of three CTC samples (CRC002.1-CTC1, 13 CTCs) reflected the molecular profile of the tissue, whereas the other CTC pools derived from the same patient only displayed the respective wildtype.

With regard to additional variations found in CTC samples, only a small fraction was recovered in other specimens from patient CRC002.1. Sixty-nine mutations were detected in CRC002.1-CTC1, including the two predominant tissue mutations in *APC* and *TP53*. Nine of 67 (13%) CTC mutations were also found at subthreshold AF in the tumor tissue. Tumor heterogeneity was also reflected by the detection of only 8 variants (12%) in another CTC, 7 (10%) in cfDNA, and 1 (1%) in cfDNA and another CTC, whereas 42 alterations (63%) were unique for CRC002.1-CTC1. Consistently, only 9 of 54 mutations (17%) detected in the two other CTC samples from patient CRC002.1 were overlapping with at least one other specimen. The analysis of cfDNA resulted in one additional mutation in the *FANCA* gene, which was not displayed by any other specimen of this patient.

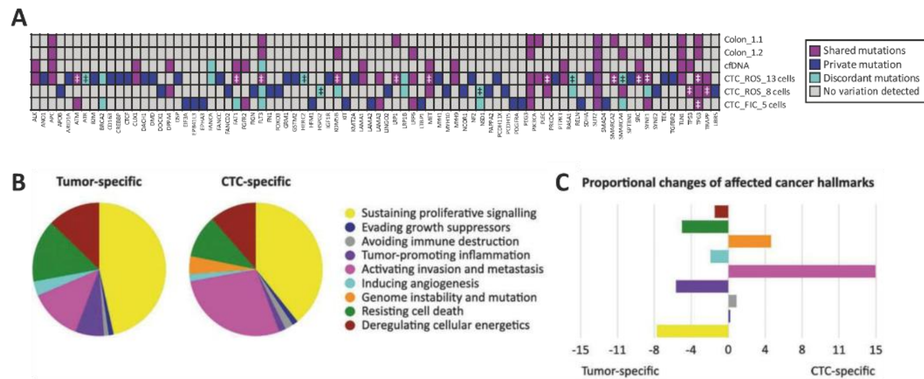


Fig. 5 Mutation profiles of corresponding CTCs, cfDNA, and tissue from a single patient reflected tumor heterogeneity, potentially affecting different cancer hallmark-related pathways. A Shared, discordant (different mutations in the same gene), and private gene alterations were identified in tumor tissue and LB from CRC002.1 and B, C assigned to cancer hallmarks, demonstrating distinct differences of involved cancer-related pathways likely to correlate with the requirements concerning tumor growth and metastasis to distant sites. ‡Multiple mutations were detected in the same gene.

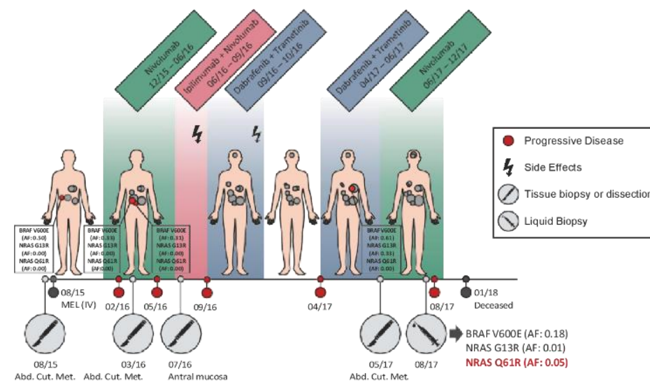


Fig. 6 Tissue and plasma genotyping of a patient with refractory melanoma to immunotherapy and BRAF-MEK-inhibition at different time points of the disease. Schematic illustration of the clinical course, including the duration of administered treatment, therapy adaption due to side effects (flash) or progressive disease (red circles), and tumor genotyping conducted on tissue (indicated by a scalpel) or plasma (indicated by a syringe). Allele frequencies (AF) of *BRAF* and *NRAS* mutations were determined by ddPCR analysis.

Kyoto Encyclopedia of Genes and Genomes mapping and further database research (COSMIC, Genetics Home References, National Institutes of Health, PubMed NCBI) was conducted to evaluate pathways possibly impaired by the detected mutations in CTCs and tissue. Pathways were assigned to one or multiple cancer hallmarks, defined by Hanahan and Weinberg [22], and proportional changes between hallmarks specific for the shared mutations of solid tumor tissue and CTC as well as those only found in CTCs were evaluated. It was demonstrated that private CTC mutations were more frequently involved in pathways correlated with invasion, genome instability and avoidance of immune destruction. In contrast, mutations shared by tissue and liquid biopsies were associated with proliferative signaling, tumor-promoting inflammation, resistance to cell death and induction of angiogenesis. A detailed summary of all mutated genes, concordance between specimens, and affected pathways is displayed in Fig. 5.

