JOHANNES KEPLER
UNIVERSITÄT LINZ

CIRCULATING TUMOR DNA

A NOVEL PROGNOSTIC MARKER FOR EARLY EVALUATION OF RESPONSE TO TREATMENT IN PANCREATIC CANCER

Eingereicht von

Patrick Kirchweger, MD

Angefertigt am

Gastrointestinal Cancer Center, Ordensklinikum Linz

First Supervisor

PD Holger Rumpold, MD

Second Supervisor

PD Helwig Wundsam, MD

Assistant Thesis Supervisor

Univ.-Prof. Reinhold Függer, MD

Juli 2023

Dissertation

zur Erlangung des akademischen Grades

Doctor of Philosophy

im Doktoratsstudium

Medical Sciences

JOHANNES KEPLER UNIVERSITÄT LINZ
Altenberger Straße 69
4040 Linz, Österreich
jku.at

I EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne fremde Hilfe verfasst, andere als die angegebenen Quellen und Hilfsmittel nicht benutzt bzw. die wörtlich oder sinngemäß entnommenen Stellen als solche kenntlich gemacht habe.

Forschungsergebnisse während des Doktoratsstudiums und dieser Dissertation wurden unter anderem im European Journal of Surgical Oncology[1], World Journal of Clinical Oncology[2] und im Frontiers in Oncology[3] veröffentlicht.

Linz, 25.07.2023

II PREFACE

This dissertation project was conducted between 2019 and 2022 at the Ordensklinikum Linz, an Austrian referral oncological and high-volume pancreatic cancer center.

Interdisciplinary management and in particular, the junction of internal and surgical oncology represent the backbone of optimal cancer treatment. As a surgical resident, my main profession consumes the majority of time available in a business day, hampering sample collection during this period. Thus, conducting this study would not have been possible without our diligent and reliable nurses at the Department of Internal Medicine I and at the Department of Surgery for their assistance in study-sample collection.

Besides expressing the warmest gratitude for having the privilege of working in the team at the Gastrointestinal Cancer Center in cooperation with the Department of Diagnostic and Interventional Radiology at the Ordensklinikum Linz or the Institute of Human Genetics at the Medical University of Innsbruck, to mention just a few, I want to especially thank Jonathan Burghofer for his supportive attitude in planning, sample processing, and transport concerns, along with his team at the Laboratory for Molecular Genetic Diagnostics at the Ordensklinikum Linz.

Studies published within this PhD program leading to this dissertation have been presented at the Austrian Congress of Surgery 2022, the ACO ASSO Congress 2022, the OeGHO FJT 2022, the ESMO TAT 2022, and the German Congress of Surgery 2023, and have been awarded the ACO ASSO Prize 2022 for the Best Publication in Surgical Oncology, the ACO ASSO Best Abstract Prize 2022, the ESMO Merit Travel Grant 2022, the OeGHO FJT Poster Prize 2022, the Dr. Walter Pilgerstorfer Prize 2022, the ESMO TAT Best Poster Prize 2023, the OeGHO FJT Best Submitted

Abstract Prize 2023, and the OeGHO Best of ASCO Innovation Award 1st Prize. Moreover, results of this dissertation have led to further hypothesis generation and consecutive project design that have earned the Hans Werner Waclawiczek Prize for the best formulation of a clinically oriented question with clinical and scientific relevance to change daily surgical routine in the future at the Austrian Congress of Surgery 2023.

Results from this PhD program leading to this dissertation have been published in the European Journal of Surgical Oncology, the World Journal of Clinical Oncology, and Frontiers in Oncology.[1–3]

I am thankful to have had the opportunity to go through the first-ever class of the PhD Medical Sciences curriculum at the Johannes Kepler University in Linz and to have enjoyed medical training as a physician at the Ordensklinikum Linz. It is a privilege to be part of these institutions.

Lastly, I want to thank my supervisors Holger Rumpold, Helwig Wundsam, and Reinhold Függer for their guidance, advice, and support in all aspects during my PhD studies, and especially Holger for affirming my belief in the dogma that science is not a sprint but a marathon. It truly was.

Dedicated to my dear family.

Thank you.

III TABLE OF CONTENTS

IV LIST OF FIGURES

Figure 14: ctDNA kinetics according to response groups in mPDAC.

Figure 15: OS and PFS according to ctDNA decrease under 57.9% of the base value at week 2 for patients with mPDAC.

Figure 16: Workflow LIQUIPANC trial.

V LIST OF TABLES

VI GERMAN SUMMARY (ZUSAMMENFASSUNG)

Einleitung und Rationale

Im Blut zirkulierende Tumor DNA (ctDNA) wird intensiv als vielversprechender Biomarker in vielen Tumorentitäten beforscht. Insbesondere die Möglichkeit einer minimalinvasiven Methode (einfache Blutabnahme) zur Anzeige der Tumorlast in Echtzeit (gleicht den Möglichkeiten einer invasiven Gewebeentnahme mittels Punktion; deshalb Liquid Biopsy) birgt großes Potenzial für die Umsetzbarkeit im klinischen Alltag. Unter den gastrointestinalen Malignomen, besteht insbesondere beim Pankreaskarzinom aufgrund der nach wie vor schlechten Prognose und steigenden Prävalenz (es wird geschätzt, dass das Pankreaskarzinom bis 2030 für die zweitmeisten tumorassoziierten Tode verantwortlich sein wird) ein hoher Bedarf an einem verlässlichen Tumormarker. Es konnte bereits gezeigt werden, dass die prätherapeutische Nachweisbarkeit von Mutationen in der Liquid Biopsy mit einem schlechteren rezidivfreien und Gesamtüberleben einhergeht. Das Hauptziel dieses Dissertationsprojekts ist es, die klinische Anwendbarkeit und das Potenzial der ctDNA, Therapieansprechen unter laufender palliativer Chemotherapie anzuzeigen und gegebenenfalls in Zukunft einen frühzeitigen Therapiewechsel zu ermöglichen.

Methoden

Dazu wurden prospektiv Proben (prätherapeutisch und alle 2 Wochen während der Chemotherapie bis zum Restaging) von insgesamt 171 Krebspatienten (75 metastasierte Pankreaskarzinome, 96 metastasierte Kolorektalkarzinome) gesammelt und mittels ddPCR getestet. Pankreaskarzinompatienten wurden auf Mutationen in KRAS G12/13 getestet; wenn diese negativ ausfielen, wurde zusätzlich auf KRAS Q61 getestet. Weiters wurden die Kolorektalpatienten hinsichtlich des histopathologischen Mutationsergebnisses und beide Tumorentitäten zusätzlich auf die derzeitigen Goldstandard-Biomarker (CA 19-9 und CEA)

getestet. Die Ergebnisse wurden mit dem radiologischen Restaging nach 3 Monaten und dem klinischen Outcome korreliert.

Ergebnisse

Die prätherapeutische Detektionsrate lag bei 64,3% (Pankreas) bzw. 88,5% (Kolorektal) und es konnte ein Grenzwert (57,9%) für das Therapieansprechen über den ctDNA Verlauf bereits bei Woche 2 mittels ROC-Analyse für beide Tumorentitäten festgestellt werden. Bezüglich des Hauptfokus des Dissertationsprojekts konnte die klinische Bedeutung der ctDNA beim Pankreaskarzinom im Folgenden erfolgreich evaluiert werden:

(1) Das Absinken unter diesen Wert nach 2 Wochen Systemtherapie zeigte eine hohe prognostische Signifikanz (OS: 5,7 vs. 11,4 Monate, p=0,006; PFS: 2,5 vs. 7,7 Monate, p<0,000). Auch der Einfluss auf das Überleben der prätherapeutischen Proben war unabhängig zur Therapielinie von signifikant hohem prognostischem Wert.

(2) Durch das Absinken unter den errechneten Grenzwert nach 2 Wochen Systemtherapie wurde einerseits ein Therapieansprechen und andererseits durch das nicht-Erreichen dieses Grenzwertes ein Progress mit einer Spezifität von 100% und einer Sensitivität von 91,67% angezeigt (AUC=0,918).

Schlussfolgerung

Die Evaluierung der Änderung der ctDNA mit unserer Methode konnte mit einem kostengünstigen, kommerziell erhältlichen und einfach in der klinischen Routine einsetzbaren Test-Kit sowohl als starker prognostischer Marker als auch als Marker für das frühe Ansprechen auf die Chemotherapie (nach 2 Wochen anstatt bisher 3 Monaten) bestätigt werden. Die Ergebnisse unserer Teststrategie sind deutlich schneller verfügbar als Breitspektrumanalysen (z.B. NGS) und somit bereits klinisch relevant, da sie sofort ohne potenziellen Therapieverzug in der Praxis umgesetzt werden könnten.

VII ABSTRACT

Introduction

Circulating tumor DNA (ctDNA) is a novel promising biomarker with the potential of minimally invasive real-time display of tumor burden that has been shown to correlate with relapse rate and overall survival in several tumor entities. Within gastrointestinal cancers, there is an especially urgent need for reliant biomarkers in pancreatic cancer as prognosis remains poor despite increasingly aggressive treatment regimes in recent years, and its prevalence is rising (estimated to become the second most common tumor-associated cause of death by 2030). The prognostic impact and potential superiority of the early change of ctDNA courses during systemic therapy to conventional radiological restaging have not yet been shown in metastatic pancreatic cancer (mPDAC). The major aim of this PhD project was to test the potential of ctDNA for a clinically applicable indicative response to treatment of palliative chemotherapy to eventually allow an early change of treatment in the future.

Material and methods

Liquid biopsy samples and patient data of 171 cancer patients (75: pancreatic cancer, 96: colorectal cancer) were prospectively collected and analyzed for ctDNA detection using commercially available ddPCR test kits (KRAS G12/13 and KRAS Q61, if initially negative for G12/13) for pancreatic cancer and individual test kits depending on the histopathologically detected mutation for colorectal cancer in addition to the current gold standard biomarkers (CA 19-9 and CEA) before treatment initiation and every two weeks thereafter until restaging during systemic therapy in the course of clinical routine.

Results

Pretherapeutic detection rates were 64.3% for pancreatic cancer and 88.5% for colorectal cancer, and a definitive cut-off of 57.9% of the base value for ctDNA kinetics at week 2 was able to be

defined using ROC analysis, which was clinically significant for both tumor entities. Regarding the main focus of this project, the clinical impact of ctDNA in pancreatic cancer, the following could be evaluated successfully:

(1) A decrease under this value after 14 days of systemic chemotherapy was of significant prognostic relevance (OS 5.7 vs. 11.4 months, p=0.006; PFS 2.5 vs. 7.7 months, p<0.000).

(2) Furthermore, ctDNA kinetics at week 2 without a decrease under 57.9% of its base value correctly predicted progressive disease, whereas a decrease under 57.9% of its base value correctly predicted response to treatment (specificity 100%, sensitivity 91.67%, AUC=0.918).

Conclusion

Aside from being of great prognostic relevance and the possibility of the early stratification of patients with a worse clinical outcome, the test strategy evaluated in this study is easily applicable in clinical routine and allows the immediate and safe ctDNA-guided evaluation of response to treatment in patients with metastatic pancreatic cancer irrespective of treatment line (after two weeks instead of the current gold standard of three months). Results from our testing regimen are available more quickly than broad spectrum analyses (e.g. NGS) and thus already clinically relevant.

VIII INTRODUCTION

Circulating tumor DNA (ctDNA) has emerged as a promising biomarker for diagnosis, prognosis, and potentially treatment monitoring in several tumor entities.[2] Deliberated into the patients' blood circulation by necrotic or apoptotic cells, ctDNA represents a tumor-specific proportion (<0.01%) of the total amount of cell-free DNA (cfDNA).[4]

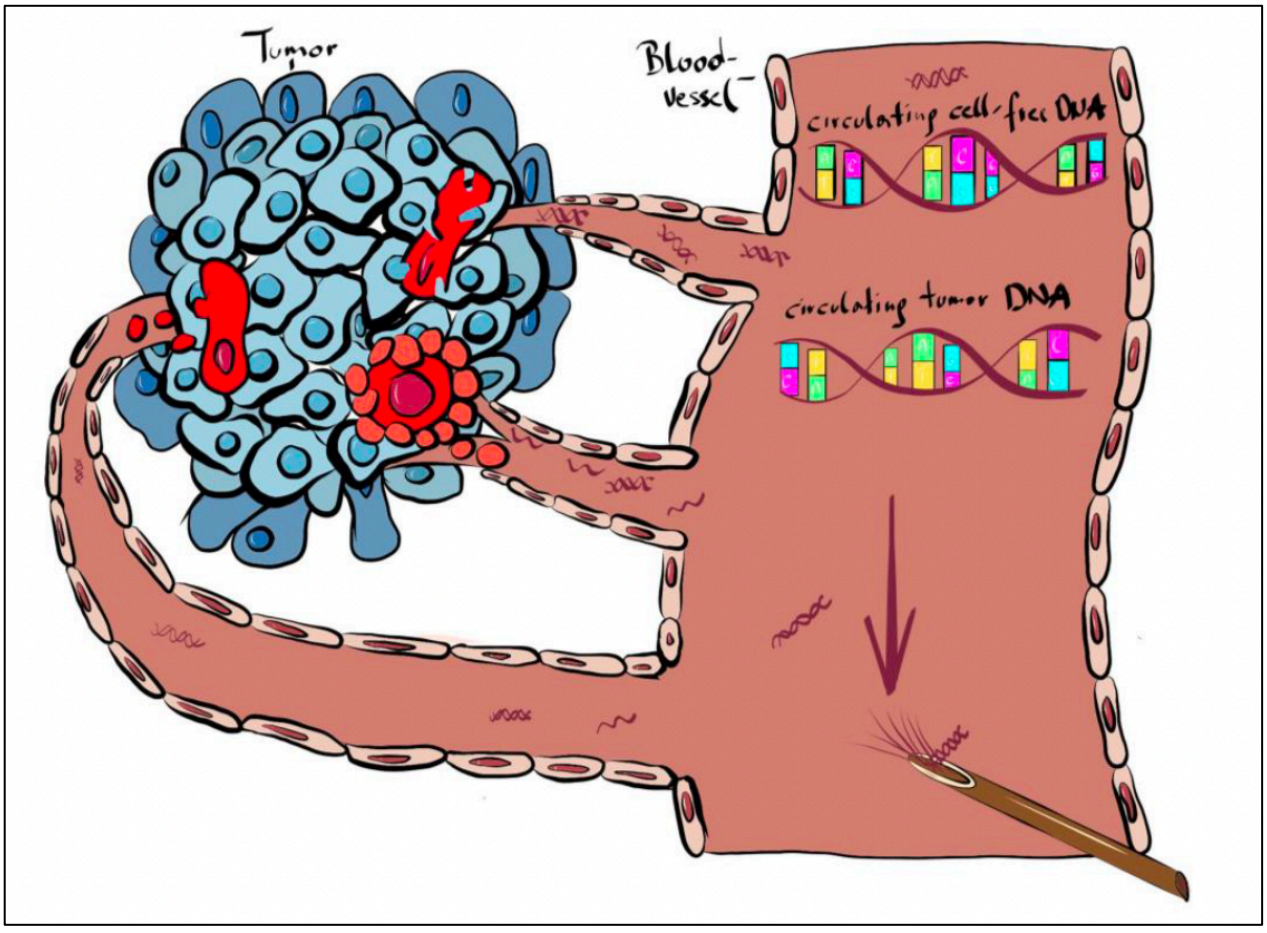


Figure 1: Schematic representation of liquid biopsy.

Circulating tumor DNA (ctDNA) is derived as a tumor-specific subset of circulating cell-free DNA (cfDNA) shed into the patients' blood circulation by necrotic or apoptotic tumor cells. ctDNA can be easily assessed through simple blood collection and later detected and quantified by e.g. PCR or DNA sequencing methods. The figure is depicted by courtesy of my collaborator Dr. Jonathan Burghofer from the Laboratory for Molecular Genetic Diagnostics at the Ordensklinikum Linz, who modified the raw image from Schwarzenbach et al.[5]

Previous studies have proven ctDNA to be a minimally invasive available marker that is robust to disturbances affecting CA 19-9 levels and confirm its property in displaying actual tumor burden and even distinguishing localized from disseminated disease in pancreatic cancer.[1,6–8]

ctDNA detection is based on mutational tests on pathogenic alterations in peripheral blood screened with the likes of polymerase chain reaction (qPCR or ddPCR) or next-generation sequencing (NGS), resulting in very low limits of detection (LOD) of 0.1-0.001%.[9–12] However, mutational detection and thus reliable confirmation of a patient as ctDNA positive are hampered by the lack of a universally accepted cut-off as some studies regard any value for mutational allele frequency (MAF) or values >0.03% or >0.1% or any positive droplet (ddPCR) or three to five mutant droplets as positive.[7,13,14] Most experts agree on three mutant positive droplets without double positives as a reasonable cut-off.[3] Furthermore, the LOD depends on the range of gene loci analyzed and thus on the test kit used. Moreover, and in addition to technical aspects, the LOD is also heavily dependent on the amount of DNA templates available for diagnostics. Detection limits of 0.1% or less claim to detect one mutated molecule for a minimum of every 1,000 molecules in the sample. A lower input of transcripts results in a higher LOD (e.g. 500 templates result in an LOD of 0.2%), whereas a higher input of transcripts reduces the LOD (e.g. 2,000 templates result in an LOD of 0.05%). In addition, there are major differences regarding the materials used for DNA extraction, as for example FFPE (formalin-fixation and paraffin-embedding) and other techniques for solid tumor fixation can yield irritating artefacts that make it difficult to differentiate them from genuine variants.[15] This effect can be overcome by the fact that high amounts of tumor cells are usually contained within an FFPE block, which usually allows for high input of DNA in the laboratory.[15] The amount of DNA harvested by liquid biopsy to represent a clinically applicable approach, on the other hand, is often limited by the amount of justifiably collected blood per sample in clinical routine (about 30 ml in Austria), tumor entity, and stage.[2] However, liquid biopsy (LB) bears significant advantages over conventional histological tumor sampling: First, LB is minimally invasive as it only requires a simple blood draw, rendering additional EUS-FNA or subcutaneous tumor punctures are obsolete. Second, LB is easily

reproducible and allows disease monitoring by way of unlimited assessments over time, whereas histological samples can usually only be assessed at one or two points in time (during surgery or EUS-FNA before surgery). Third, LB provides information on the total systemic tumor burden and can give comprehensive information on the stage of the disease (disseminated vs. localized disease) compared to histological samples that only provide information on a small piece of the disease.[1]