Identification of subclonal resistance through LB

One patient with refractory MEL to immunotherapy and BRAF-MEK inhibition was of special interest due to the mutation spectrum detected in tissue and cfDNA (Fig. 6 schematically depicts the clinical course of MEL003.1, including genotyping of tissue and plasma). Whole exome sequencing (WES) of a subcutaneous metastasis was performed as part of a precision oncology program of the Charité, revealing the *BRAF* V600E (AF: 0.64) and secondary *NRAS* G13R (AF: 0.41) mutation. After few months of nivolumab treatment, the patient presented with new pulmonary, hepatic and cerebral metastases. At that time, cfDNA displayed the previously reported *BRAF* V600E mutation (AF: 0.26) as well as the *NRAS* G13R mutation at subthreshold level (AF: 0.02). In addition, cfDNA revealed the emergence of the *NRAS* Q61R mutation with an AF of 0.15.

Validation by the highly sensitive Droplet Digital™ PCR (method description in Supplementary information and Supplementary

Table 3) confirmed the presence of all three mutations in cfDNA at slightly different AF (V600E: 0.18, G13R: 0.01, Q61R: 0.05). In four metastases resected between 2015 and 2017, *BRAF* V600E was found at AF of 0.31–0.61 in all samples, *NRAS* G13R (AF: 0.33) was only present in one subcutaneous lesion as already known from WES, whereas *NRAS* Q61R was not even detected on subclonal level. All three mutations were proven to be tumor-derived based on their absence in the respective germline sample of the patient (mutant AF: 0.00). Five CTCs were isolated from MEL003.1; however, QC-PCR demonstrated insufficient DNA integrity of cells for sequencing.

DISCUSSION

Previous studies have suggested superiority of cfDNA as compared to CTC-based mutation profiling [23, 24]. However, mutation detection in cfDNA reflecting the overall profile of cancer cells may differ in informative value from the subset of CTCs, representing a population of cells possibly evading therapy. In this pilot study, we evaluated the feasibility of CTCs and cfDNA in representing the mutational landscape of corresponding tumor tissue in cancer types with distinct metastatic routes. Thus, only patients with advanced disease were enrolled in this study and blood samples were collected shortly after recurrent tumor dissection when highest concordance in the mutation profiles of the solid tumor and LB can be expected.

We observed no difference regarding the informative value of liquid biopsies between tumor entities with distinct metastatic tropism. However, best overall concordance was achieved in CRC followed by MEL, whereas LB-based cancer profiling in HNSCC was less concordant possibly explained by the notoriously heterogeneous mutation profile of this cancer type [25]. A limitation of our study was the small patient cohort, requiring further validation of our observations with a sufficient sample size. In spite of the small number of cases covered per tumor entity, patients were not obviously different from other cohorts with regard to age, sex, clinical course, and metastatic tumor location.

CfDNA outperformed CTC analysis not only with regard to convenience of sample handling, but primarily in reflecting the genomic profile of the solid tissue more closely. Despite the slightly lower cfDNA concentrations isolated in our study compared to previously published data [26–28], tissue mutations were detected in 69% of cfDNA samples. In contrast, Lebofsky et al. found matching mutations in cfDNA and tumor biopsies in 79% of patients with metastatic cancer, recovering 28 of 29 (97%) tissue mutations in plasma [29]. In our study, concordance between tumor tissue and cfDNA was 63%, 55%, and 11% in CRC, MEL, and HNSCC, respectively. In contrast, different groups reported detection of 56–87% of tissue mutations in plasma of CRC patients [20, 26, 30], 73–85% in MEL [17, 31], and 42–92% in HNSCC patients [32, 33]. However, it should be considered that sequencing of high-input cfDNA samples (8 of 18 samples with 30–100 ng) allowed the detection of 21 of 27 (78%) tissue mutations in plasma from our cohort. Thus, concordance rates in highly concentrated cfDNA samples achieved comparable values as previously reported (92% in CRC, 100% in MEL, and 50% in HNSCC). Another interesting exploratory finding was that higher cfDNA concentrations were associated with a shorter OS in CRC and MEL, which is in line with previous results from Bettegowda et al. [20]. The opposite was observed in HNSCC patients; however, this observation must be validated in a bigger patient cohort.

Achieved CTC detection rates were consistent with previous publications or even exceeded reported detection levels (100% of CRC patients: 1–33 CTCs, 67% of HNSCC: 1–6 CTCs, and 50% of MEL: 1–5 CTCs). This might be explained by high tumor aggressiveness in our patient cohort, since ten patients deceased within 6 months after LB collection, whereas only three patients showed an OS of 3.5–4 years to date. In comparison, multiple studies reported detection levels of 20–60% in stage IV CRC patients (1–61 CTCs) [19–21], 41–43% in advanced HNSCC (1 CTC) [15, 34], and 25% in patients with metastatic MEL (≥2 CTCs) [35, 36]. CTC counts were associated with shorter OS in CRC patients, using the cutoff of ≥3 CTCs/7.5 ml [37]. In contrast, application of reported prognostic CTC counts of ≥2 CTCs/7.5 ml blood did not show any association with OS in MEL [35] and HNSCC [15] patients. However, these analyses are exploratory in nature due to the limited sample size and only validation with a larger patient cohort would allow interpretation of this preliminary observation.

The fact that in a significant portion of patients no or only few CTCs were detected might be explained by the limited set of markers applied to identify tumor cells in the peripheral circulation. Different CTC phenotypes express a subset of proteins on their surfaces, hampering the isolation of epithelial, mesenchymal, and hybrid phenotypes of CTCs [38, 39]. Sequencing of solely living CTCs further limited the approach to extracellular markers. Low frequency and quantity of detectable CTCs may limit their diagnostic potential in clinical practice. Even if the analysis of those cells might increase our understanding of the mechanisms of metastatic spread, only 0.01% of CTCs are reported to harbor the potential for colonization in a secondary organ [9], which in turn might contribute to the low concordance between CTCs and metastatic tissue in our study. In addition, technical limitations should not be dismissed when working with single cells. Uniform WGA of CTCs might be impaired by insufficient DNA integrity or allelic imbalance and dropout, reducing the informative value of CTCs for cancer profiling, as seen in our study. Only one sequenced CTC sample reflected the tissue mutation, highlighting the potential of CTCs to reflect tumor heterogeneity, which was particularly evident in patient CRC002.1.

In CRC002.1, tumor heterogeneity was depicted in partially complementary mutation profiles of three CTC samples compared to two spatial areas of the colon tumor tissue and the respective cfDNA sample. Multiple studies determined diversity of mutational status and gene rearrangements in CTCs from the same individual in several cancerous diseases [40–42]. De Luca et al. even reported almost all of the detectable aberrations to be private to each single CTC isolated from breast cancer patients [42]. Interestingly, we demonstrated that gene mutations detected in CTCs were cumulatively assigned to cancer hallmarks matching the requirements of tumor cells circulating in the periphery, including activation of invasion and metastasis [43] as well as avoidance of immune destruction [44]. In contrast, alterations shared by CTCs and tumor tissue were rather associated with requirements of progressing tumor lesions, such as sustaining proliferative signaling, inducing angiogenesis and deregulating cellular energetics [22]. In contrast to cfDNA analysis, only expression profiles of CTCs may provide insight into altered pathways to possibly identify new therapeutically targetable CTC signatures. For example, in the study of Miyamoto et al., CTC heterogeneity in the non-canonical Wnt signaling pathway was linked to resistance against androgen receptor inhibition in a small cohort of prostate cancer patients [45].

In addition, we investigated cancer-clone dynamics in several tumor tissues and the corresponding cfDNA sample from MEL003.1. Here, cfDNA analysis displayed the occurrence of a *NRAS* Q61R mutation after multiple lines of treatment, possibly mediating drug resistance. This case report indicated the pivotal role of clonal evolution under therapeutic pressure and the advantages of LB analysis to detect predominant tissue mutations in plasma when lesions are not accessible for biopsies or only insufficient DNA quantities and/or qualities are available for tissue profiling. Especially in MEL, rapid adaption of the mutational signature in response to selective treatment has been reported [46]. In the study by Gorges et al., continuous changes of the mutation profiles were detected in CTCs from MEL patients

regarding the genes *BRAF*, *NRAS*, *EGFR*, and *MAP2K1*. Cancer plasticity during targeted therapy has not only been evident from the analysis of cfDNA and CTCs but also from studies of blood-derived extracellular vesicles as previously published by our group. In the study of Yap et al., a change in the mutant variant profile from *BRAF* V600E to V600K was detected in extracellular vesicles from a MEL patient under BRAF-MEK inhibition, and the emergence of *KRAS* G12D mutation was found after cetuximab treatment of a CRC patient with a *KRAS* wildtype primary tumor [47]. This highlights how promising LB analysis is to detect escape mutations prior to clinical manifestation of cancer progression.

Simultaneous analysis of multiple LB components such as CTCs and cfDNA might improve patient surveillance. Comparable to our results, previous analysis of our group demonstrated an independence of CTC and cfDNA levels in CRC patients (stage I–IV), indicating the great potential for complementary analysis of both fractions [26]. Similarly, synergy was also discussed by Gorges et al., demonstrating that the parallel analysis of CTCs and cfDNA in MEL patients provided supplementary information for monitoring of the underlying disease [46]. This was supported by Onidani et al., who performed NGS analysis of CTCs and cfDNA from patients with HNSCC, CRC, esophageal, and gastric cancer [48]. Low concordance indicated that both biomarkers exhibit private mutation footprints, allowing an increased sensitivity of tissue profiling when analyzing both constituents. Multiple studies reported that 26–52% of variants were solely seen in liquid biopsies [32, 46, 49]. Concerning our pilot project, a possible explanation for the exclusive detection of gene alterations in liquid biopsies but not in tissue is that most of the patients presented with multiple cancer foci, of which only one or two were sequenced and compared to LB. Therefore, analysis of all metastases might reveal increased concordance rates. More importantly, this is again consistent with a high influence of tumor heterogeneity on mutation prevalence in a spatial and temporal manner [3].