KRAS, TP53, SMAD4, or CDKN2A mutations are found in the tissue of pancreatic cancer in over 90% of patients, indicating that pancreatic cancer (PC) development may depend on ERK pathway activation, WNT signaling activation, and the escape mechanisms of apoptosis.[16,17] Nevertheless, actual detection rates for PDAC in peripheral blood are significantly lower and vary between 10–75% (10–60% without knowledge on the mutational pattern through prior tissue analysis) depending on the tumor stage, assay used for detection, and metastasis distribution in disseminated disease.[18–23]

Amounts of cf/ctDNA in patients with metastatic pancreatic cancer reported in the literature are heavily heterogenous and levels vary from two-fold to ten-fold higher than in localized disease, but are mostly reported to be about three-fold higher (e.g. 17.5 ng/µl by Adamo et al). [2,18,24]

Early results within this project that were the subject of the dissertation of my collaborator Jonathan Burghofer revealed mean cfDNA concentrations of 3.13 (95% CI 2.63–3.62) ng/µl in 50 µl of elution of 40 patients with localized PC.[25] The respective proportion of extracted ctDNA (MAF) was 0.57% (95% CI 0.05–1.09).[25]

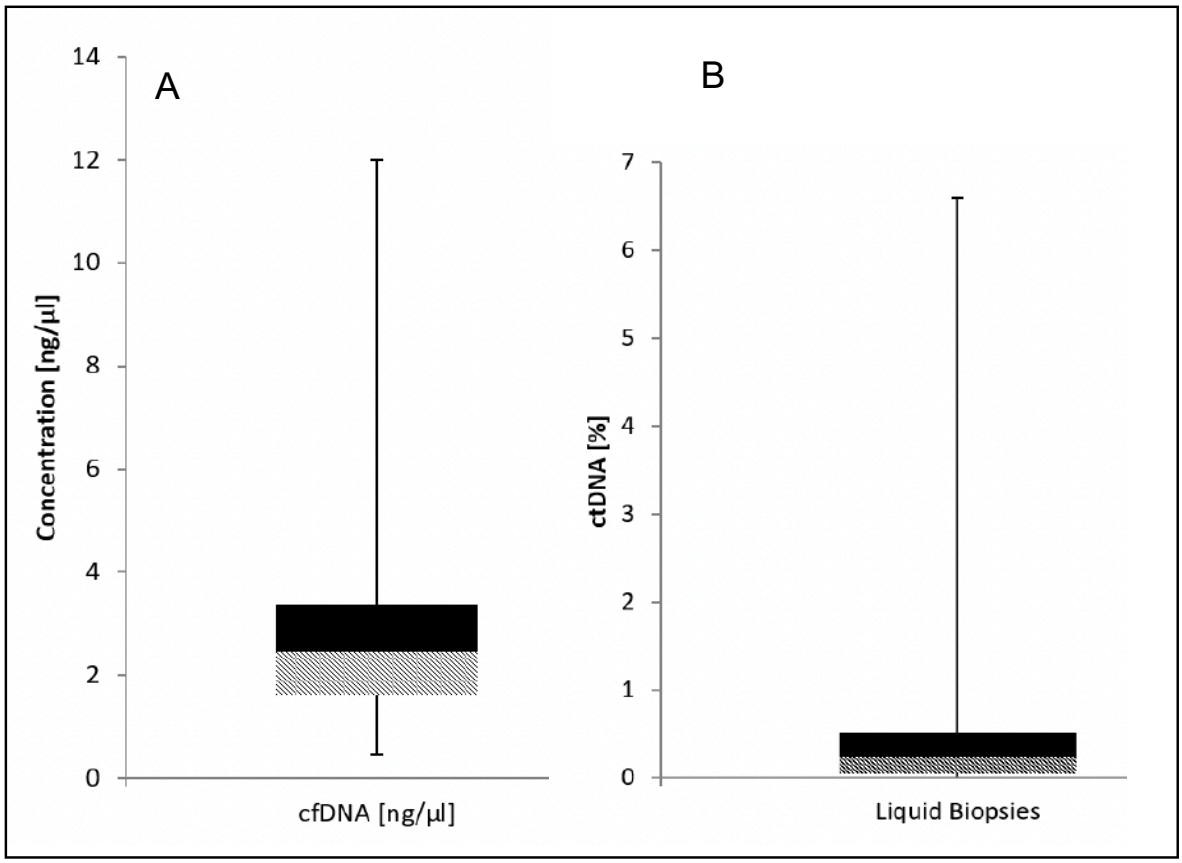


Figure 3: cfDNA (A) and ctDNA (B) concentration in localized pancreatic cancer.

Mean extracted (A) cell-free DNA (cfDNA) and respective (B) circulating tumor DNA (ctDNA) in patients with localized pancreatic cancer. This figure is depicted by courtesy of my collaborator Dr. Jonathan Burghofer from the Laboratory for Molecular Genetic Diagnostics at the Ordensklinikum Linz.[25]

However, although thresholds of ctDNA for considering a sample positive vary from author to author and are controversially reported in the literature, approximate detection rates can be assumed as follows: stage I (10–30%), stage II-III (43–54%), stage IV (about 60–67%), and up to over 90% in some studies, if only samples showing histologically verified mutation prior to testing of the periphery were evaluated.[2,18,9,19–22,26]

Aside from the stage of the disease, ctDNA detection rates vary between the different tumor entities.[2] Compared to other solid gastrointestinal tumors, but especially colorectal cancer (see

Table 3), pancreatic cancer is known to shed significantly fewer tumor-specific DNA fragments into the patients' blood, hampering detectability using liquid biopsy if not adjustable by increasing DNA input or more sensitive detecting approaches.[2,9]

Thus, the definition and evaluation of a treatment-relevant cut-off represented one major aim of this dissertation project. Furthermore, applied methods should be clinically applicable and therefore (I) time from diagnosis to potential clinical realization (e.g. early change of treatment) is required to be as short as possible (NGS and individual assay design can take up to several weeks depending on the underlying mutation) and (II) the applied method should be cost-effective to enable broad implementation beyond study conditions.

Currently, multiphase high-quality pancreas protocol computed tomography (CT) represents the gold standard for detection of response to treatment during palliative chemotherapy. However, this technique bears substantial limits of detection, as about one-third of patients with locoregionally advanced but visually non-disseminated PDAC in CT staging ultimately show peritoneal or hepatic metastases during upfront surgery or preoperative diagnostic laparoscopy.[27,28] In disseminated disease, CT restaging reveals progressive disease (PD) in about 23% of patients who have received an mFOLFIRINOX treatment and thus suffer from the side effects of an insufficient cytotoxic treatment such as neutropenia (23%), fatigue (12%), diarrhea (10%), nausea (9%), or febrile neutropenia (5%).[29] However, other studies have shown the potential of CA 19-9 in relapse detection by indicating patients with a worse clinical outcome as the increase of its levels from the baseline to weeks 6–8 after treatment initiation compared to declining or normalized values could indicate response to treatment.[30] At any rate, the predictive value of this plasma protein-based biomarker is limited as its change at month 2 did predict response to treatment, but the respective changes at month 1 did not.[31,32]

The current restaging strategy using CA 19-9 and CT is usually performed at three months after palliative chemotherapy.

Carbohydrate antigen 19-9 (CA 19-9) represents the only blood-based biomarker used for clinical application that is recommended by the NCCN (National Comprehensive Cancer Network).[18,33] Nevertheless, its usage is controversial as CA 19-9 levels can easily be irritated by common complications in pancreatic cancer care such as cholangitis or other inflammatory processes.[18]

Accounting for over 90% of all malignant pancreatic neoplasms, ductal adenocarcinoma (PDAC) represents the most common malignant disease of this organ, which is currently the fourth leading cause of cancer-associated death.[34,35] Independent of the patient's sex, pancreatic cancer represents a deadly and increasingly common tumor entity that is likely to become second most responsible for tumor-associated deaths worldwide within the next ten years.[34,36]

Despite major improvements in perioperative chemotherapy regimens and increasingly aggressive surgical approaches in recent years to enable complete tumor removal, real-world data evaluated from the SEER database showed a 31.7% five-year-overall survival rate in stage Ia and 11.8% in stage Ib respectively.[37] However, upfront resection is only possible in about 10% of patients, leaving 30% of patients classified as borderline resectable and 60% of patients diagnosed at a metastatic stage.[27] Thus, the five-year survival rate for patients with PDAC across the whole population is reported to be about 4.2%, whereas patients with disseminated disease show extremely low five-year survival rates of 0.5%.[27,37,38] Furthermore, in stage IV patients, median survival does not exceed one year despite the application of FOLFIRINOX or Gemcitabine/nab-Paclitaxel-based approaches.[18,37]

Based on this, the primary aim of this study was to evaluate ctDNA's potential to detect response to treatment earlier than the current gold standard and to evaluate the potential survival benefit in pancreatic cancer patients.

Second, this study aimed to evaluate the differences in different tumor entities (colorectal cancer and pancreatic cancer) and their association of ctDNA levels with metastasis distribution and tumor volume subsets.

In detail, the aim of this PhD project was the evaluation of:

(I) cancer-specific ctDNA detection rates (pancreatic cancer vs. colorectal cancer),

(II) correlation of ctDNA levels with tumor volume and established biomarkers (CEA, CA 19-9), and

(III) the prognostic impact of ctDNA change in pancreatic cancer.

IX MATERIAL AND METHODS

This PhD study was conducted at the Ordensklinikum Linz in the Gastrointestinal Cancer Center in cooperation with the Department of Internal Medicine I for Hematology with Stem Cell Transplantation, Hemostaseology, and Medical Oncology, and the Department of Surgery from 2019–2022.

Patients

Patients with metastatic pancreatic and colorectal cancer receiving palliative chemotherapy, irrespective of treatment line, were prospectively included at the Ordensklinikum Linz, a referral tertiary oncological center. The study was approved by the local Ethics Committee (EK 70/90) and written informed consent was collected from all participants. Patient treatment was unaffected by participation in the study, which was blinded to study data including ctDNA status. Clinical routine data including follow-up and survival status was collected from the medical records and prospective cancer registry of the oncological center.

Sampling and processing of plasma samples

For all patients and at every point in time (pretherapeutic on the day of chemotherapy start immediately before treatment initiation and analogously every two weeks until restaging), liquid biopsy samples (28.5 mL blood) for later ddPCR analysis were collected on the day before treatment application using cell-free DNA collection tubes (Roche, Basel, Switzerland). To prevent tumor-specific contamination with leukocyte DNA, leukocyte necrotic cell death was impeded by cell membrane stabilizers within the collection tubes.

Following a first centrifugation (200 g for ten minutes) and transfer of the supernatant into new 15 mL tubes (Sarstedt, Nürnbrecht, Germany), a second centrifugation (1500 g for ten minutes) yielded a total of 10 mL plasma. These tubes were stored at -20 °C in another set of fresh 15 mL tubes until further DNA preparation.

Processing of circulating cell-free DNA

Using the test kit CMG-1304 (Perkin Elmer, Waltham, Massachusetts, USA), which is a completely automated bead-based kit, 10 mL plasma was used for the preparation of circulating cell-free DNA (cfDNA) on the Chemagic 360. The DNA elution was prepared further using 70 µL of CMG-844 buffer from the same manufacturer. Omitting a natural loss as residual liquid in the beads, a total volume of 40–50 µL DNA was obtained per sample. Quantus fluorometers (Promega, Madison, Wisconsin, USA) were used for quantification and afterward, samples were stored at -20 °C again until actual ddPCR.

Droplet Digital PCR (ddPCR)

KRAS alteration screening was performed using the QX200™ Droplet Digital™ PCR system from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA). Initially, all samples were tested for KRAS G12/13 alterations using a commercially available multiplex screening kit from Bio-Rad Laboratories. Further testing for KRAS Q61 was conducted if the initial screening for KRAS G12/13 was negative. For analysis, 20 µL each were used for two reactions per sample resulting in 5 ng of cfDNA, whenever possible. The maximum available amount of cfDNA was used if this amount could not be assessed. Analysis took place with QuantaSoft™ Analysis Pro software (version 1.0.596) and the detection limit for a positive classification was defined as three mutant droplets (double-positive droplets did not count).

Radiological analysis

One and the same trained specialist in radiology performed all volumetric measurements and restaging assessments unaware of the actual laboratory, treatment, or outcome data. The MM Oncology Workflow configuration in the radiological program Syngo.via (Siemens Healthcare, Forchheim, Germany) was used for semiautomated lesion detection analysis of multiphase enhanced computed tomography imaging. Every automatic calculation performed by the software itself was checked by the radiologist and if the lesion margin was not set accurately, it was

corrected by hand. Lymph nodes were classified as pathological if the short axis diameter was larger than 10 mm (RECIST) and all solid organ lesions accounted for the sum of the largest tumor diameter (SLD) and the total tumor volume.[39]

Statistics

Statistics were prepared on R version 4.1.2 using the R survival package (survdiff, survfit, and coxph). Reverse Kaplan-Meier methods were used to calculate the median FUP (follow-up). Time from start of treatment until death was defined as OS (overall survival), whereas time from start of treatment until progressive disease was defined as PFS (progression-free survival). Continuous variables (e.g. age) as well as discrete variables (e.g. sex) were analyzed using the Mann-Whitney-U test for group comparison (ctDNA detectability). The individual's change in MAF (mutant allele frequency) as a quotient of the baseline value and the value at defined points in time (two weeks, four weeks, restaging) were classified as ctDNA kinetics. AUC (area under the curve) analyses from R package pROC (roc function) were used for identification of the ideal cut-off for ctDNA kinetics, representing the ideal combination possibility of specificity and sensitivity. For all analyses, a two-sided level of significance of 5% and a 95% confidence interval were applied. SPSS 26.0 was used to visualize survival data.

X RESULTS

Patient cohort

Within the scope of the program, patient data and liquid biopsy samples were collected from 211 cancer patients (75: pancreatic cancer, 96: colorectal cancer, 40: stomach/esophagus cancer).

The results of the dissertation focus on patients with pancreatic cancer and the possibility of predicting early response to treatment when undergoing palliative systemic chemotherapy, but will also elucidate the impact of ctDNA in metastasized colorectal cancer and its derivation depending on the tumor subsets for better understanding of the applicability of liquid biopsies in clinical routine.

The study design of the main focus of this dissertation project (early response to treatment during palliative chemotherapy for patients with metastatic pancreatic cancer[3]) is represented as a consort diagram in Figure 2.

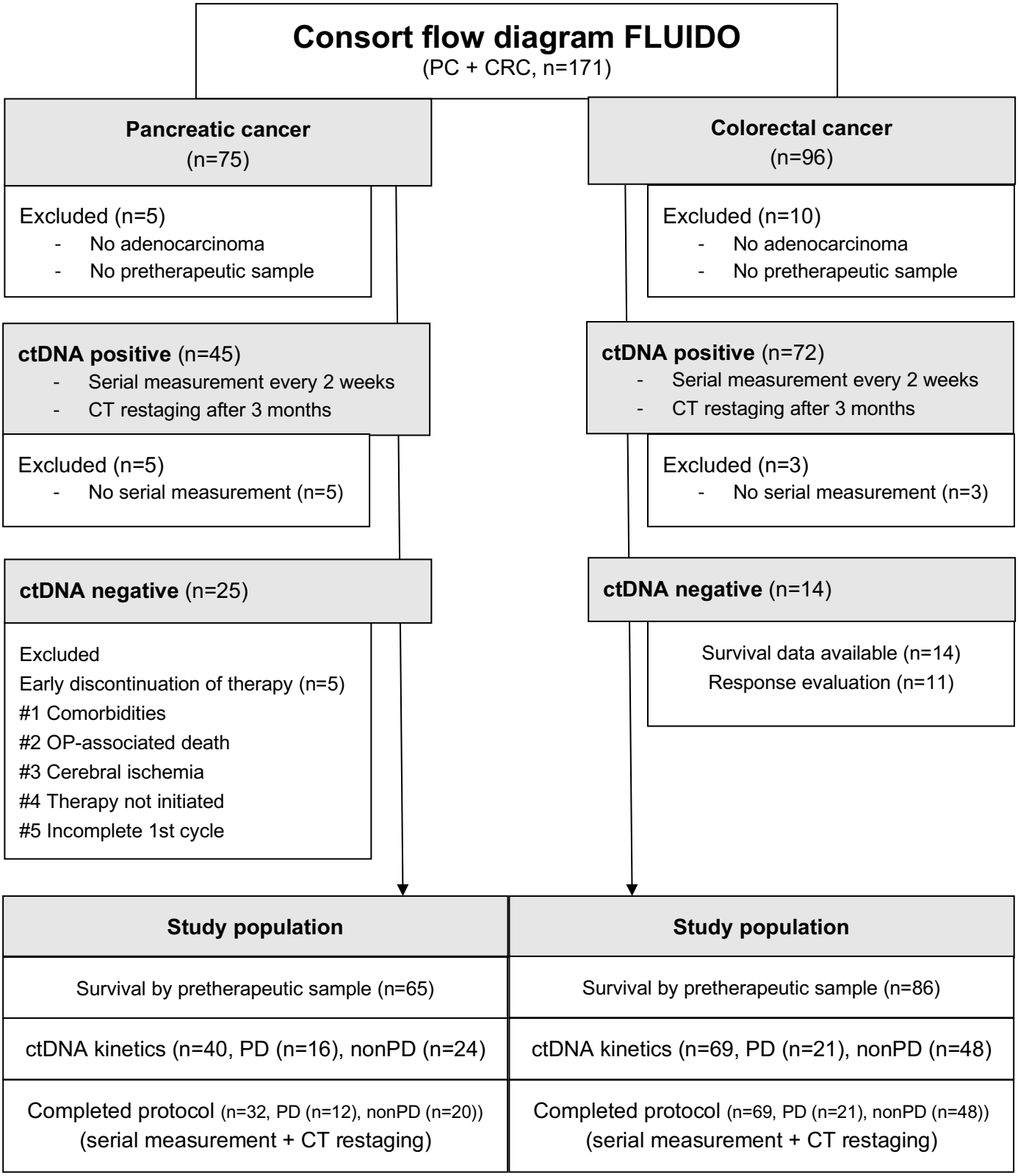


Figure 2: Consort flow diagram FLUIDO.