From a clinical perspective, adequate diagnostic tools to closely monitor changes in clonal cancer architecture toward disease progression are urgently needed, since most patients develop recurrent or progressive disease despite the many advances in patient management. Therefore, LB should be recognized not as a surrogate for standard tissue profiling but rather as a relevant complementary biomarker to depict the molecular profile of the underlying disease and reveal therapy-induced emergence of cancer subclones. Our results clearly emphasized the advantages of cfDNA-based cancer profiling, indicating a superior utility in CRC and MEL compared to HNSCC. It was furthermore demonstrated that in some patients CTCs may serve as an additional means to detect rare subclones and more closely investigate tumor heterogeneity, possibly leading to treatment resistance. Prior to clinical application, however, standardization of isolation and analysis procedures remains a prerequisite.

MATERIAL AND METHODS

Patient recruitment and study cohort

Eighteen patients diagnosed with metastasized HNSCC, CRC, and MEL were enrolled in our study at the Charité University Hospital. Patients' informed written consent was obtained prior to sample collection, which included blood and archival FFPE tissue. Our study was approved by the local ethics committee (EA 4/087/15).

CTC isolation and whole genome amplification

CTCs were enriched using the RosetteSep™ Human CD45 Depletion kit (Stemcell Technologies, Vancouver, Canada). During the preliminary recruiting phase, an additional blood sample was processed in parallel, from which peripheral blood mononuclear cells were isolated together with CTCs by a density gradient centrifugation protocol using Ficoll-Paque PLUS (GE Healthcare Life Sciences/Merck KGaA, Darmstadt, Germany). CRC- and HNSCC-derived tumor cells were stained for EpCAM and EGFR, whereas MCSP was detected on MEL-CTCs. Leucocytes were identified based on their CD45 expression. In addition, a viability staining was performed using the LIVE/DEAD™ Fixable Blue Dead Cell Stain (Thermo Fisher Scientific, protocol details in Supplementary information). Using Leica's DMI 3000B inverted microscope for visualization (Leica Biosystems, Wetzlar, Germany), viable CTCs were identified as CD45-negative and tumor marker-positive cells and isolated using the Microinjector IM-9B (Narishige Group, Tokyo, Japan). CTC samples were subjected to an overnight WGA as single or pooled cells according to the manufacturer's instructions of the REPLI-g Single Cell kit (Qiagen). To evaluate DNA integrity of CTCs and thus effective WGA procedure, a QC-PCR was performed according to the manufacturer's protocol (Ampli1™ QC kit from Menarini Silicon Biosystems, Castel Maggiore, Italy). The Ampli1™ QC kit amplifies up to four DNA fragments of different size and chromosomal location to predict successful downstream application.

Library preparation and targeted sequencing

A detailed description of the DNA isolation from whole blood, plasma, and FFPE specimens is given in the Supplementary information. The HaloPlex™ HS target enrichment system for Illumina sequencing (Agilent Technologies, Santa Clara, USA) was used for mutational profiling of archival tumor tissue from CRC and HNSCC patients as well as the LB-derived samples from the entire cohort. Our in-house panel was designed to detect frequently mutated genes of functional relevance in cancer [50], targeting the exonic sequence of 327 genes (1.47 Mb). Library preparation was performed as previously described following manufacturer's protocol (Agilent, protocol version C1, December 2016) [50]. DNA input varied depending on the available DNA concentrations isolated from different starting material, such as FFPE tissue, whole blood, cfDNA, or CTCs, ranging between 10 and 100 ng. Paired-end sequencing was carried out on the Illumina NextSeq500 platform with the High Output v2 sequencing kit (300 cycles, Illumina, San Diego, USA).

Sequencing of the metastatic tissue of the MEL-cohort was performed as part of the Treat20plus study conducted at the Max Planck Institute in partnership with the Charité Comprehensive Cancer Center. WES was performed on the HiSeq™ system following the protocol of the Nextera Rapid Capture Exome and Expanded Exome kit (Illumina), which covers 201,121 target regions and comprises approximately 62 Mb of DNA.

Sequencing data analysis and variant calling

Raw fastq files were processed with the Agilent SureCall Software (version 3.5.1.46). A median sequencing depth of 158-fold, 83-fold, 74-fold, and 47-fold was achieved in germline, FFPE, cfDNA, and CTC samples, respectively. Personal alterations were excluded when detected in the individual whole blood sample. Remaining alterations were sieved based on their predicted deleterious effect annotated in the COSMIC database [51, 52] and the Cancer Genome Interpreter [53, 54]. Further analysis was performed as previously described [50]. The detailed procedure of variant calling and data analysis is described in the Supplementary information and depicted in Supplementary Fig. 2. Sequencing data will be available from the corresponding author upon reasonable request.

Statistical analysis

Continuous variables were summarized by median and range, and categorical variables by frequency. Due to the small sample size, no statistical comparisons were made.