Consort flow diagram for patients with metastatic colorectal and pancreatic cancer enrolled in FLUIDO who were analyzed as part of this PhD project (excluding GEC patients).

Detection of ctDNA in different cancer types

The detection rate for pretherapeutic ctDNA in metastatic pancreatic cancer was 64.4%, whereas this figure was 88.5% in metastatic colorectal cancer (knowing the underlying tissue mutation). Moreover, 10-fold higher proportions of ctDNA were found in patients with colorectal cancer (CRC) than in those with pancreatic cancer. Furthermore, there is a difference in the amount of ctDNA in patients regarded as ctDNA negative compared to ctDNA positive within those entities (four-fold in PC, ten-fold in CRC), which leaves pancreatic cancer as a tumor entity with narrow limits of detection and a need for improvement in liquid biopsy analysis or even definitive treatment relevant cut-offs.

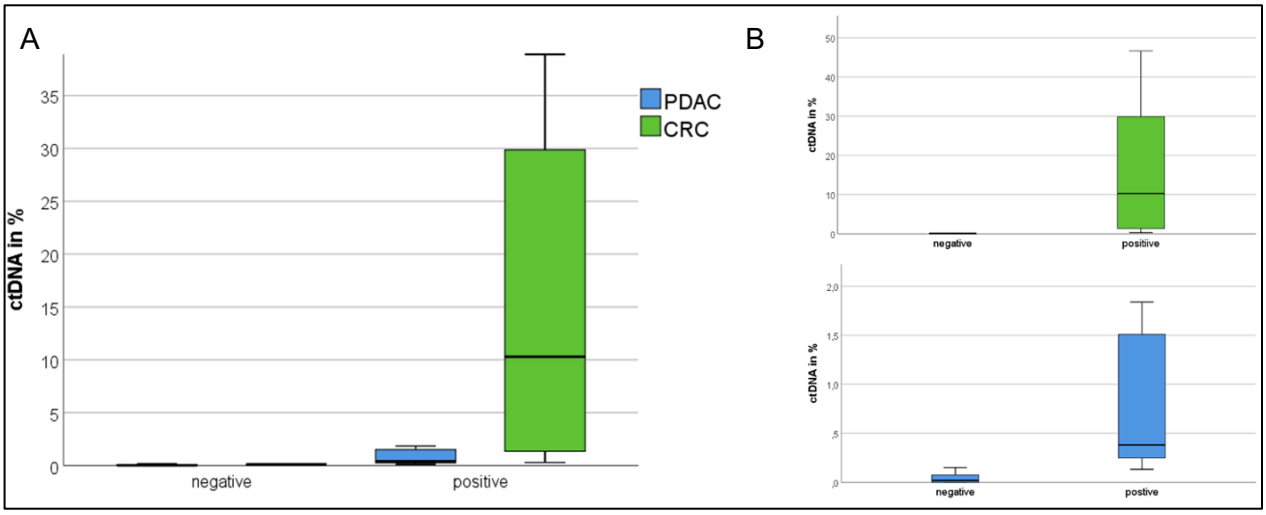


Figure 4: Proportion of extracted ctDNA in PDAC compared to CRC.

ctDNA fraction (ctDNA/cfDNA) of patients with metastatic colorectal (CRC) compared to pancreatic (PDAC) carcinoma in liquid biopsy.

Correlation of ctDNA with tumor volume, metastasis localization, and tumor markers

In contrast to local recurrence or lung metastases, significant results were found for total tumor volume (R=0.294, p=0.002) and liver metastasis volume (R=0.302, p=0.002), although the respective R values indicate weak correlations. The lung metastasis volume and tumor mass

volume of local recurrence did not show a significant correlation with the respective CEA values. Results are given in Figure 5.

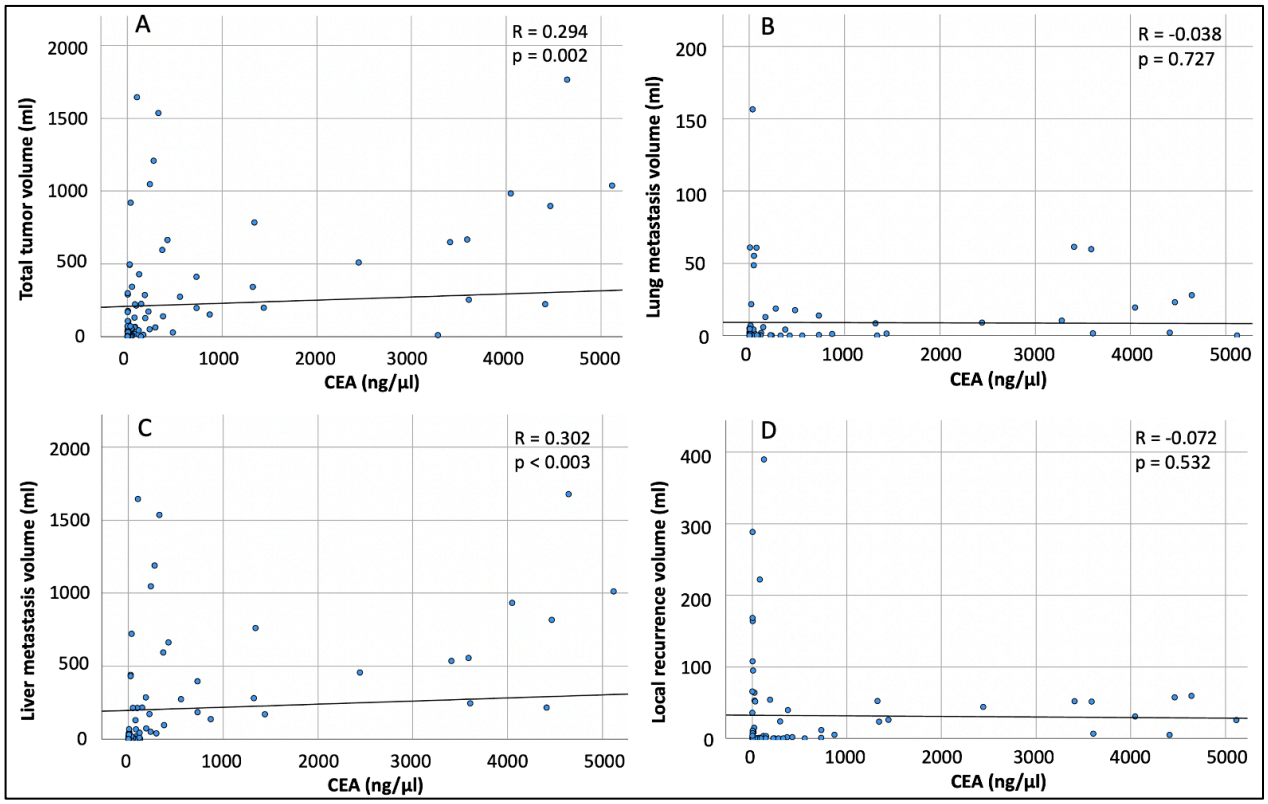


Figure 5: Correlation of CEA and actual morphometric tumor volume in CRC.

Correlation (Spearman's Rho) of radiologically assessed total tumor volume (A) and respective tumor volume subsets (lung (B), liver (C), local recurrence (D)) with conventional biomarker CEA values.

CtDNA, however, has turned out to be more tumor-specific than the current gold standard biomarker in colorectal cancer (CEA), displayed as actual volumetric tumor burden, with a significant correlation of MAF (%) with total tumor volume (R=0.507, p<0.000) and its respective subsets (Lung: R=0.351, p<0.000, Liver: R=0.520, p<0.000). The tumor volume of the actual primary tumor or its local recurrence did not correlate with ctDNA levels (R=-0.082, p=0.438). Results are given in Figure 6.

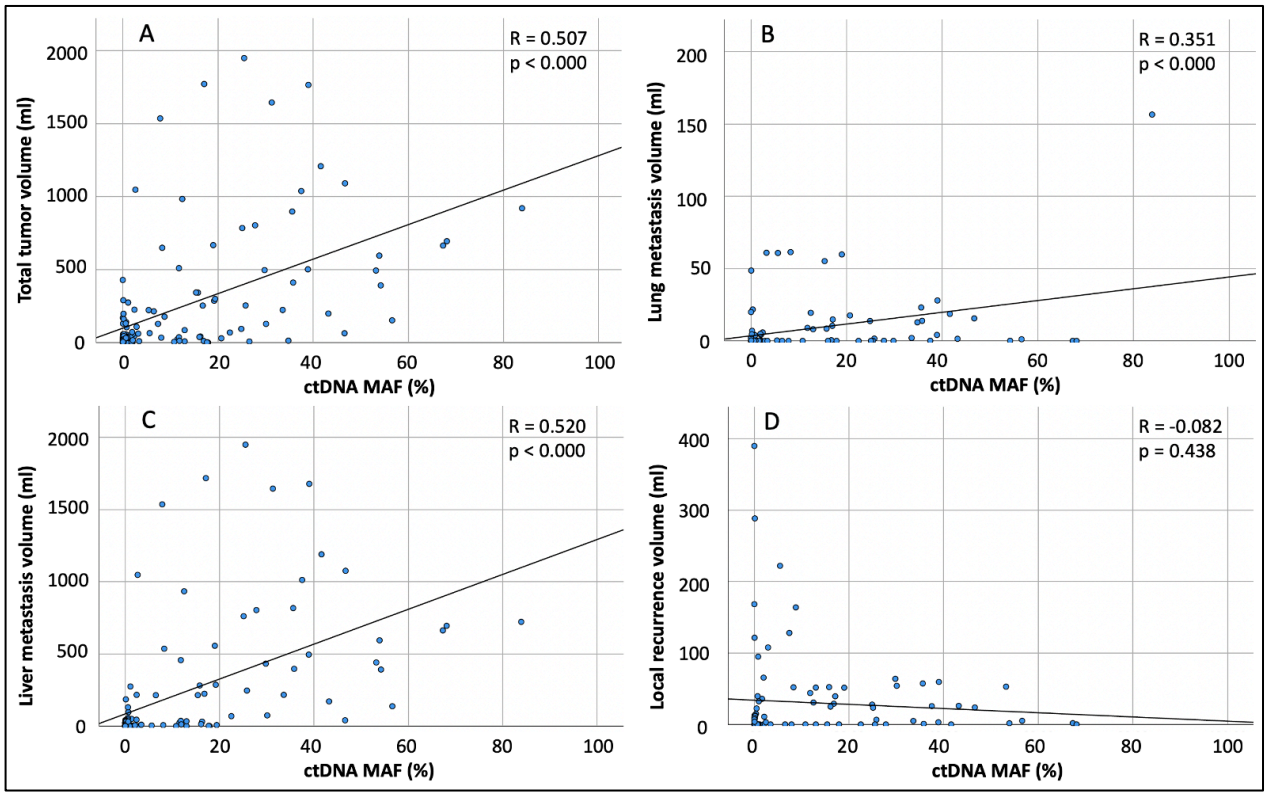
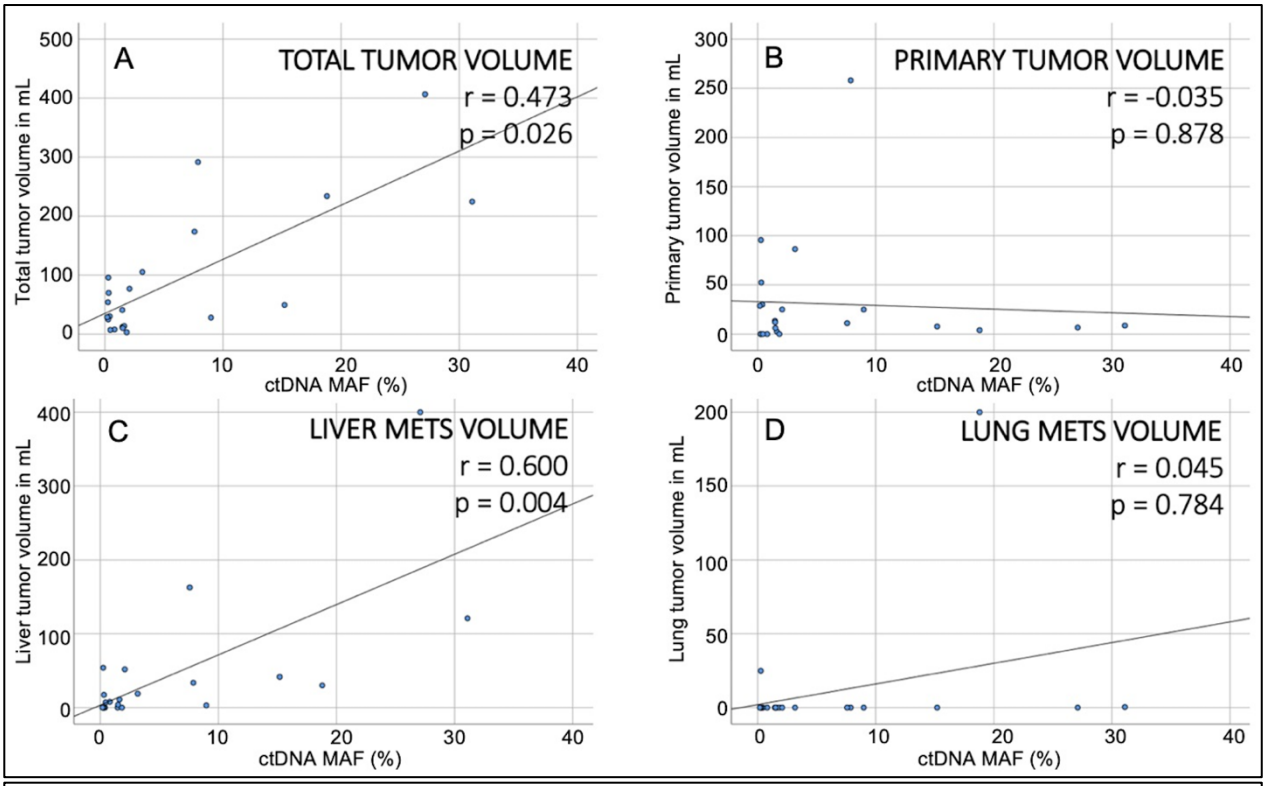


Figure 6: Correlation of ctDNA and actual morphometric tumor volume in CRC.

Correlation (Spearman's Rho) of radiologically assessed total tumor volume (A) and respective tumor volume subsets (lung (B), liver (C), local recurrence (D)) with circulating tumor DNA MAF (%) values.

Similarly, ctDNA significantly reflected total tumor volume (R=0.473, p=0.026) and liver metastasis volume (R=0.600, p=0.004) but not local recurrence volume (R=-0.035, p=0.878). Results for correlation with lung metastasis were hampered by a small sample size in this cohort but showed no significant correlation with ctDNA in mPDAC (R=0.045, p=0.784). CA 19-9, as the current gold standard biomarker in pancreatic cancer, was incapable of reflecting actual tumor volume or its subsets. Furthermore, significant correlation with positive lymph nodes (R=0.331, p=0.30) and lymph node ratio (R=0.393, p=0.009) could be observed harvested from the surgical specimen of localized pancreatic cancer with MAF (%). Results are given in Figure 7.



Localized	Volume (total)	p	Volume (PRIM)	p	LN (pos)	p	LN (ratio)	p
cfDNA (ng/µL)	.081	.619	.081	.619	.116	.391	.111	.413
ctDNA MAF (%)	.077	.573	.077	.573	.331*	.030*	.393*	.009*
CA 19-9 (U/mL)	.104	.488	.104	.488	.145	.320	.146	.318

Metastasized	Volume (total)	p	Volume (PRIM)	p	Volume (HEP)	p	Volume (PUL)	p	Volume (OTH)	p
cfDNA (ng/µL)	.397*	.009*	.034	.841	.391*	.011*	.017	.918	.011	.945
ctDNA MAF (%)	.473*	.026*	-.035	.878	.600**	.004*	.045	.784	.182	.254
CA 19-9 (U/mL)	.061	.709	.105	.543	.197	.391	.094	.574	.094	.574

Figure 7: Correlation of biomarkers with tumor volume subsets in PDAC.[1]

Correlation of cfDNA, ctDNA, and CA 19-9 with total tumor volume (total), primary tumor volume (PRIM), and metastatic lesion volume (HEP: liver, PUL: lung, OTH: other) in patients with localized and metastasized pancreatic cancer.

Levels of circulating tumor DNA correlated with total tumor volume (R^2=0.504, p=0.004), especially carried by the volume of liver metastases (R^2=0.543, p=0.002) in contrast to current gold standard biomarker CA 19-9 (total tumor volume: R^2=0.042, p=0.796; liver volume: R^2=0.160, p=0.307). Nevertheless, with both biomarkers, ctDNA (R^2=0.821, p<0.000) and CA 19-9 (R^2=0.720, p<0.000), the dynamics showed a correlation to the change in total tumor volume during the start of chemotherapy and restaging. However, unlike CA 19-9, whose change does not facilitate a prediction of response to treatment before six to eight weeks of chemotherapy, ctDNA dynamics between treatment initiation and week 2 (second chemotherapeutic administration) already did correlate with the dynamics of ctDNA until restaging (R^2=0.882, p<0.000) and of CA 19-9 until restaging (R^2=0.889, p<0.000). The respective scatter diagrams are depicted in Figure 8.