REFERENCES

1. Kidess E, Jeffrey SS. Circulating tumor cells versus tumor-derived cell-free DNA: rivals or partners in cancer care in the era of single-cell analysis? Genome Med. 2013;5:70.

2. Prasetyanti PR, Medema JP. Intra-tumor heterogeneity from a cancer stem cell perspective. Mol Cancer. 2017;16:41.
3. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med. 2012;366:883–92.
4. Fisher R, Pusztai L, Swanton C. Cancer heterogeneity: implications for targeted therapeutics. Br J Cancer. 2013;108:479–85.
5. Misale S, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature. 2012;486:532–6.
6. Martelotto LG, Ng CKY, Piscuoglio S, Weigelt B, Reis-Filho JS. Breast cancer intra-tumor heterogeneity. Breast Cancer Res. 2014;16:210.
7. Castro-Giner F, Aceto N. Tracking cancer progression: from circulating tumor cells to metastasis. Genome Med. 2020;12:31.
8. Tayoun T, Faugeroux V, Oulhen M, Aberlenc A, Pawlikowska P, Farace F. CTC-derived models: a window into the seeding capacity of circulating tumor cells (CTCs). Cells. 2019;8:1145.
9. Luzzi KJ, MacDonald IC, Schmidt EE, Kerkvliet N, Morris VL, Chambers AF, et al. Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. Am J Pathol. 1998;153:865–73.
10. Diaz LA Jr, Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. J Clin Onco. 2014;32:579–86.
11. Siravegna G, Mussolin B, Buscarino M, Corti G, Cassingena A, Crisafulli G, et al. Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. Nat Med. 2015;21:795–801.
12. Khan KH, Cunningham D, Werner B, Vlachogiannis G, Spiteri I, Heide T, et al. Longitudinal liquid biopsy and mathematical modeling of clonal evolution forecast time to treatment failure in the PROSPECT-C Phase II Colorectal Cancer Clinical Trial. Cancer Discov. 2018;8:1270–85.
13. Kuhlmann JD, Wimberger P, Bankfalvi A, Keller T, Schöler S, Aktas B, et al. ERCC1-positive circulating tumor cells in the blood of ovarian cancer patients as a predictive biomarker for platinum resistance. Clin Chem. 2020;60:1282–9.
14. Fernandez-Garcia D, Hills A, Page K, Hastings RK, Toghill B, Goddard KS, et al. Plasma cell-free DNA (cfDNA) as a predictive and prognostic marker in patients with metastatic breast cancer. Breast Cancer Res. 2019;21:149.
15. Grisanti S, Almici C, Consoli F, Buglione M, Verardi R, Bolzoni-Villaret A, et al. Circulating tumor cells in patients with recurrent or metastatic head and neck carcinoma: prognostic and predictive significance. PLoS One. 2014;9:e103918.
16. Oellerich M, Schütz E, Beck J, Walson PD. Circulating cell-free DNA-diagnostic and prognostic applications in personalized cancer therapy. Ther Drug Monit. 2019;41:115–20.
17. Gray ES, Rizos H, Reid AL, Boyd SC, Pereira MR, Lo J, et al. Circulating tumor DNA to monitor treatment response and detect acquired resistance in patients with metastatic melanoma. Oncotarget. 2015;6:42008–18.
18. Manier S, Park J, Capelletti M, Bustoros M, Freeman SS, Ha G, et al. Whole-exome sequencing of cell-free DNA and circulating tumor cells in multiple myeloma. Nat Commun. 2018;9:1691.
19. Lee S-Y, Chae D-K, An J, Jung S, Chae CH, Bhak J, et al. Comprehensive mutation profiling of both cfDNA and CTCs in gynecological cancer patients. bioRxiv. 2019:566786.
20. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med. 2014;6:224ra24.
21. Haber DA, Velculescu VE. Blood-based analyses of cancer: circulating tumor cells and circulating tumor DNA. Cancer Disco. 2014;4:650–61.
22. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646–74.
23. Freidin MB, Freydina DV, Leung M, Montero Fernandez A, Nicholson AG, Lim E. Circulating tumor DNA outperforms circulating tumor cells for KRAS mutation detection in thoracic malignancies. Clin Chem. 2015;61:1299–304.
24. Dawson SJ, Tsui DW, Murtaza M, Biggs H, Rueda OM, Chin SF, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med. 2013;368:1199–209.
25. Leemans CR, Snijders PJF, Brakenhoff RH. The molecular landscape of head and neck cancer. Nat Rev Cancer. 2018;18:269–82.
26. Liebs S, Keilholz U, Kehler I, Schweiger C, Haybäck J, Nonnenmacher A. Detection of mutations in circulating cell-free DNA in relation to disease stage in colorectal cancer. Cancer Med. 2019;8:3761–9.
27. Salvianti F, Pinzani P, Verderio P, Ciniselli CM, Massi D, De Giorgi V, et al. Multi-parametric analysis of cell-free DNA in melanoma patients. PLoS One. 2012;7:e49843.
28. Mazurek AM, Rutkowski T, Fiszer-Kierzkowska A, Małusecka E, Składowski K. Assessment of the total cfDNA and HPV16/18 detection in plasma samples of head and neck squamous cell carcinoma patients. Oral Oncol. 2016;54:36–41.
29. Lebofsky R, Decraene C, Bernard V, Kamal M, Blin A, Leroy Q, et al. Circulating tumor DNA as a non-invasive substitute to metastasis biopsy for tumor genotyping and personalized medicine in a prospective trial across all tumor types. Mol Oncol. 2015;9:783–90.