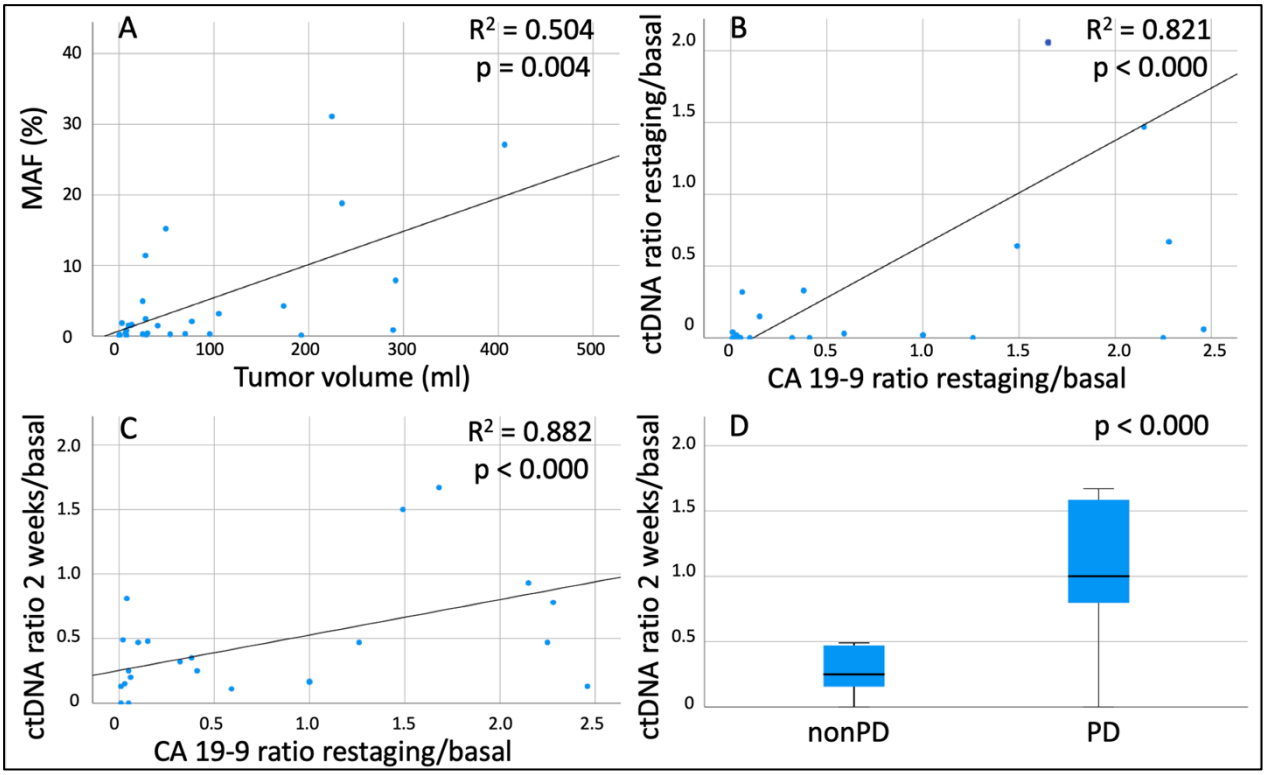


Figure 8: Correlation of ctDNA and tumor burden in patients with mPDAC.[3]

Correlation of MAF at the baseline with total tumor volume (A), the dynamic change of MAF from the baseline to restaging with the respective CA19-9 change (B), and similar relation for MAF change from the baseline to two weeks after treatment initiation with the change of CA 19-9 from the baseline to restaging (C), with ctDNA as an indicator for predicting tumor burden response after two weeks of antineoplastic treatment (D).

Abbreviations: ctDNA: circulating tumor DNA, MAF: mutant allele frequency, nonPD: non-progressive disease, PD: progressive disease, R^2: Spearman's Rho.

In localized pancreatic cancer (n=60), median CA 19-9 levels did not differ (p=0.334) between patients with pretherapeutic detectable ctDNA (169.9 U/ml, IQR 40.8–459.9) and patients who were regarded as ctDNA negative (397.4 U/ml, IQR 64.5–1357.6). Similarly, median cfDNA levels did not differ (p=0.165) between the patient groups regarding ctDNA detectability (3.42 ng/µl, IQR 0.58–12.93 vs. 0.92 ng/µl, IQR 0.61–1.51). Concentrations were 11.84 ng/ml (IQR 4.99–17.99) in positive compared to 2.12 ng/ml (IQR 1.14–4.81) in ctDNA negative patients (p=0.012), and the respective median MAF was 0.225 % (IQR 0.12–1.03) vs. 0% (IQR 0–0.008, p<0.000).

In metastasized disease, however, CA 19-9 levels were significantly higher (p<0.000) in ctDNA positive (3074.7 U/ml, IQR 983.2 – 32498.5) compared to ctDNA negative (267.2 U/ml, IQR 54.6–647) patients. In contrast, cfDNA concentrations did not differ between the groups (0.99 ng/µl, IQR 0.75–5.19 vs. 0.79 ng/µl, IQR 0.56–1.6, p=0.155). However, concentration of ctDNA differed significantly (p<0.000) depending on whether ctDNA was regarded as detectable (16.04 ng/µl, IQR 3.96–741.78) prior to treatment initiation or not (1.11 ng/µl, IQR 0.7–1.54). Ultimately, median MAF was 1.47% (IQR 0.25–7.58) when in patients regarded as positive compared to 0.05 (IQR 0.003–0.07) when regarded as negative (p<0.000).

Mutational spectrum detected in metastasized pancreatic cancer

The initial screening for KRAS G12/13 in liquid biopsy was able to detect mutations in 39 patients (55.7%), which increased by 8.6% (n=6) when testing initially negative patients for KRAS Q61. This resulted in an overall detection rate of 64.3% (n=45/70) within this study. The observed median MAF was 1.6%. The mutational distribution pattern is depicted in Figure 9.

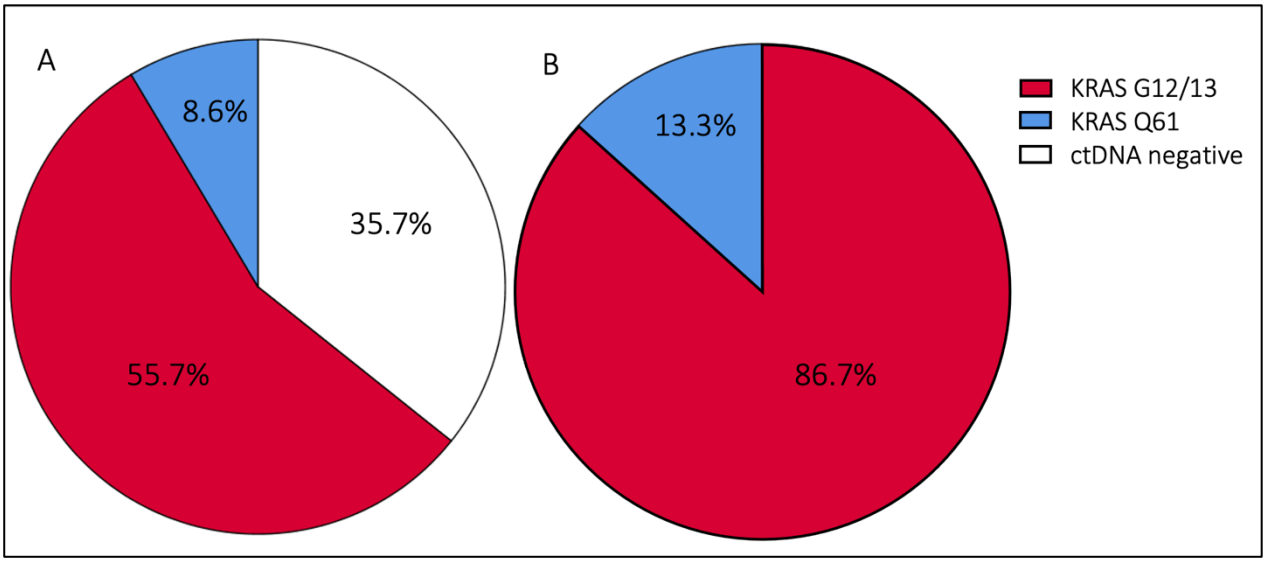


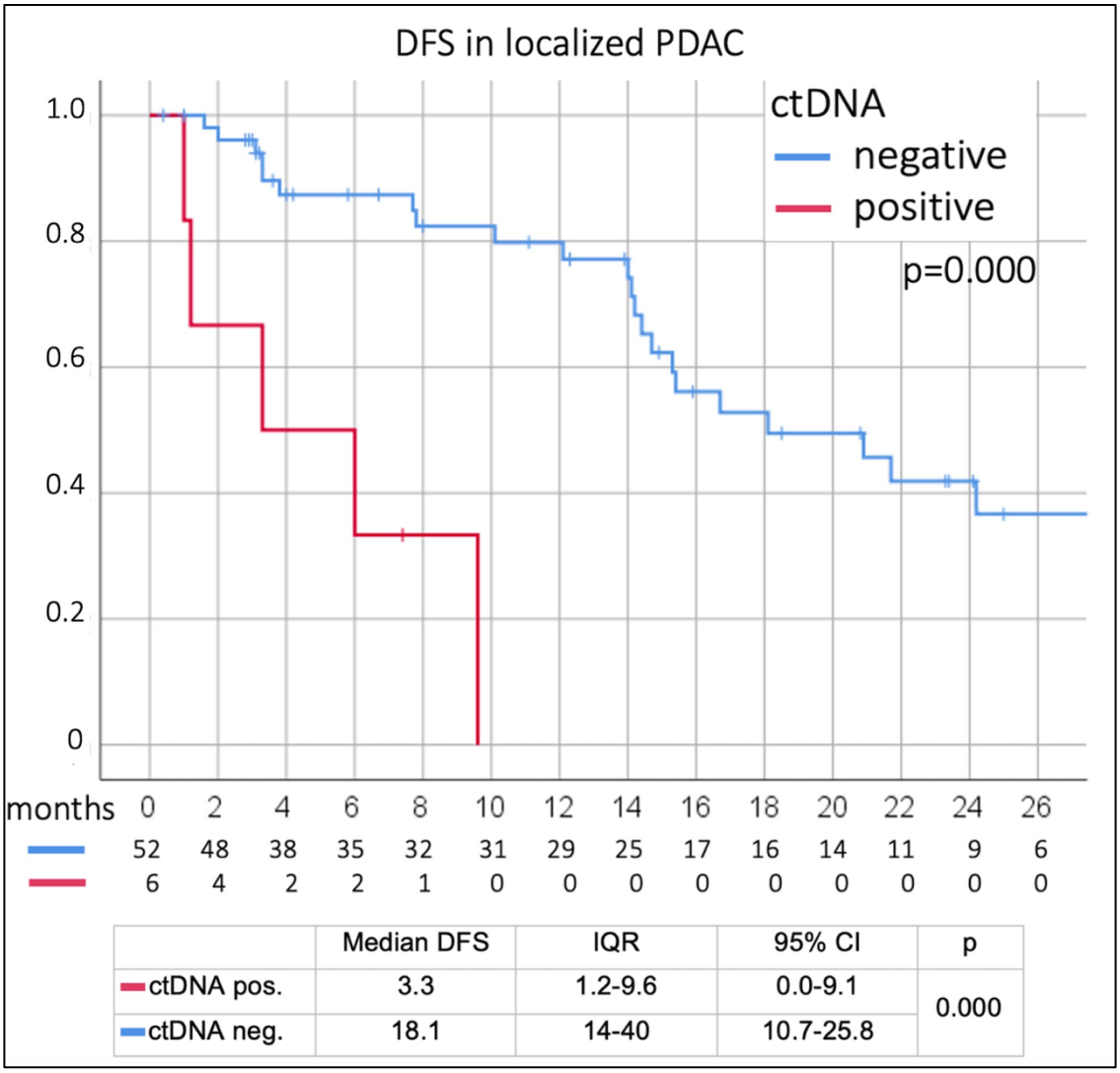
Figure 9: Mutational pattern in liquid biopsies of patients with mPDAC.[3]

Mutational distribution pattern of mPDAC patients (A) in the overall cohort and (B) in ctDNA positive patients. Additional testing for KRAS Q61 away from the most common KRAS G12/13 testing increases the detection rate by a further 8.6% (13.3% of ctDNA positive patients without KRAS G12/13 mutation bear Q61 alterations).

Results regarding the amount and proportion of CA 19-9, cfDNA, and ctDNA extracted from localized and metastasized pancreatic cancer patients within our cohort are given in Table 1.

Prognostic impact of ctDNA in localized pancreatic cancer

The prognostic impact of ctDNA in localized pancreatic cancer was particularly promising. Patients with detectable ctDNA prior to pancreatic resection for curative purposes suffered from early distant relapse (in the liver) within one year despite no radiological evidence of advanced disease prior to surgery, with DFS of 3.3 months (95% CI 0–9.1) compared to 18.1 months (95% CI 10.7–25.8) in patients without detectable ctDNA.[1]



	Median DFS	IQR	95% CI	p
ctDNA pos.	3.3	1.2-9.6	0.0-9.1	0.000
ctDNA neg.	18.1	14-40	10.7-25.8	

Figure 10: Disease-free survival of patients with pretherapeutic detectable ctDNA.[1]

Patients with localized pancreatic cancer undergoing pancreatic surgery for curative purposes and with preoperative detectable ctDNA experienced relapse at a median of 3.3 months compared to 18.1 months (p<0.000).

Prediction of response to treatment and prognostic impact in mPDAC

The primary aim of this thesis was to predict treatment response in patients with metastasized pancreatic cancer undergoing palliative chemotherapy. Results from the primary aim have been published in Front Oncol.[3]

A total of 70 patients with metastatic pancreatic cancer received systemic chemotherapy with a palliative intention, 71.4% of which as a first line treatment. Regarding ctDNA detectability, the cohort showed comparable demographics (sex, age, ECOG performance index, point in time of metastasis (syn/metachronous), treatment administration time, or time until CT restaging), which are depicted in Table 2. Higher ctDNA detection rates were observed in patients with liver dissemination (p=0.001), larger total tumor volume (p=0.016), largest tumor lesion diameter (p=0.078), or liver lesion volume (p=0.014), as well as increased levels of CA 19-9 (p<0.000) and advanced treatment lines (p=0.023).

In adherence to our clinic's guidelines, CT restaging was conducted at a median of 12.1 weeks (IQR 9.6–13.0). Restaging data and complete ctDNA courses including MAF (%) immediately before treatment initiation, at 14 days after treatment initiation, and at the time of restaging were available for 32 patients. Progressive disease (PD) was observed in 12 patients, whereas 20 patients experienced non-progressive disease.

ctDNA detectability before treatment initiation correlated with a worse clinical outcome in both median overall survival (7 months IQR 2.2–12.8 vs. 11.3 months IQR 7.2–not reached, p=0.045) and median progression-free survival (3.4 months IQR 2.1–9.2 vs. 10.8 months IQR 2.9–13.6), independent of treatment lines. In contrast, the mere presence of ctDNA in liquid biopsy prior to treatment initiation in 78 colorectal cancer patients appeared to have no impact on overall survival (p=0.653) and even did not reach statistical significance for progression-free survival (p=0.073), which is likely due to the higher proportion of mCRC (metastasized colorectal cancer) patients being ctDNA detectable (88.5%) compared to mPDAC (64.3%) and, of course, lPDAC (localized pancreatic cancer) (10–30%). A higher proportion of ctDNA detectability in the overall cohort increases the need for a definitive prognostic cut-off rather than the mere presence of ctDNA to identify patients at a higher risk for progressive disease and has been addressed as the major issue of this PhD project (Fig. 15–17). The impact of pretherapeutic ctDNA detectability on clinical outcome in metastatic pancreatic cancer patients is depicted in Figure 11.

Compared to patients who had detectable ctDNA at start of treatment but became ctDNA negative during the therapy, patients who stayed ctDNA positive throughout the entire therapy until restaging showed significantly worse OS (5.7 (IQR 4.2–7.0) vs. 12.8 (IQR 9.7–13.5) months, p=0.001) and PFS (2.9 (IQR 2.2–3.4) vs. 5.6 (IQR 4.0–11.3) months, p=0.019), independent of the treatment line applied.

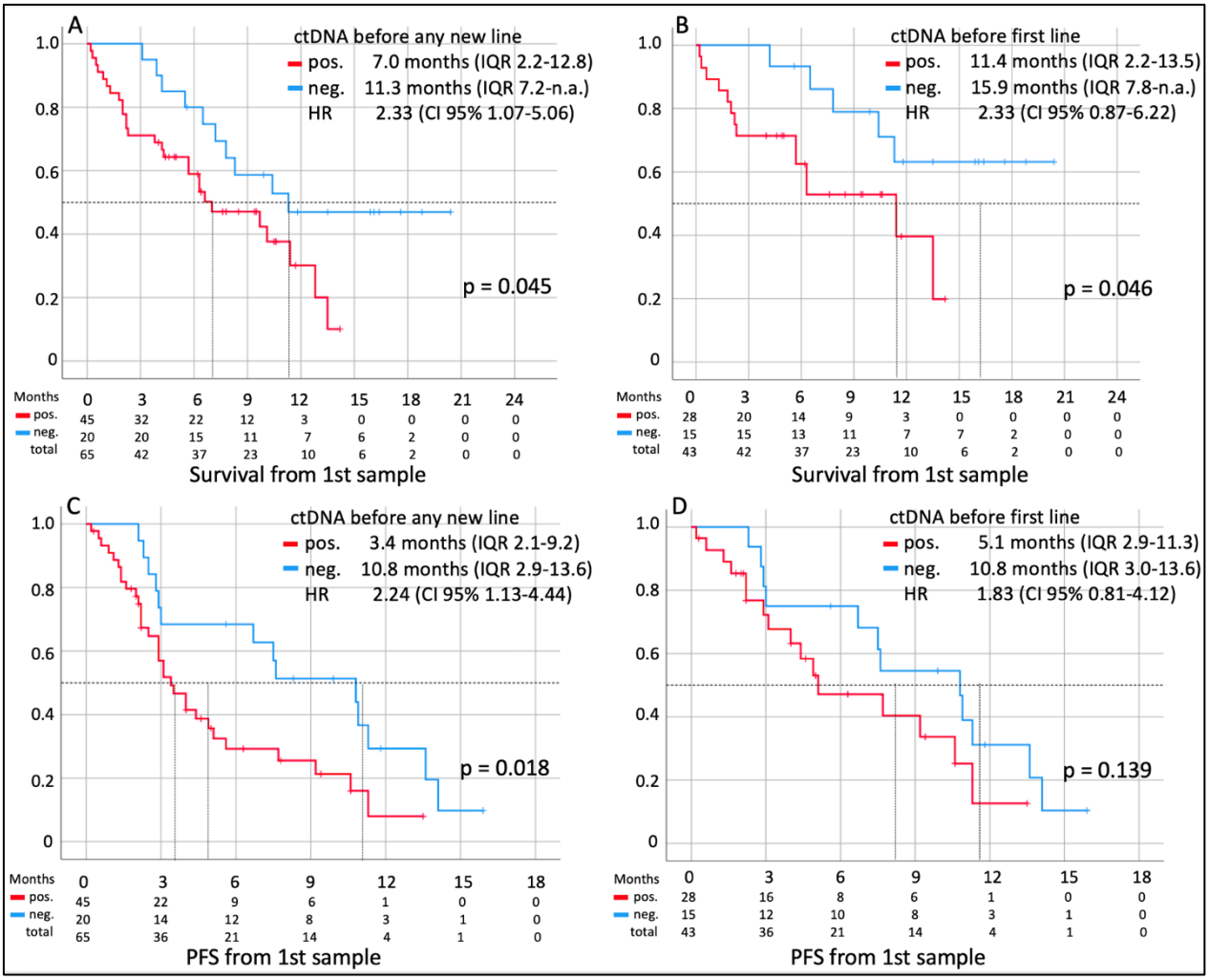


Figure 11: Outcome of pretherapeutic ctDNA detection in mPDAC.[3]

Pretherapeutic ctDNA detectability correlates with a worse OS (A–B) and PFS (C–D) in first line chemotherapy and regardless of treatment line.

Abbreviations: HR: hazard ratio, IQR: interquartile range, OS: overall survival, PFS: progression-free survival.

Furthermore, patients with detectable ctDNA (KRAS G12/Q61) at the time of treatment initiation but who turned negative during the course of treatment showed similar mean overall survival rates to patients without detectable ctDNA at the start of treatment in OS (12.8 (IQR 9.7–13.5 months) and PFS (10.6 (IQR 5.1–11.3 months). The prognostic impact of adherence to initial ctDNA course or the change of detectability under chemotherapy is visualized in Figure 12.

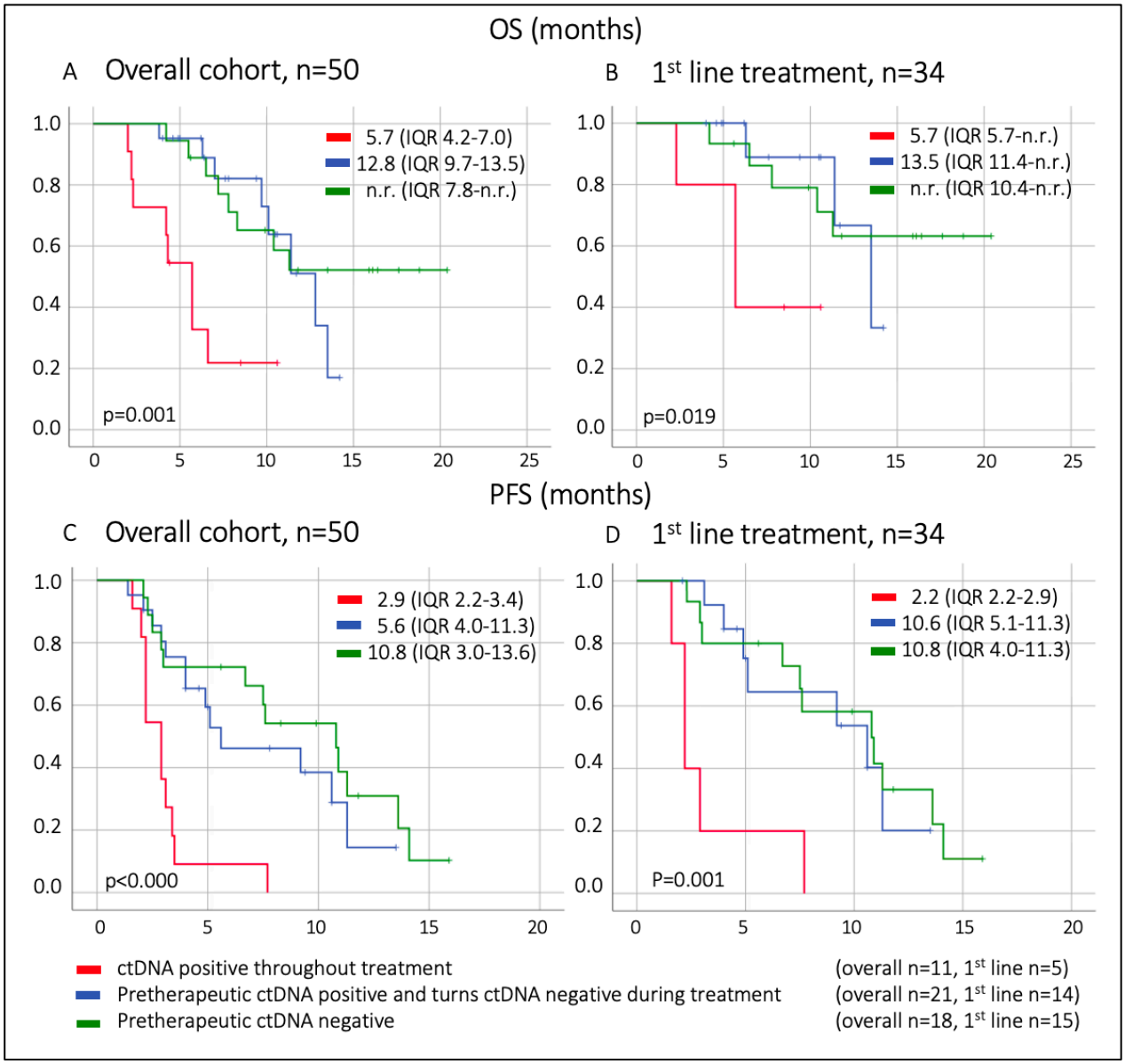


Figure 12: Prognostic impact of ctDNA change in mPDAC during treatment.[3]

Patients with pretherapeutic positive ctDNA who turned ctDNA negative during palliative chemotherapy (blue) had significantly improved OS and PFS compared to patients whose ctDNA was positive throughout the course of therapy (red) in the overall cohort (A, C) and in patients receiving first line treatment (B, D). Furthermore, it appears that patients turning ctDNA negative

during treatment showed similar OS and PFS to patients with pretherapeutic negative ctDNA (green).

Abbreviations: ctDNA: circulating tumor DNA, IQR: interquartile range, OS: overall survival, PFS: progression-free survival.

There was a significant difference in the ctDNA courses of patients with PD compared to nonPD at restaging (p<0.000), but also already after 14 days of systemic chemotherapy (p<0.000). Furthermore, a decrease of MAF under 57.9% of the initial value before treatment initiation was able to predict response to treatment in correctly distinguishing PD from nonPD with a sensitivity of 91.7% and a specificity of 100%. The corresponding ROC analysis, showing similar predictive values irrespective of treatment lines, is depicted in Figure 13.

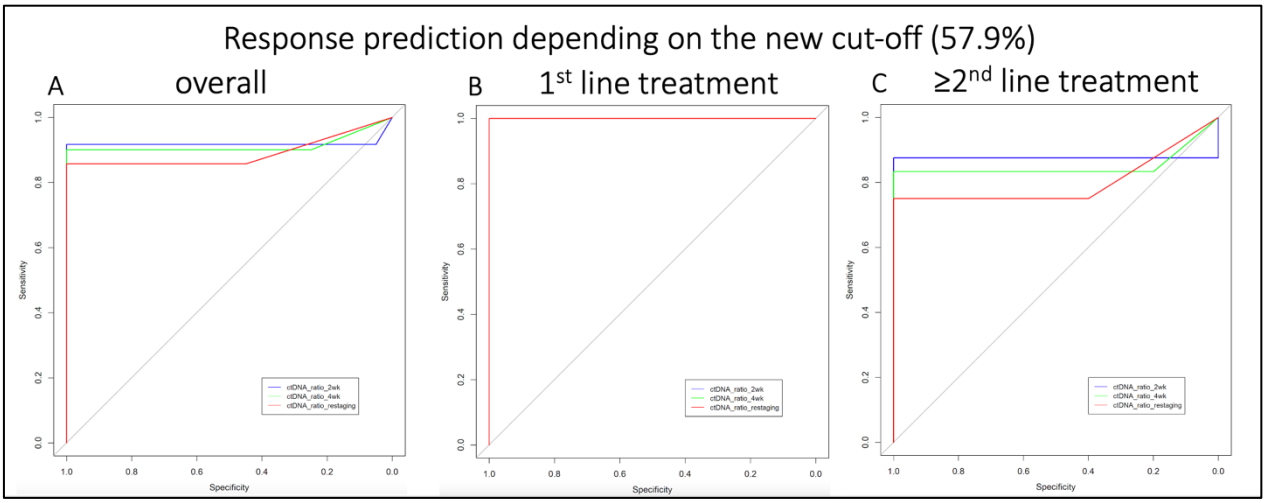


Figure 13: ROC analysis for the ideal cut-off of ctDNA kinetics for prediction of response to treatment in mPDAC undergoing palliative chemotherapy.[3]

Sensitivity and specificity for response prediction during systemic treatment by relative ctDNA reductions at two or four weeks or at restaging for the overall population (A), the first line treatment population (B) and for patients with > 1 treatment line (C).

Abbreviations: AUC: area under the curve, ctDNA: circulating tumor DNA, MAF: mutant allele frequency, ROC: receiver operating characteristics.

Application of the same cut-off (decrease of under 58% of the respective baseline value) for ctDNA kinetics in colorectal cancer patients undergoing palliative chemotherapy revealed a concordance with radiological response to treatment (PD vs. nonPD) in 69 out of 72 (95.8%) patients, whereas methylation of WIF1 or NPY did so in 85.1% of patients. Nevertheless, the current gold standard biomarker for colorectal cancer (CEA) achieved this in just under three quarters of patients (74.5%). Moreover, early assessment of response to treatment at the first possible point in time (14–21 days) depending on the applied chemotherapy regimen resulted in significantly higher rates of correct response prediction of ctDNA (87.5%) and even methylation (64.2%) compared to a mere 5.5% for CEA. Median time until correct prediction was 16 days (IQR 14–28) for ctDNA, 17 days (IQR 14–32) for methylation, and 56 days (IQR 42–71) for CEA respectively. Results are depicted in Table 3.

Prediction of response to treatment	**n**	**%**	**d**	
CEA	41/55	74.5%	56	(42–71)
CEA 1st possible point of time	3/55	5.5%		
Methylation	57/67	85.1%	17	(14–32)
Methylation 1st possible point of time	43/67	64.2%		
ctDNA	69/72	95.8%	16	(14–28)
ctDNA 1st possible point of time	63/72	87.5%		

Table 3: Rates of response prediction for CEA, methylation, and ctDNA in mCRC.

Rates of correct prediction of response to treatment using CEA, methylation (of NPY and WIF1), and ctDNA in correlation with radiological findings (CT) and the respective point in time for treatment evaluation (median days and IQR in brackets).

Applying the new cut-off (decrease under 57.9% of the base value), non-progressive disease was correctly observed in 19 out of 20 (95%) patients already after 14 days of treatment, whereas progressive disease was correctly observed in 100% (n=12) of patients after 14 days of treatment. Overall, the median time until reliable response prediction by the novel liquid biopsy approach was

14 days (IQR 8–15) compared to 97 days (IQR 75–107) by the current gold standard multiphase computed tomography. Thus, liquid biopsy was proven to be superior (p<0.000) in terms of predicting response to treatment by a median of 78 days (IQR 60–89), equivalent to a reduction of 84.8% (IQR 81.6–86.5) exposure time to cytotoxic treatment without evaluation of treatment success. MAF level courses are depicted in Figure 14.

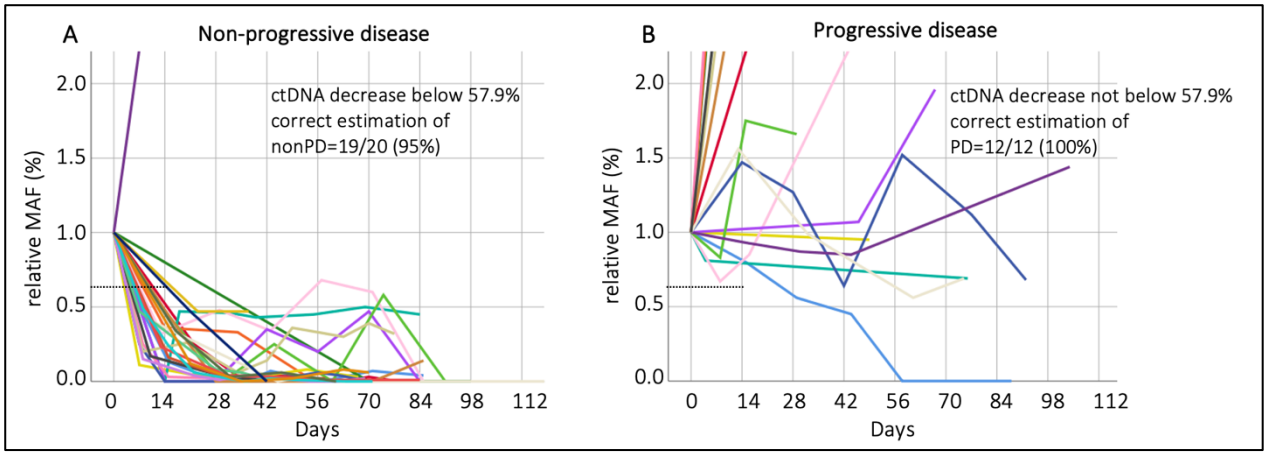


Figure 14: ctDNA kinetics according to response groups in mPDAC.

Non-progressive disease **(**nonPD; A) and progressive disease (PD; B). Different patients (ctDNA kinetics) are symbolized by different colors.

Abbreviations: ctDNA: circulating tumor DNA, nonPD: non-progressive disease (complete response, partial response, stable disease), MAF: mutant allele frequency, PD: progressive disease.

Aside from the prognostic influence on overall survival that could be read by ctDNA normalization (change from detectable to undetectable during treatment) at restaging after three months, ctDNA kinetics showed an even greater impact on survival rates when applying the decrease of under 57.9% of the pretherapeutic base value at already 14 days after treatment initiation. Independent of the treatment line applied, patients who did not experience a decrease under the threshold showed significantly worse OS (5.7 (IQR 4.2–7.0) vs. 11.4 (IQR 9.7–13.5) months, p=0.006) compared to patients who did (HR 3.7 (CI 95% 1.4–9.9)). This difference was even more substantial in the first line cohort (5.7 (IQR 2.3–6.5) vs. 13.5 (IQR 11.4–n.r.) months, p=0.026, HR

5.86 (CI 95% 1.0–35.1)). Of even greater importance and in contrast to the limited practical suitability of ctDNA normalization on the influence on progression-free survival, a decrease under the threshold was associated with significantly better PFS in treatment-naïve patients (2.2 (IQR 1.6–2.2) vs. 9.2 (IQR 5.1–11.3) months, p<0.000, HR 8.5 (CI 95% n.a.)), and independently of treatment line (2.5 (IQR 2.2–2.9) vs. 7.7 (IQR 4.0–11.3) months, p<0.000, HR 9.1 (CI 95% 3.0–28.0)). A visualization is given in Figure 15.

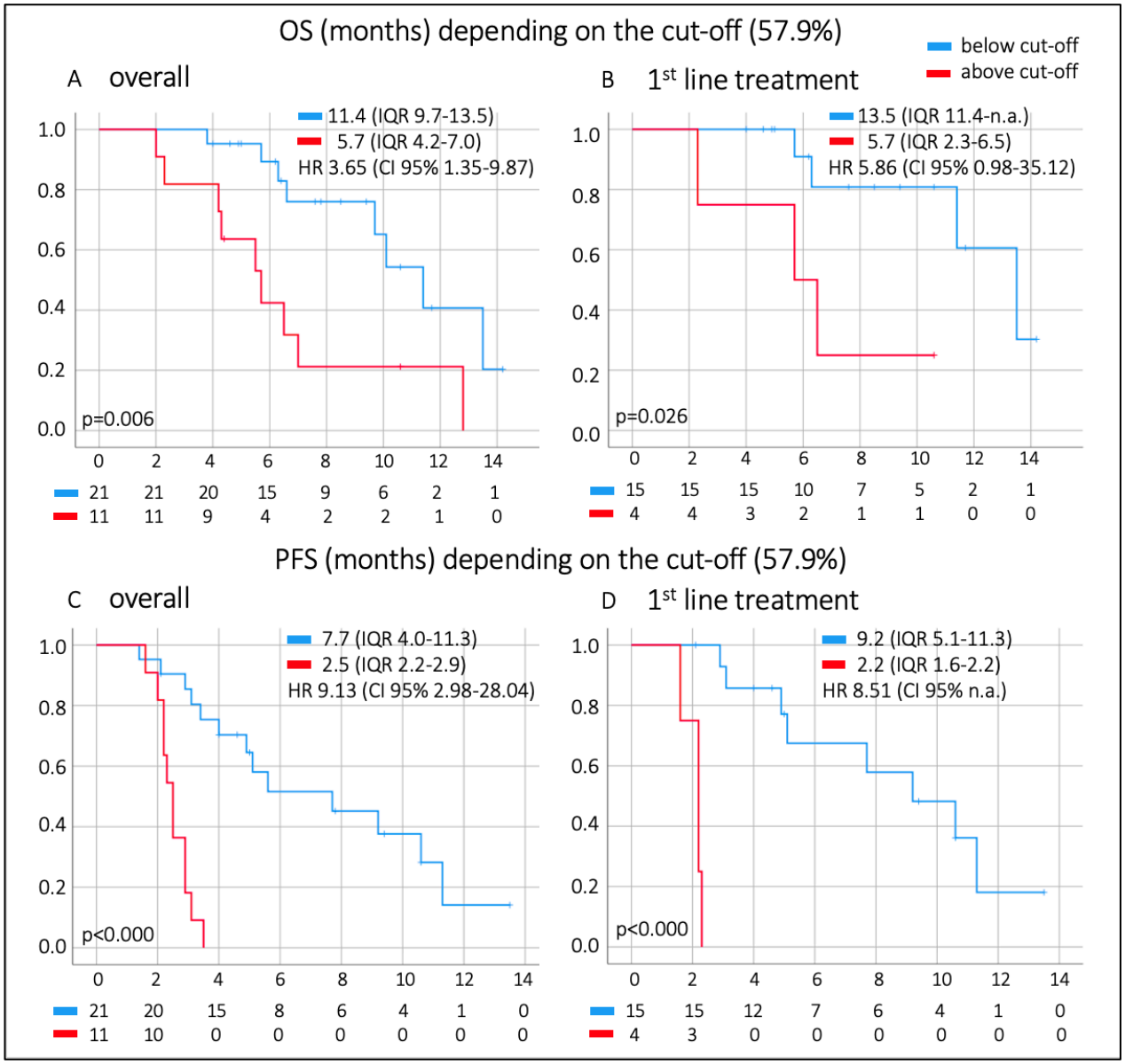


Figure 15: OS and PFS according to ctDNA decrease under 57.9% of the base value at week 2 for patients with mPDAC.