30. Kidess E, Heirich K, Wiggin M, Vysotskaia V, Visser BC, Marziali A, et al. Mutation profiling of tumor DNA from plasma and tumor tissue of colorectal cancer patients with a novel, high-sensitivity multiplexed mutation detection platform. Oncotarget. 2015;6:2549–61.
31. Lin SY, Huang SK, Huynh KT, Salomon MP, Chang S-C, Marzese DM, et al. Multiplex gene profiling of cell-free DNA in patients with metastatic melanoma for monitoring disease. JCO Precis Oncol. 2018;2:1–30.
32. Galot R, van Marcke C, Helaers R, Mendola A, Goebbels RM, Caignet X, et al. Liquid biopsy for mutational profiling of locoregional recurrent and/or metastatic head and neck squamous cell carcinoma. Oral Oncol. 2020;104:104631.
33. Wang Y, Springer S, Mulvey CL, Silliman N, Schaefer J, Sausen M, et al. Detection of somatic mutations and HPV in the saliva and plasma of patients with head and neck squamous cell carcinomas. Sci Transl Med. 2015;7:293ra104.
34. Hristozova T, Konschak R, Stromberger C, Fusi A, Liu Z, Weichert W, et al. The presence of circulating tumor cells (CTCs) correlates with lymph node metastasis in nonresectable squamous cell carcinoma of the head and neck region (SCCHN). Ann Oncol. 2011;22:1878–85.
35. Khoja L, Lorigan P, Zhou C, Lancashire M, Booth J, Cummings J, et al. Biomarker utility of circulating tumor cells in metastatic cutaneous melanoma. J Invest Dermatol. 2013;133:1582–90.
36. Cayrefourcq L, De Roeck A, Garcia C, Stoebner P-E, Fichel F, Garima F, et al. S100-EPISPOT: a new tool to detect viable circulating melanoma cells. Cells. 2019;8:755.
37. Cohen SJ, Punt CJ, Iannotti N, Saidman BH, Sabbath KD, Gabrail NY, et al. Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. J Clin Oncol. 2008;26:3213–21.
38. Genna A, Vanwynsberghe AM, Villard AV, Pottier C, Ancel J, Polette M, et al. EMT-associated heterogeneity in circulating tumor cells: sticky friends on the road to metastasis. Cancers (Basel). 2020;12:1632.
39. Yu M, Bardia A, Wittner BS, Stott SL, Smas ME, Ting DT, et al. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. Science. 2013;339:580–4.
40. Pestrin M, Salvianti F, Galardi F, De Luca F, Turner N, Malorni L, et al. Heterogeneity of PIK3CA mutational status at the single cell level in circulating tumor cells from metastatic breast cancer patients. Mol Oncol. 2015;9:749–57.
41. Pailler E, Auger N, Lindsay CR, Vielh P, Islas-Morris-Hernandez A, Borget I, et al. High level of chromosomal instability in circulating tumor cells of ROS1-rearranged non-small-cell lung cancer. Ann Oncol. 2015;26:1408–15.
42. De Luca F, Rotunno G, Salvianti F, Galardi F, Pestrin M, Gabellini S, et al. Mutational analysis of single circulating tumor cells by next generation sequencing in metastatic breast cancer. Oncotarget. 2016;7:26107–19.
43. Majidpoor J, Mortezaee K. Steps in metastasis: an updated review. Med Oncol. 2021;38:3.
44. Hu B, Tian X, Li Y, Liu Y, Yang T, Han Z, et al. Epithelial-mesenchymal transition may be involved in the immune evasion of circulating gastric tumor cells via downregulation of ULBP1. Cancer Med. 2020;9:2686–97.
45. Miyamoto DT, Zheng Y, Wittner BS, Lee RJ, Zhu H, Broderick KT, et al. RNA-Seq of single prostate CTCs implicates noncanonical Wnt signaling in antiandrogen resistance. Science. 2015;349:1351–6.
46. Gorges K, Wiltfang L, Gorges TM, Sartori A, Hildebrandt L, Keller L, et al. Intra-patient heterogeneity of circulating tumor cells and circulating tumor DNA in blood of melanoma patients. Cancers (Basel). 2019;11:1685.
47. Yap SA, Münster-Wandowski A, Nonnenmacher A, Keilholz U, Liebs S. Analysis of cancer-related mutations in extracellular vesicles RNA by Droplet Digital™ PCR. Biotechniques. 2020;69:99–107.
48. Onidani K, Shoji H, Kakizaki T, Yoshimoto S, Okaya S, Miura N, et al. Monitoring of cancer patients via next-generation sequencing of patient-derived circulating tumor cells and tumor DNA. Cancer Sci. 2019;110:2590–9.
49. Lack J, Gillard M, Cam M, Paner GP, VanderWeele DJ. Circulating tumor cells capture disease evolution in advanced prostate cancer. J Transl Med. 2017;15:44.
50. Eder T, Hess AK, Konschak R, Stromberger C, Johrens K, Fleischer V, et al. Interference of tumour mutational burden with outcome of patients with head and neck cancer treated with definitive chemoradiation: a multicentre retrospective study of the German Cancer Consortium Radiation Oncology Group. Eur J Cancer. 2019;116:67–76.
51. Tate JG, Bamford S, Jubb HC, Sondka Z, Beare DM, Bindal N, et al. COSMIC: the catalogue of somatic mutations in cancer. Nucleic Acids Res. 2019;47:D941–d7.
52. COSMIC – Catalogue of somatic mutations in cancer. cancer.sanger.ac.uk. 2004. accessed date 17.07.2019.