The change in ctDNA levels below or above 57.9% (i.e. a reduction more or less than 42.1%) of

the baseline value after two weeks of treatment correlates with overall survival (A–B) and progression-free survival (C–D) in ctDNA positive patients.

Abbreviations: CI: confidence interval, HR: hazard ratio, IQR: interquartile range, OS: overall survival, PFS: progression-free survival.

XI DISCUSSION

Detection of ctDNA in different cancer types

Sensitivity problems have always appeared regarding ctDNA detection testing in pancreatic cancer, especially in less advanced tumor stages, compared to other gastrointestinal tumor entities.[2] To tackle this issue, new technologies (e.g. advanced precision medicine approaches with tissue-informed NGS or analysis of alterations in multiple cancer-related genes from a broad range) have been invented by several companies (e.g. Signatera, Personalis, or Inivata).[3] However, although these technologies have so far only been tested on an experimental base, they show potential to achieve improvements in molecular residual disease (MRD) detection, not restricted to pancreatic cancer, but in various tumor subsets.[40,41] Eventually, the latest technical developments could result in ancillary detection of a further 25% of patients not recognized by liquid biopsy, but co-existing tissue mutations.[3] However, the clinical advantage of broader ctDNA detection has yet to be proven, as while evidence of ctDNA in the periphery happens to be of significant prognostic value with current detection limits, it could be diminished if non-clinically relevant amounts are certified. Our findings in metastatic colorectal cancer affirm this hypothesis. In this case, reliable cut-offs for the clinical applicability of the amount of ctDNA found or its kinetics might become of even greater value in the future, taking advantage of the clinical impact of improving testing methods. Besides this, application of these techniques is further hampered by the time needed from individual panel design after tissue sampling to actual liquid biopsy to test the mutation harbored in the periphery, which currently takes up to eight weeks, making them currently not ready for clinical use.[3,40]

In what is to date the biggest study on this topic (n=255), HOXD8 and POU4F1, two methylated markers from Prodige 35 and 37 studies, were retrospectively analyzed by ddPCR and revealed detection rates of 56.8%.[42] Without knowledge on the actual mutational pattern of an individual by prior alteration screening of the respective tumor tissue, detection rates in disseminated pancreatic cancer reported in the literature vary by about 40–65% depending on the screening method (ddPCR to NGS) and can be increased to 75% in tissue-informed approaches (BEAMing).[3,13,18]

Following the test strategy presented in this PhD project by screening for KRAS G12/13 and, if negative, further screening for KRAS Q61, resulted in a detection rate of approximately 64.3%.[3] These findings are comparable to COSMIC database results, revealing about 64% of over 9,000 PC patients harboring KRAS mutations.[43]

Different amounts of tumor DNA shed into the patients' blood depending on the tumor entity result in different detection rates for liquid biopsy depending on the respective tumor entity.

However, the detection rates reported are highly heterogenous and vary heavily depending on the source. For example, for localized pancreatic cancer, detection rates in LB vary from 10–69%, whereas rates of 43–80% are reported for localized colorectal cancer.[2,20,44,45]

We published a review article on this topic and depicted a summary of the different detection rates and the respective prognostic impact of ctDNA in different cancer types in Table 4.[2]

Correlation of ctDNA with tumor volume, metastasis localization, and tumor markers

Conventional tumor markers, such as CEA in colorectal cancer, only show a very slight correlation to the actual tumor lesion volume measured by radiological volumetric analysis from computed tomography.

Until recently, little was known about the actual derivation of circulating tumor DNA and its correlation with measurable primary tumor or metastasis lesion volume in patients with pancreatic cancer.[1] In addition to Strijker et al, who were the first to show the correlation of ctDNA and total tumor volume in pancreatic cancer, we were able to break down the derivation of ctDNA from the actual tumor volume subsets (liver, lung, primary tumor volume, lymph nodes, etc.) in a precursor study within this PhD project.[1,13]

Furthermore, as ctDNA detection showed a strong correlation with liver lesion volume in metastatic disease (mainly contributing to total tumor burden) but not with primary tumor volume, which was especially true in localized disease with correlation to locoregionally advanced lymph node status in this stage respectively, the use of ctDNA as a marker for dissemination or advanced disease vs. localized disease could be indicated.[1]

Results further suggest that the presence of detectable pretherapeutic ctDNA indicates an advanced lymph node status or even metastases undetectable by radiological staging and could therefore help to distinguish localized from disseminated disease in radiologically assumed localized pancreatic cancer and provide additional assistance in personalized decision-making for neoadjuvant chemotherapy or upfront surgery based on ctDNA status in the future.[1]

Mutational spectrum detected in metastasized pancreatic cancer

Despite suffering from lower detection rates than, for example, colorectal cancer, PC bears a major advantage over other tumor entities, with a majority of patients harboring KRAS mutations that enable small spectrum analysis (see Table 4).[2]

Following the test strategy presented in this PhD project by screening for KRAS G12/13 and, if negative, further screening for KRAS Q61, seems to be an appropriate approach for sensitive and cost-effective testing in a clinically applicable setting, resulting in a ctDNA detection of approximately 64.3%.[3] The screening kits used are commercially available and affordable and have been proven to be applicable for everyday clinical use in the study published in Frontiers in Oncology.[3] Furthermore, these findings are comparable to historically proven and still well established COSMIC database results revealing about 64% of over 9,000 PC patients harboring KRAS mutations.[43] Second, and not assessed by our procedure, TP53 is reported to have been found in 43% out of approximately 3,150 PC patients in the COSMIC database.[46] TP53 was revealed to be detectable in our precursor studies in about 34% of patients but bears an enormous heterogeneity of different subtypes, hampering clinical applicability using small spectrum tests and instead making prior NGS testing mandatory.[46] Furthermore, respective assays to test the periphery for these rather rare subsets are significantly more expensive than KRAS G12D/C and Q61 test kits depending on the underlying mutation.

Bearing only a few but very common mutations seems to have a significant advantage over other gastrointestinal tumors like colorectal cancer, which on the one hand, bear higher detection rates

but on the other hand, diminish clinical applicability by possessing a wide range of potential mutations.

Prognostic impact of pretherapeutic ctDNA

Previous studies have agreed on the significant negative prognostic impact of pretherapeutic ctDNA detectability on OS.[18,42,47] However, the reported outcome varies between the studies and depends on the underlying method used for mutation detection, ranging from about 8.2 compared to 12.6 months using highly sensitive methods (ddPCR) to about 16.8 months compared to not reached using methods with lower sensitivity but cheaper by contrast and more easily assessable in clinical routine (Idylla™ kits).[42,47] Our study results therefore resemble the literature with OS of 11.4 compared to 15.9 months (p=0.046) in the first-line-only cohort and 7 compared to 11.3 months (p=0.045) in the overall cohort, irrespective of the treatment line.[3]

In contrast, our results regarding PFS in the first line treatment (3.4 vs. 10.8 months) did not resemble the findings of the 354 patients analyzed by Pietrasz et al., who did not confirm the same great prognostic impact in the first line cohort (5.3 vs. 6.2 months).[42] A relatively smaller patient cohort or the patients between the different treatment lines may have contributed to this discrepancy, in addition to the broad variety of the screening methods applied in the different studies, as there is a significant correlation between progression-free survival and the covered alteration in gene loci, making it difficult to compare studies of different methodologies.[3,48] Thereto, Botrus et al. found a reduction of PFS to 3.7 months when patients bearing KRAS mutations harbored ≥2 alterations, subsequently to the different impact on PFS by the harbored mutation (KRAS: 5.8 vs. 12.9 months, TP53: 5.9 vs. 10.9 months, CCND2: 3.7 vs. 8.2 months) compared to ctDNA negative patients.[48]

Nevertheless, the prognostic value of ctDNA detectability in metastatic disease at a single point in time (e.g. pretherapeutic) vs. non-detectability (HR 1.6 95% CI 1.1–2.5, p=0.029) is, similarly to the prognostic impact of CA 19-9, greater than 1366 U/mL (HR 1.7 95% CI 1.2–2.5, p=0.006), which is far easier to assess and already established in clinical routine.[42] From my perspective, the broader clinical value of pretherapeutic ctDNA detection in pancreatic cancer may instead be

occupied by the supposed localized disease because of its high prognostic impact on PFS in lPDAC and the underlying derivation from metastatic or advanced lymphoregional disease, and therefore should not be detectable in a true localized stage of the disease. Thus, pretherapeutic ctDNA detection in pancreatic cancer could assist in distinguishing localized from disseminated disease and help in clinical decision-making (CTX vs. surgery), which is to be addressed by our follow-up project, described briefly below (LIQUIPANC).

Prognostic information of ctDNA dynamics and response to treatment evaluation

By contrast, the change of ctDNA detectability during treatment harbors even greater prognostic information for patients at an advanced stage of the disease. Patients with pretherapeutic detectable ctDNA who turned non-detectable under palliative chemotherapy showed similar overall and progression-free survival rates to patients who were regarded as ctDNA negative before treatment initiation, whereas patients who remained ctDNA positive despite systemic treatment suffered from significantly worse progression-free and overall survival (Figure 12). These findings were independent of treatment lines and support the previous findings of Kruger et. Al, who first described the impact of ctDNA normalization in metastatic pancreatic cancer in 2018.[18]

Moreover, ctDNA kinetics by means of our new established cut-off (decrease of >42% of the baseline value at week 2) were of yet further prognostic impact (overall survival of 5.7 months vs. 11.4–13.5 months in the first line and in higher lines respectively) and were recognizable ahead of the previously earliest feasible point of four weeks after treatment initiation.[3]

While other studies have suggested time until pretherapeutic detectable ctDNA turns undetectable during the course of systemic treatment (ctDNA normalization) as a predictive endpoint for treatment evaluation that found a correlation with treatment success after four weeks following initiation, our findings enabled response to treatment to be foreseen at a specific cut-off value after already two weeks of treatment.[18] The threshold of a decrease of 42% compared to the baseline at week 2 was accompanied by a difference in progression-free survival of 2.2 vs. 9.2 months in

the first line cohort and 2.5 vs. 7.7 months regardless of the treatment line. Furthermore, a decrease of the baseline ctDNA value below 57.9% at week 2 could predict treatment success and absence of this decline was able to indicate progressive disease with a specificity of 100% and sensitivity of nearly 92%, leading to a remarkable superiority to CA 19-9 or computed tomography.[3]

Moreover, such thresholds for liquid biopsy-guided therapy in metastatic pancreatic cancer patients undergoing systemic chemotherapy did not exist until this point.[3]

Thus, our findings prove that the continuous measurement of ctDNA levels is clinically applicable and could open the door to early change of treatment under palliative chemotherapy to not only spare patients from the administration of insufficient cytotoxic drugs (potentially >80% less exposure time) but also eventually provide them with efficient regimens (10 weeks before the current gold standard treatment evaluation via CT) to prevent progressive disease and enable survival benefit.[3]

Potential limitations of this study

By only using small spectrum mutation analysis for the most common germline mutations in pancreatic cancer (KRAS G12/13, KRAS Q61), we cannot rule out the potential of having missed other possible common mutations such as SMAD4, TP53, or CDKN2A, which could have introduced bias by resulting in lower detection rates compared to whole genome sequencing. However, this was done on purpose in order to test a method immediately applicable in clinical routine without being restricted to the experimental and expensive methods used within study conditions (e.g. NGS) and to overcome prolonged turnaround times. Furthermore, having not found a mutation in e.g. KRAS G12/13 by testing peripheral blood, it cannot be stated that the respective patient is actually wild-type in the tested gene without having performed paired tissue analysis. We can therefore only speak of undetectable ctDNA or being ctDNA negative when solely using liquid biopsy.

For the sake of testing a robust biomarker independent of treatment line, the evaluation of different treatment lines (from treatment-naïve to up to third line treatment) as a whole study cohort cannot rule out potential evaluation bias by the introduction of heterogeneity. Nevertheless, this issue was addressed by describing the survival analyses as an unselected study cohort and for treatment-naïve patients and patients who had received higher treatment lines separately.
In addition, actual clinical treatment was unaffected by the study results and blinded to ctDNA detectability. Thus, survival could have been improved with the knowledge of certain pathogenic germline variants and the consecutive application of, for example, platinum-based treatment in BRCA mutated patients.

Lastly, this study did not have a control group and therefore did not undergo randomization but rather tried to give a full census of all available patients at a single study center.
These findings should be validated in larger prospective multicentric randomized controlled clinical trials to compensate the relatively small sample size of this monocentric study and definitively prove the survival benefit of ctDNA dynamic-based targeted therapy through early change of treatment.

Summary and outlook

First, the results of this study could suggest the immediate implementation of a low-cost ctDNA testing procedure (€100-150/patient and point in time; €200-250/patient to calculate ctDNA dynamics) in clinical care as commercially available test kits have been used without prior tissue testing by NGS (which would prolong turnaround time from sample processing until analysis by approximately two weeks or more). The turnaround time of ddPCR alone is about two days. Thus, evaluation of treatment success during systemic therapy in neoadjuvant, adjuvant, or palliative chemotherapy could be assessed between two treatment cycles without losing time compared to the standard of care and would already allow early change of treatment in clinical care 80% faster than the current gold standard. However, this procedural approach is hampered by the methodologically limited detection rate of approximately 64% in disseminated disease and only

10–30% in localized disease for pancreatic cancer, which could be addressed by prior tissue testing (NGS) and personalized assay design to improve detection rates. Nevertheless, assay design could take further days to weeks based on the rarity of the underlying mutation until a decision is made. This could further thwart the claim of clinical applicability (in localized disease) as treatment should be initiated within about two weeks from diagnosis and diminishes the clinical impact (in metastasized disease) as lead time is reduced when compared to the current gold standard computed tomography, which is being performed increasingly earlier (after two months) because of improved resolutions.

Thus, improvement of available ctDNA test kits or faster turnaround times for individual assay design are mandatory steps for making this approach clinically applicable for all patients who bear mutational targets.

Second, our results have demonstrated significant improvement in survival and detecting response to treatment by the method applied in this study. In our view and according to positive feedback by interdisciplinary discussion on various occasions at international congresses (e.g. ESMO TAT 23 in Paris), this allows immediate implementation of this method for the sake of change of treatment by ctDNA kinetics within two weeks of treatment initiation in a prospective interventional study.

Third, as I am a surgical resident, my major interest focusses on improving the outcome of potentially resectable pancreatic cancer patients. Ideally, individualized assays could be designed from tissue testing by EUS-FNA (endosonographic ultrasound fine needle aspiration), placing ctDNA testing in the periphery as a potential supplement for routine staging procedures in the future. However, localized disease may not necessarily need immediate improvements in sensitivity before clinical implementation, as current detection limits may perhaps already determine clinical impact. In other words, if ctDNA is detectable with the current limit of detection in radiologically classified localized disease, this approach could highlight patients with a heavy biological (yet radiologically undetectable) tumor burden and who could profit from systemic treatment because they are already suffering from biologically advanced/disseminated disease.

Our current knowledge on ctDNA in pancreatic cancer may not be sufficient to overturn a decision for systemic treatment when there are signs of advanced disease or even dissemination in conventional staging (and would be nonsense anyway), but ctDNA may play a role as a sensitive addition to biological tumor burden display and as a marker for the need for additional systemic treatment when being estimated in a localized disease free of systemic tumor burden.

LIQUIPANC

Based on the results of the projects within this PhD study, our study group is currently planning a larger-scale prospective multicentric interventional study to address the issue of early recurrence following resection for pancreatic cancer for curative purposes, despite no evidence of dissemination by current gold standard staging procedures including high resolution CT and CA 19-9. To guarantee validity and uniformity, sample processing and preparation will be standardized for every center and definitive molecular analysis by ddPCR will be performed at one and the same laboratory (Medical University of Innsbruck) to minimize heterogeneity in molecular profiling.

Patients with non-disseminated pancreatic cancer will be staged during clinical routine. The Tumor Board will recommend either neoadjuvant chemotherapy or upfront surgery. All patients will be addressed for ctDNA observation during the course of the study for retrospective evaluation. However, the prospective treatment decision will partially depend on the ctDNA status in addition to conventional staging results. If upfront surgery is recommended and ctDNA is detectable, patients will either be enrolled in the observation group (standard of care as recommended by the Tumor Board) or in the intervention group (additional neoadjuvant chemotherapy due to ctDNA positivity followed by pancreatic resection), because we consider the presence of ctDNA as a sensitive sign of dissemination/advanced disease not depicted by CT and CA 19-9. This approach adds criteria for biological resectability to the conventional radiological resectability on which we completely base our current standard of care.

Aside from the applied treatment method, there is no change in the standard of care treatment when participating in the study. Although tissue-agnostic ctDNA testing of the periphery will be used, FFPE for whole exome sequencing is harbored via routine endosonographic fine needle aspiration to comprehensively evaluate the detection rate of this method and determine whether patients are really ctDNA negative or our approach has simply missed out a significant mutational target.

Liquid biopsy will be collected during clinical routine. No additional puncture outside of the clinic's standard of care will be needed. Follow-up is also planned during clinical routine. Thus, no additional appointments or interventions outside of the clinic's standard of care are needed. All patients will be followed up on for 24 months. The primary endpoint will be PFS.

The aim of this project is to provide a proof of concept of the (i) clinical applicability and the (ii) impact on overall survival/disease-free survival of a novel and potentially universally usable biomarker approach (ctDNA) for early prediction of relapse and thus individualized treatment decisions (upfront surgery vs. neoadjuvant/adjuvant chemotherapy) in order to implement guided treatment matched to personalized molecular profiling in localized pancreatic cancer care.

This project has been awarded the Hans Werner Waclawiczek Prize for the best formulation of a clinically oriented question with clinical and scientific relevance to change daily surgical routine in the future at the Austrian Congress of Surgery 2023.