53. Tamborero D, Rubio-Perez C, Deu-Pons J, Schroeder MP, Vivancos A, Rovira A, et al. Cancer Genome Interpreter annotates the biological and clinical relevance of tumor alterations. Genome Med. 2018;10:25.
54. Institute for Research in Biomedicine. Cancer Genome Interpreter. 2018. accessed date 17.07.2019. https://www.cancergenomeinterpreter.org.

ACKNOWLEDGEMENTS

We thank Dr Med. Claas Ulrich and the Hauttumorcentrum Charité for providing an additional tumor tissue specimen from patient MEL003.1, allowing a broader analysis with regard to NRAS and BRAF profiling. EK-S is participant in the BIH-Charité Clinician Scientist Program funded by the Charité – Universitätsmedizin Berlin and the Berlin Institute of Health.

FUNDING

Our analysis was financed by the Berliner Krebsgesellschaft (BRFF201520) and partly supported by the German Cancer Consortium (DKTK), which is funded as one of the National German Health Centers by the Federal German Ministry of Education and Research. Open Access funding enabled and organized by Projekt DEAL.

COMPETING INTERESTS

The authors declare no competing financial interests. MS is an employee of Alacris Theranostics GmbH.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41388-021-01928-w.

Correspondence and requests for materials should be addressed to S.L.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

Publication list

Tinhofer I, Niehr F, Konschak R, **Liebs S**, Munz M, Stenzinger A, Weichert W, Keilholz U, Budach V. Next-generation sequencing: hype and hope for development or personalized radiation therapy? Radiation Oncology 2015; 10:183. *(Impact Factor: 2.466)*

Boehnke K, Iversen PW, Schumacher D, Lallena MJ, Haro R, Amat J, Haybaeck J, **Liebs S**, Lange M, Schäfer R, Regenbrecht CRA, Reinhard C, Velasco JA. Assay establishment and validation of a high-throughput screening platform for three-dimensional patient-derived colon cancer organoid cultures. Journal of Biomolecular Screening 2016; 21(9):931-41. *(Impact Factor: 2.444)*

Schütte M, Risch T, Abdavi-Azar N, Boehnke K, Schumacher D, Keil M, Yildiriman R, Jandrasits C, Borodina T, Amstislavskiy V, Worth CL, Schweiger C, **Liebs S**, Lange M, Warnatz HJ, Butcher LM, Barrett JE, Sultan M, Wierling C, Golob-Schwarzl N, Lax S, Uranitsch S, Becker M, Welte Y, Regan JL, Silvestrov M, Kehler I, Fusi A, Kessler T, Herwig R, Landegren U, Wienke D, Nilsson M, Velasco JA, Garin-Chesa P, Reinhard C, Beck S, Schäfer R, Regenbrecht CRA, Henderson D, Lange B, Haybaeck J, Keilholz U, Hoffmann J, Lehrach H, Yaspo ML. Molecular dissection of colorectal cancer in pre-clinical models identifies biomarkers predicting sensitivity to EGFR inhibitors. Nature Communications 2017. 8:14262. *(Impact Factor: 12.353)*

Liebs S, Keilholz U, Kehler I, Schweiger C, Haybäck J, Nonnenmacher A. Detection of mutations in circulating cell-free DNA in relation to disease stage in colorectal cancer. Cancer Medicine 2019; 8(8):3761-3769. *(Impact Factor: 3.491)*

Liebs S, Nonnenmacher A, Klauschen F, Keilholz U, Vecchione L. Liquid biopsy assessment of synchronous malignancies: a case report and review of the literature. ESMO Open 2019; 4:e000528. *(Impact Factor: 5.329)*