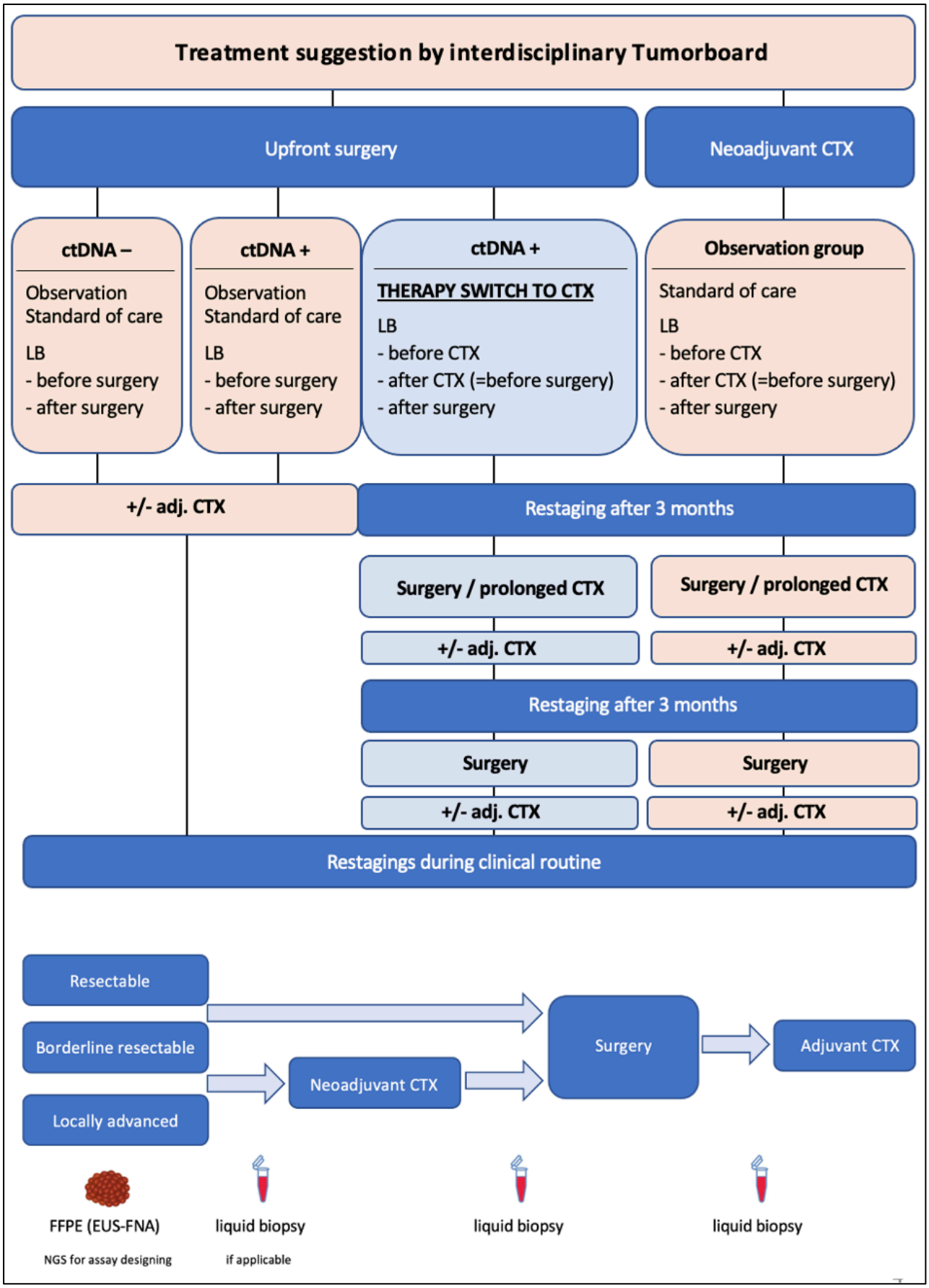


Figure 16: Workflow LIQUIPANC trial.

XII CONCLUSION

Clinical decisions for cancer patients in general, and especially pancreatic cancer patients as evaluated in this study, could be guided by the mutational testing of circulating tumor DNA in liquid biopsy in the future. We have proven the detectability of ctDNA to be associated with advanced disease, whether depicted by the presence of locoregional lymph nodes or distant metastases (mostly liver), which we published as a precursor study in the European Journal of Surgical Oncology.[1] We have further enriched knowledge on this topic by demonstrating the clinical applicability and superiority to current gold standard restaging methods (CA 19-9 and computed tomography) in early relapse detection (84.8% faster) of a cost-effective and readily available minimally invasive method when evaluating ctDNA kinetics with serial measurements during clinical routine in this dissertation project. Furthermore, we have found a practical cut-off for early ctDNA change with a significant impact on outcome and relapse estimation after two weeks of systemic treatment in this study. The findings of this project were published in Frontiers in Oncology.[3] These outcome results were blinded to the study's findings (ctDNA status) and should be evaluated on a larger scale when comparing ctDNA-guided treatment (early change of treatment according to the ctDNA course) with current state-of-the-art treatment. However, our results suggest an improvement in chance of survival by early change of treatment with consecutive avoidance of unnecessary cytotoxic side effects from insufficient therapy. These implications and even the testing strategy and applied cut-offs for metastasized disease are not limited to pancreatic cancer but are reproducible and similar in colorectal cancer as we have proven within this PhD project. However, the clinical applicability displayed by the cost-effectiveness and simplicity of necessary tests for gaining clinical impact highlights pancreatic cancer as a tumor entity ready for the implementation of a ctDNA-guided treatment decision under clinical study conditions.

We strongly believe in a revolutionary change to cancer patients' treatment by using the possibility of non-invasive real-time display of tumor burden in liquid biopsy for highly individual precision oncological patient care in the next few years.

XIII REFERENCES

1. Kirchweger P, Kupferthaler A, Burghofer J, et al. Circulating tumor DNA correlates with tumor burden and predicts outcome in pancreatic cancer irrespective of tumor stage. *Eur J Surg Oncol*. December 2021. doi:10.1016/J.EJSO.2021.11.138

2. Kirchweger P, Wundsam HV, Rumpold H. Circulating tumor DNA for diagnosis, prognosis and treatment of gastrointestinal malignancies. *World J Clin Oncol*. 2022;13(6):473-484. doi:10.5306/WJCO.V13.I6.473

3. Kirchweger P, Kupferthaler A, Burghofer J, et al. Prediction of response to systemic treatment by kinetics of circulating tumor DNA in metastatic pancreatic cancer. *Front Oncol*. 2022;12. doi:10.3389/FONC.2022.902177

4. Park G, Park JK, Son DS, et al. Utility of targeted deep sequencing for detecting circulating tumor DNA in pancreatic cancer patients. *Sci Rep*. 2018;8(1). doi:10.1038/S41598-018-30100-W

5. Schwarzenbach H, Hoon DSB, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. *Nat Rev Cancer*. 2011;11(6):426-437. doi:10.1038/NRC3066

6. Stroun M, Anker P, Maurice P, Lyautey J, Lederrey C, Beljanski M. Neoplastic characteristics of the DNA found in the plasma of cancer patients. *Oncology*. 1989;46(5):318-322. doi:10.1159/000226740

7. Lee B, Lipton L, Cohen J, et al. Circulating tumor DNA as a potential marker of adjuvant chemotherapy benefit following surgery for localized pancreatic cancer. *Ann Oncol Off J Eur Soc Med Oncol*. 2019;30(9):1472-1478. doi:10.1093/ANNONC/MDZ200

8. Dvir K, Galarza-Fortuna GM, Haines JM, Gines P, Ruiz AL, Rodriguez E. Real-World Data on Liquid Biopsy Use in Non-Small Cell Lung Cancer in the Community Setting. *J Immunother Precis Oncol*. 2021;4(1):1-5. doi:10.36401/JIPO-20-26

9. Lee JS, Park SS, Lee YK, Norton JA, Jeffrey SS. Liquid biopsy in pancreatic ductal

adenocarcinoma: current status of circulating tumor cells and circulating tumor DNA. *Mol Oncol*. 2019;13(8):1623-1650. doi:10.1002/1878-0261.12537

10. Brown P. The Cobas® EGFR Mutation Test v2 assay. *Future Oncol*. 2016;12(4):451-452. doi:10.2217/FON.15.311

11. Vogelstein B, Kinzler KW. Digital PCR. *Proc Natl Acad Sci U S A*. 1999;96(16):9236-9241. doi:10.1073/PNAS.96.16.9236

12. Gorgannezhad L, Umer M, Islam MN, Nguyen NT, Shiddiky MJA. Circulating tumor DNA and liquid biopsy: opportunities, challenges, and recent advances in detection technologies. *Lab Chip*. 2018;18(8):1174-1196. doi:10.1039/C8LC00100F

13. Strijker M, Soer EC, de Pastena M, et al. Circulating tumor DNA quantity is related to tumor volume and both predict survival in metastatic pancreatic ductal adenocarcinoma. *Int J cancer*. 2020;146(5):1445-1456. doi:10.1002/IJC.32586

14. Moser T, Waldispuehl-Geigl J, Belic J, et al. On-treatment measurements of circulating tumor DNA during FOLFOX therapy in patients with colorectal cancer. *npj Precis Oncol*. 2020;4(1). doi:10.1038/s41698-020-00134-3

15. Eijkelenboom A, Kamping EJ, Kastner-van Raaij AW, et al. Reliable Next-Generation Sequencing of Formalin-Fixed, Paraffin-Embedded Tissue Using Single Molecule Tags. *J Mol Diagn*. 2016;18(6):851-863. doi:10.1016/J.JMOLDX.2016.06.010

16. Kanda M, Matthaei H, Wu J, et al. Presence of somatic mutations in most early-stage pancreatic intraepithelial neoplasia. *Gastroenterology*. 2012. doi:10.1053/j.gastro.2011.12.042

17. Waddell N, Pajic M, Patch AM, et al. Whole genomes redefine the mutational landscape of pancreatic cancer. *Nature*. 2015;518(7540):495-501. doi:10.1038/NATURE14169

18. Kruger S, Heinemann V, Ross C, et al. Repeated mut KRAS ctDNA measurements represent a novel and promising tool for early response prediction and therapy monitoring

in advanced pancreatic cancer. *Ann Oncol*. 2018. doi:10.1093/annonc/mdy417

19. Strijker M, Soer EC, de Pastena M, et al. Circulating tumor DNA quantity is related to tumor volume and both predict survival in metastatic pancreatic ductal adenocarcinoma. *Int J cancer*. 2020;146(5):1445-1456. doi:10.1002/IJC.32586

20. Wong R, Tie J, Lee M, et al. The potential role of circulating tumor DNA (ctDNA) in the further investigation of colorectal cancer patients with nonspecific findings on standard investigations. *Int J cancer*. 2019;145(2):540-547. doi:10.1002/IJC.32117

21. Berger AW, Schwerdel D, Ettrich TJ, et al. Targeted deep sequencing of circulating tumor DNA in metastatic pancreatic cancer. *Oncotarget*. 2017;9(2):2076-2085. doi:10.18632/ONCOTARGET.23330

22. Groot VP, Mosier S, Javed AA, et al. Circulating Tumor DNA as a Clinical Test in Resected Pancreatic Cancer. *Clin Cancer Res*. 2019;25(16):4973-4984. doi:10.1158/1078-0432.CCR-19-0197

23. Uesato Y, Sasahira N, Ozaka M, Sasaki T, Takatsuki M, Zembutsu H. Evaluation of circulating tumor DNA as a biomarker in pancreatic cancer with liver metastasis. *PLoS One*. 2020;15(7). doi:10.1371/journal.pone.0235623

24. Jones RP, Pugh SA, Graham J, Primrose JN, Barriuso J. Circulating tumour DNA as a biomarker in resectable and irresectable stage IV colorectal cancer; a systematic review and meta-analysis. *Eur J Cancer*. 2021;144:368-381. doi:10.1016/J.EJCA.2020.11.025

25. Burghofer J. Highly Sensitive NGS Methods to Monitor Clonal Dynamics in Tumors During Therapy. Unpublished dissertation. Paris-Lodron University Salzburg. 2021.

26. Ueberroth BE, Jones JC, Bekaii-Saab TS. Circulating tumor DNA (ctDNA) to evaluate minimal residual disease (MRD), treatment response, and posttreatment prognosis in pancreatic adenocarcinoma. *Pancreatology*. 2022;22(6). doi:10.1016/J.PAN.2022.06.009

27. Strobel O, Neoptolemos J, Jäger D, Büchler MW. Optimizing the outcomes of pancreatic

cancer surgery. *Nat Rev Clin Oncol*. 2019. doi:10.1038/s41571-018-0112-1

28. Liu RC, Traverso LW. Diagnostic laparoscopy improves staging of pancreatic cancer deemed locally unresectable by Computed Tomography. *Surg Endosc Other Interv Tech*. 2005;19(5):638-642. doi:10.1007/s00464-004-8165-x

29. Tong H, Fan Z, Liu B, Lu T. The benefits of modified FOLFIRINOX for advanced pancreatic cancer and its induced adverse events: a systematic review and meta-analysis. *Sci Rep*. 2018;8(1). doi:10.1038/S41598-018-26811-9

30. Pelzer U, Hilbig A, Sinns M, et al. Value of carbohydrate antigen 19-9 in predicting response and therapy control in patients with metastatic pancreatic cancer undergoing first-line therapy. *Front Oncol*. 2013;3. doi:10.3389/FONC.2013.00155

31. Diaz CL, Cinar P, Hwang J, Ko AH, Tempero MA. CA 19-9 Response: A Surrogate to Predict Survival in Patients With Metastatic Pancreatic Adenocarcinoma. *Am J Clin Oncol*. 2019;42(12):898-902. doi:10.1097/COC.0000000000000620

32. Hammad N, Heilbrun LK, Philip PA, et al. CA19-9 as a predictor of tumor response and survival in patients with advanced pancreatic cancer treated with gemcitabine based chemotherapy. *Asia Pac J Clin Oncol*. 2010;6(2):98-105. doi:10.1111/J.1743-7563.2010.01290.X

33. Tempero MA, Malafa MP, Al-Hawary M, et al. Pancreatic Adenocarcinoma, Version 2.2021, NCCN Clinical Practice Guidelines in Oncology. *J Natl Compr Canc Netw*. 2021;19(4):439-457. doi:10.6004/JNCCN.2021.0017

34. Quante AS, Ming C, Rottmann M, et al. Projections of cancer incidence and cancer-related deaths in Germany by 2020 and 2030. *Cancer Med*. 2016;5(9):2649-2656. doi:10.1002/CAM4.767

35. Kleeff J, Korc M, Apte M, et al. Pancreatic cancer. *Nat Rev Dis Prim*. 2016;2. doi:10.1038/NRDP.2016.22

36. Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res*. 2014;74(11):2913-2921. doi:10.1158/0008-5472.CAN-14-0155

37. Bengtsson A, Andersson R, Ansari D. The actual 5-year survivors of pancreatic ductal adenocarcinoma based on real-world data. *Sci Rep*. 2020. doi:10.1038/s41598-020-73525-y

38. Neoptolemos JP, Palmer DH, Ghaneh P, et al. Comparison of adjuvant gemcitabine and capecitabine with gemcitabine monotherapy in patients with resected pancreatic cancer (ESPAC-4): a multicentre, open-label, randomised, phase 3 trial. *Lancet*. 2017. doi:10.1016/S0140-6736(16)32409-6

39. Eisenhauer EA, Therasse P, Bogaerts J, et al. New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). *Eur J Cancer*. 2009;45(2):228-247. doi:10.1016/j.ejca.2008.10.026

40. Knapp B, Mezquita L, Devarakonda S, et al. Exploring the Feasibility of Utilizing Limited Gene Panel Circulating Tumor DNA Clearance as a Biomarker in Patients With Locally Advanced Non-Small Cell Lung Cancer. *Front Oncol*. 2022;12:856132. doi:10.3389/FONC.2022.856132

41. Fakih M, Sandhu J, Wang C, et al. Evaluation of Comparative Surveillance Strategies of Circulating Tumor DNA, Imaging, and Carcinoembryonic Antigen Levels in Patients With Resected Colorectal Cancer. *JAMA Netw Open*. 2022;5(3):E221093. doi:10.1001/JAMANETWORKOPEN.2022.1093

42. Pietrasz D, Wang-Renault S, Taieb J, et al. Prognostic value of circulating tumour DNA in metastatic pancreatic cancer patients: post-hoc analyses of two clinical trials. *Br J Cancer*. 2022;126(3). doi:10.1038/S41416-021-01624-2

43. Di Magliano MP, Logsdon CD. Roles for KRAS in pancreatic tumor development and

progression. *Gastroenterology*. 2013;144(6):1220-1229. doi:10.1053/J.GASTRO.2013.01.071

44. 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). doi:10.1126/SCITRANSLMED.3007094

45. Tie J, Cohen JD, Lo SN, et al. Prognostic significance of postsurgery circulating tumor DNA in nonmetastatic colorectal cancer: Individual patient pooled analysis of three cohort studies. *Int J cancer*. 2021;148(4):1014-1026. doi:10.1002/IJC.33312

46. Weissmueller S, Manchado E, Saborowski M, et al. Mutant p53 drives pancreatic cancer metastasis through cell-autonomous PDGF receptor β signaling. *Cell*. 2014;157(2):382-394. doi:10.1016/J.CELL.2014.01.066

47. Schlick K, Markus S, Huemer F, et al. Evaluation of circulating cell-free KRAS mutational status as a molecular monitoring tool in patients with pancreatic cancer. *Pancreatology*. 2021;21(8):1466-1471. doi:10.1016/J.PAN.2021.09.004

48. Botrus G, Uson Junior PLS, Raman P, et al. Circulating Cell-Free Tumor DNA in Advanced Pancreatic Adenocarcinoma Identifies Patients With Worse Overall Survival. *Front Oncol*. 2022;11. doi:10.3389/FONC.2021.794009

49. Tie J, Cohen JD, Wang Y, et al. Serial circulating tumour DNA analysis during multimodality treatment of locally advanced rectal cancer: a prospective biomarker study. *Gut*. 2019;68(4):663-671. doi:10.1136/GUTJNL-2017-315852

50. García-Foncillas J, Tabernero J, Élez E, et al. Prospective multicenter real-world RAS mutation comparison between OncoBEAM-based liquid biopsy and tissue analysis in metastatic colorectal cancer. *Br J Cancer*. 2018;119(12):1464-1470. doi:10.1038/S41416-018-0293-5

51. Manca P, Corallo S, Busico A, et al. The Added Value of Baseline Circulating Tumor DNA Profiling in Patients with Molecularly Hyperselected, Left-sided Metastatic Colorectal

Cancer. *Clin Cancer Res*. 2021;27(9):2505-2514. doi:10.1158/1078-0432.CCR-20-4699