Yap SA, Münster-Wandowski A, Nonnenmacher A, Keilholz U, **Liebs S**. Analysis of cancer-related mutations in extracellular vesicles RNA by Droplet DigitalTM PCR. BioTechniques 2020; 69:2, 99-107. *(Impact Factor: 1.993)*

Liebs S, Eder T, Klauschen F, Schütte M, Yaspo ML, Keilholz U, Tinhofer I, Kidess-Sigal E, Braunholz D. Applicability of liquid biopsies to represent the mutational profile of tumor tissue from different cancer entities. Oncogene. 2021 Jul; doi: 10.1038/s41388-021-01928-w. *(Impact Factor: 9.867)*

Staudte S, Klinghammer K, Jurmeister PS, Jank P, Blohmer JU, **Liebs S**, Rhein P, Hauser AE, Tinhofer I. Multiparametric Phenotyping of Circulating Tumor Cells for Analysis of Therapeutic Targets, Oncogenic Signaling Pathways and DNA Repair Markers. Cancers. 2022 Jun; doi: 10.3390/cancers14112810. *(Impact Factor: 6.575)*

Acknowledgments

Nach vielen Jahren intensiver Arbeit ist es nun an der Zeit mich bei denjenigen Menschen zu bedanken, die mich in dieser herausfordernden Phase meines Lebens begleitet haben und ohne welche die Anfertigung dieser Dissertation nicht zustande gekommen wäre.

Zu besonderem Dank bin ich meinem Doktorvater Prof. Dr. Ulrich Keilholz für die gezielte fachliche Unterstützung während meiner gesamten Promotion verpflichtet. Ihr klinisches und wissenschaftliches Fachwissen trugen maßgeblich zur Konzeption und Fertigstellung dieser Arbeit bei. Ihr Vertrauen in mein Können hat mich stets menschlich und beruflich dazulernen und wachsen lassen. Ebenso möchte ich mich bei Prof. Dr. Ingeborg Tinhofer-Keilholz bedanken, die mit ihren kritischen Betrachtungen wichtige Aspekte zu meiner Arbeit beigetragen und sie somit auf ein höheres wissenschaftliches Niveau angehoben hat.

Meinen wissenschaftlichen Kollegen am Charité Comprehensive Cancer Center danke ich für die jahrelange Unterstützung im Labor, der kritischen Auseinandersetzung mit meinen Daten, das mir entgegengebrachte Vertrauen und die gute Zeit während der Mittagspausen. Besonders hervorzuheben sind hier Loredana Vecchione und Anika Nonnenmacher.

Außerdem möchte ich dem Deutschen Krebsforschungszentrum, der Berliner Krebsgesellschaft und der Sonnenfeld-Stiftung für die finanzielle Förderung unserer Arbeit danken, als auch allen Kooperationspartner, die einen nicht zu vernachlässigbaren Teil zu meinen Projekten beigetragen haben. Meinen herzlichen Dank möchte ich zudem auch allen Studienteilnehmern für ihre Bereitschaft zur Teilnahme aussprechen.

Mein besonderer Dank gilt meinen ehemaligen und aktuellen Kollegen, die mir in den vergangenen Jahren nicht nur auf wissenschaftlicher Ebene weitergeholfen, sondern vor allem Kraft und Mut zur Vollendung meiner Promotionsschrift gegeben haben. Ihrer Unterstützung gebührt hier mein voller und besonders herauszustellender Dank und ich schätze mich glücklich, viele wunderbare Menschen als Freunde in dieser Zeit dazu gewonnen zu haben. Hierzu zählen Dr. Franziska Niehr und Robert Konschak, die mir den Einstieg in meine akademische Laufbahn vereinfacht haben. Dr. Diana Schoppe und Dr. Evelyn Kidess-Sigal haben mir nicht nur die Möglichkeit gegeben in dem von

ihnen geplanten Projekt einen wissenschaftlichen Beitrag zu leisten, sondern haben mich stets motiviert und unterstützt meine Ziele trotz einiger Rückschläge nicht aus den Augen zu verlieren. Mein ganz besonderer Dank gilt Christoph Hapke, Stephanie Staudte und Oliver Beyer, die meine Erfolge mit mir gefeiert und meiner Frustration ein offenes Ohr geschenkt haben. Ihr seid eine unverzichtbare Stütze in all den Jahren gewesen, die ich in meinem Leben nicht mehr missen möchte. Das Gleiche gilt für diejenigen Freunde in meinem Leben, die mich bereits aus der Schule oder dem Studium kennen und auch diese Etappe in meinem Leben mit mir zusammen durchgestanden haben.

Eine herausragende Stellung in jeglicher Hinsicht nimmt meine Familie und insbesondere meine Mama, Kerstin Liebs, ein. Mein größter Dank gilt ihr für ihre uneingeschränkte Unterstützung, Liebe und ungebrochenen Zuversicht in jeder Lebenslage. Du warst immer der größte Rückhalt für mich, weshalb ich dir diese Arbeit widme. Ich kann dir gar nicht genug danken für alles, was du bereits für mich getan hast und in der Zukunft wohl auch noch tun wirst.