52. Cabel L, Decraene C, Bieche I, et al. Limited Sensitivity of Circulating Tumor DNA Detection by Droplet Digital PCR in Non-Metastatic Operable Gastric Cancer Patients. *Cancers (Basel)*. 2019;11(3). doi:10.3390/CANCERS11030396

53. Maron SB, Chase LM, Lomnicki S, et al. Circulating Tumor DNA Sequencing Analysis of Gastroesophageal Adenocarcinoma. *Clin Cancer Res*. 2019;25(23):7098-7112. doi:10.1158/1078-0432.CCR-19-1704

54. Saluja H, Karapetis CS, Pedersen SK, Young GP, Symonds EL. The Use of Circulating Tumor DNA for Prognosis of Gastrointestinal Cancers. *Front Oncol*. 2018;8(JUL). doi:10.3389/FONC.2018.00275

55. Pectasides E, Stachler MD, Derks S, et al. Genomic Heterogeneity as a Barrier to Precision Medicine in Gastroesophageal Adenocarcinoma. *Cancer Discov*. 2018;8(1):37-48. doi:10.1158/2159-8290.CD-17-0395

56. Jin Y, Chen DL, Wang F, et al. The predicting role of circulating tumor DNA landscape in gastric cancer patients treated with immune checkpoint inhibitors. *Mol Cancer*. 2020;19(1). doi:10.1186/S12943-020-01274-7

57. Huang A, Zhang X, Zhou SL, et al. Detecting Circulating Tumor DNA in Hepatocellular Carcinoma Patients Using Droplet Digital PCR Is Feasible and Reflects Intratumoral Heterogeneity. *J Cancer*. 2016;7(13):1907-1914. doi:10.7150/JCA.15823

58. Ren N, Ye QH, Qin LX, Zhang BH, Liu YK, Tang ZY. Circulating DNA level is negatively associated with the long-term survival of hepatocellular carcinoma patients. *World J Gastroenterol*. 2006;12(24):3911-3914. doi:10.3748/WJG.V12.I24.3911

59. Ettrich TJ, Schwerdel D, Dolnik A, et al. Genotyping of circulating tumor DNA in cholangiocarcinoma reveals diagnostic and prognostic information. *Sci Rep*. 2019;9(1). doi:10.1038/S41598-019-49860-0

60. Lamarca A, Kapacee Z, Breeze M, et al. Molecular Profiling in Daily Clinical Practice: Practicalities in Advanced Cholangiocarcinoma and Other Biliary Tract Cancers. *J Clin Med*. 2020;9(9):1-18. doi:10.3390/JCM9092854

XIV TABLES

Localized PC	**Overall (n=60)**	**ctDNA + (n=6)**	**ctDNA – (n=54)**	**p**
CA 19-9 (U/mL) Median (IQR)	364.2 (48.1–1174.7)	169.9 (40.8–459.9)	397.4 (64.5–1357.6)	0.334
cfDNA (ng/µL) Median (IQR)	0.95 (0.61–1.6)	3.42 (0.58–12.93)	0.92 (0.61–1.51)	0.165
ctDNA (ng/mL) Median (IQR)	2.64 (1.47–11.55)	11.84 (4.99–17.99)	2.12 (1.14–4.81)	0.012*
ctDNA (MAF %) Median (IQR)	0 (0–0.11)	0.225 (0.12–1.03)	0 (0–0.08)	0.000**
Metastasized PC	**Overall (n=70)**	**ctDNA + (n=45)**	**ctDNA – (n=25)**	**p**
CA 19-9 (U/mL) Median (IQR)	995.1 (228.4–5447.5)	3074.7 (983.2–32498.5)	267.2 (54.6–647)	0.000**
cfDNA (ng/µL) Median (IQR)	0.97 (0.63–1.77)	0.99 (0.75–5.19)	0.79 (0.56–1.6)	0.155
ctDNA (ng/µL) Median (IQR)	5.33 (1.3–38.74)	16.04 (3.96–741.78)	1.11 (0.7–1.54)	0.000**
ctDNA (MAF %) Median (IQR)	0.15 (0.05–1.51)	1.47 (0.25–7.58)	0.05 (0.0025–0.07)	0.000**

Table 1: Amount and proportion of extracted cfDNA/ctDNA depending on the ctDNA detectability and stage of the disease in pancreatic cancer.

Patient characteristics	**Number of patients (%)** **Age, CA19-9, SLD, volumes, MAF: median (IQR)**					
	Overall (n=70)		**ctDNA + (n=45)**		**ctDNA – (n=25)**	**p**
Age (y)	66	(58–73)	66	(59–73)	67 (60–73)	0.825
Male sex	43	(61.4)	28	(62.2)	15 (60.0)	0.856
ECOG-PS						
0	48	(67.4)	33	(80.5)	15 (60.0)	0.114
1	13	(23.9)	5	(12.2)	8 (32.0)	0.041*
≥2	4	(8.7)	3	(7.3)	1 (4.0)	0.613
Treatment line						
1	50	(71.4)	28	(62.2)	22 (88.0)	0.023*
≥2	20	(28.6)	17	(37.8)	3 (12.0)	
Treatment regimen						
Folfirinox	13	(18.6)	8	(17.8)	5 (20.0)	0.682
5FU/Naliri	12	(17.1)	8	(17.8)	4 (16.0)	0.992
GnP	38	(54.3)	24	(53.3)	14 (56.0)	0.478
Others	7	(10.0)	5	(11.1)	2 (8.0)	0.680
Discontinuation of therapy	10	(14.3)	5	(11.1)	5 (20.0)	0.236
Time of treatment	10.8	(5.1–12.6)	9.6	(4.7–12.5)	11.0 (5.1–12.7)	0.741
Time until restaging	12.1	(9.6–13.0)	12.2	(9.7–13.1)	11.4 (9–12.7)	0.264
Site of metastasis						
Liver	53	(75.7)	40	(88.9)	13 (52.0)	0.001*
Lung	20	(28.6)	12	(26.7)	8 (32.0)	0.638

Lymph nodes	11	(15.7)	7	(15.6)	4	(16.0)	0.961
Peritoneum	15	(21.4)	8	(17.8)	7	(28.0)	0.321
Metachronous dissemination	31	(44.3)	18	(40.0)	13	(52.0)	0.336
SLD (mm)	44	(30.8–85.4)	49.9	(34–103.4)	42.3	(17.6–57.7)	0.078
Total tumor volume (mm^3)	27.9	(7.8–98.7)	30	(11–139.4)	11.7	(4.9–58.5)	0.016*
Liver metastasis volume (mm^3)	2.64	(0–23.6)	10.9	(0–46.7)	0	(0–2.9)	0.014*
CA19-9 (kU/L)	1014	(252–5608)	3074	(983–32499)	286	(48–650)	0.000*
MAF (%)	1.6	(0.3–5.1)	1.6	(0.3–5.1)			
KRAS G12/13	39	(55.7)	39	(86.7)			
KRAS Q61	6	(8.6)	6	(13.3)			

Table 2: Demographics of patients with metastatic pancreatic cancer.

Abbreviations: ECOG-PS: Eastern Cooperative Oncology Group performance status, Naliri: nanoliposomal irinotecan, GnP: gemcitabine nab-paclitaxel, SLD: sum of the largest tumor diameter, PD: progressive disease, MAF: mutant allele frequency (%), *KRAS*: Kirsten rat sarcoma, treatment time and time until restaging in weeks.

Prediction of response to treatment	n	%	d	
CEA	41/55	74.5%	56	(42–71)
CEA 1st possible point of time	3/55	5.5%		
Methylation	57/67	85.1%	17	(14–32)
Methylation 1st possible point of time	43/67	64.2%		
ctDNA	69/72	95.8%	16	(14–28)
ctDNA 1st possible point of time	63/72	87.5%		

Table 3: Rates of correct response prediction for CEA, methylation, and ctDNA in mCRC. Rate of correct prediction of response to treatment using CEA, methylation (of NPY and WIF1), and ctDNA in correlation with radiological findings (CT) and the respective point in time for treatment evaluation (median days and IQR in brackets).

Tumor entity	Frequent target	Detection rate	PFS ctDNA positive	OS ctDNA positive
lPDAC	KRAS, TP53, SMAD4, CDKN2A	10–69%[20]	8 vs. 19[7]	5.8 vs. 16.3[7]
mPDAC		60–75%[18]	3.9 vs. 5[18]	3.2 vs. 8.4[19]
lCRC	KRAS, NRAS, BRAF, PIK3CA, APC, TP53, EGFR, ERBB3/4	73% (43–80%)[44,45]	33 vs. 87% 3y[49]	-
mCRC		>90%[50]	4.5 (BRAF) vs. 8.3 (RAS) vs. 22.9 (wt)[20]	17.1 vs. 36.5[51]
lGEC	TP53, HER2, MET, EGFR, KRAS	20%[52]	12.5 vs. n.r.[53]	37.3 vs. 66.9[54]
mGEC		87.5%[55]	4.9 vs. 7.4[56]	8.6 vs. 13.7[53]

HCC	TP53, CTNNB1, TERT	56.3%[57]	22 vs. 47% 3y[58]	24 vs. 61%. 3y[58]
mIHCC	TP53, KRAS, ARID1A	92%[59]	4.6 vs. 8.2[60]	7.4 vs. 16.4[60]
mEHCC		55%[59]	-[59]	-[59]

Table 4: Comparison of common targets, detection rates, and prognostic impact of pretherapeutic ctDNA detectability of different gastrointestinal tumor entities.

Abbreviations: lPDAC: localized pancreatic ductal adenocarcinoma, mPDAC: metastasized pancreatic ductal adenocarcinoma, lCRC: localized colorectal carcinoma, mCRC: metastasized colorectal cancer, lGEC: localized gastroesophageal cancer, mGEC: metastasized gastroesophageal cancer, HCC: hepatocellular carcinoma, mIHCC: metastasized intrahepatic cholangiocarcinoma, mEHCC: metastasized extrahepatic cholangiocarcinoma, PFS: progression free survival in months, OS: overall survival in months, n.r.: not reached.[2]

Mutational targets ctDNA			**LB negative**	
KRAS	52	66.7%	6	66.7%
TP	14	17.9%	2	22.2%
BRAF	9	11.5%	1	11.1%
NRAS	2	2.6%	0	
no target available	1	1.3%		
LB positive	69	88.5%		
LB negative	9	11.5%		

Mutational targets ctDNA			**LB negative**	
KRAS	**52**	**66.7%**		
G12D	19	24.4%	2	10.5%

G12V	16	20.5%	2	12.5%
G12C	6	7.7%	1	16.7%
G13D	3	3.8%		
G12S	2	2.6%	1	50%
G12A	2	2.6%		
G13C	1	1.3%		
A146T	1	1.3%		
A59G	1	1.3%		
K117N	1	1.3%		
Q61L	1	1.3%		
Q61R	1	1.3%		
G12?	1	1.3%		
TP	**14**	**17.9%**		
R213*	3	3.8%	1	33.3%
R175H	2	2.6%		
C242Afs*5	1	1.3%	1	100%
C275Y	1	1.3%		
D281Y	1	1.3%		
E336*	1	1.3%		
L289Pfs*5	1	1.3%		
L35F*9	1	1.3%		
R248W	1	1.3%		
R249K	1	1.3%		
R432*	1	1.3%		
S215N	1	1.3%		

Y220C	1	1.3%		
BRAF V600E	**9**	**11.5%**	**1**	**11.1%**
NRAS G12D	**2**	**2.6%**		
no target available	**1**	**1.3%**		

Mutational targets methylation		
WIF	56	72.7%
NPY	6	7.8%
LB positive	62	80.5%
LB negative	15	19.5%

Table 5: Mutational distribution pattern of metastatic colorectal cancer patients.

Quantity of extracted DNA using liquid biopsy – overview			
	cfDNA [ng/µl]	**ctDNA [ng/ml]**	**MAF [%]**
Pat. #1	1.35	6.70	0.25
Pat. #2	0.54		
Pat. #3	11.70	741.78	3.17
Pat. #4	0.90	7.20	0.40
Pat. #5	0.99	16.04	0.81
Pat. #6	1.92	10.37	0.27
Pat. #7	0.90	26.46	1.47
Pat. #8	191.00		
Pat. #9	0.74		
Pat. #10	6.28	12.56	0.10

Pat. #11	1.41	42.02	1.49
Pat. #12	0.97	31.62	1.63
Pat. #13	0.74		
Pat. #14	0.88		
Pat. #15	0.88	138.69	7.88
Pat. #16	0.84		
Pat. #17	0.81	7.34	0.45
Pat. #18	0.75	27.60	1.84
Pat. #19	0.46		
Pat. #20	1.92		
Pat. #21	0.45	2.80	0.31
Pat. #22	1.63		
Pat. #23	0.63	3.53	0.28
Pat. #24	1.00		
Pat. #25	0.48	1.82	0.19
Pat. #26	8.32	3128.32	18.80
Pat. #27	1.08		
Pat. #28	0.45		
Pat. #29	0.47		
Pat. #30	0.49		
Pat. #31	1.77		
Pat. #32	0.42	12.68	1.51
Pat. #33	0.63		
Pat. #34	17.50	9485.00	27.10
Pat. #35	4.80	1459.20	15.20

Pat. #36	0.66		
Pat. #37	0.97	40.35	2.08
Pat. #38	1.68		
Pat. #39	11.40	2599.20	11.40
Pat. #40	31.70	19717.40	31.10
Pat. #41	5.19	442.19	4.26
Pat. #42	1.52		
Pat. #43	1.05	3.15	0.15
Pat. #44	0.58	1.08	0.09
Pat. #45	0.60	59.40	4.95
Pat. #46	1.32	3.96	0.15
Pat. #47	2.42	40.90	0.85
Pat. #48	0.88	42.59	2.42
Pat. #49	7.00	4060.00	29.00
Pat. #50	0.70		
Pat. #51	1.25	2.50	0.10
Pat. #52	0.32	0.64	0.10
Pat. #53	0.82	138.91	8.47
Pat. #54	0.65	88.40	6.80
Pat. #55	0.25	18.85	3.77
Pat. #56	33.30		
Pat. #57	1.17		
Pat. #58	1.81	124.17	3.43
Pat. #59	1.09	112.92	5.18
Pat. #60	0.95	1.90	0.10

Pat. #61	1.27	3.81	0.15
Pat. #62	0.20		
Pat. #63	0.73	44.53	3.05
Pat. #64	1.79	397.38	11.10
Pat. #65	5.80		
Pat. #66	2.16	146.02	3.38
Pat. #67	0.82		
Pat. #68	1.66	19.46	0.59
Pat. #69	1.24	28.02	1.13
Pat. #70	1.86	101.56	2.73

Table 6: Amount of extracted DNA in liquid biopsies of metastatic pancreatic cancer.

Abbreviations: Pat. #: patient number, cfDNA: cell-free DNA in ng/µl, ctDNA: circulating tumor DNA in ng/ml, MAF: mutant allele frequency (%).

XV DISCLOSURES

Parts of this thesis have been published in the articles depicted in the following:

Circulating tumor DNA correlates with tumor burden and predicts outcome in pancreatic cancer irrespective of tumor stage

Kirchweger Patrick,[1,2,3] Kupferthaler Alexander,[4] Burghofer Jonathan,[5] Webersinke Gerald,[5] Jukic Emina,[6] Schwendinger Simon,[6] Weitzendorfer Michael,[7] Petzer Andreas,[3,8] Függer Reinhold,[2,3] Rumpold Holger,[1,3] Wundsam Helwig[2]

[1] Gastrointestinal Cancer Center, Linz, Austria

[2] Department of Surgery, Ordensklinikum Linz, Austria

[3] Johannes Kepler University Linz, Medical Faculty, Linz, Austria

[4] Department of Diagnostic and Interventional Radiology, Ordensklinikum Linz, Austria

[5] Laboratory for Molecular Genetic Diagnostics, Ordensklinikum Linz, Austria

[6] Institute of Human Genetics, Medical University of Innsbruck, Austria

[7] Department of Surgery, Paracelsus Medical University Salzburg, Salzburg, Austria

[8] Department of Internal Medicine I for Hematology with Stem Cell Transplantation, Hemostaseology and Medical Oncology, Ordensklinikum Linz, Austria

Citation: Kirchweger P, Kupferthaler A, Burghofer J, Webersinke G, Jukic E, Schwendinger S, Weitzendorfer M, Petzer A, Függer R, Rumpold H, Wundsam H. Circulating tumor DNA correlates with tumor burden and predicts outcome in pancreatic cancer irrespective of tumor stage. Eur J Surg Oncol. 2022 May;48(5):1046-1053. doi: 10.1016/j.ejso.2021.11.138. Epub 2021 Dec 1. PMID: 34876329.

Access link: https://www.ejso.com/article/S0748-7983(21)00947-1/fulltext

Permission for the implementation of the data of this publication to this dissertation has been given by all authors.

Prediction of response to systemic treatment by kinetics of circulating tumor dna in metastatic pancreatic cancer

Patrick Kirchweger[1,2,3], Alexander Kupferthaler[4], Jonathan Burghofer[5], Gerald Webersinke[5], Emina Jukic[6], Simon Schwendinger[6], Helwig Wundsam[2], Matthias Biebl [2,3], Andreas Petzer[3,7], Holger Rumpold [1,3]

[1] Gastrointestinal Cancer Center, Linz, Austria

[2] Department of Surgery, Ordensklinikum Linz, Austria

[3] Johannes Kepler University Linz, Medical Faculty, Linz, Austria

[4] Department of Diagnostic and Interventional Radiology, Ordensklinikum Linz, Austria

[5] Laboratory for Molecular Genetic Diagnostics, Ordensklinikum Linz, Austria

[6] Institute of Human Genetics, Medical University of Innsbruck, Austria

[7] Department of Internal Medicine I for Hematology with Stem Cell Transplantation, Hemostaseology and Medical Oncology, Ordensklinikum Linz, Austria

Citation: Kirchweger P, Kupferthaler A, Burghofer J, Webersinke G, Jukic E, Schwendinger S, Wundsam H, Biebl M, Petzer A, Rumpold H. Prediction of response to systemic treatment by kinetics of circulating tumor DNA in metastatic pancreatic cancer. Front Oncol. 2022 Aug 30;12:902177. doi: 10.3389/fonc.2022.902177. PMID: 36110940; PMCID: PMC9468369.

Access link: https://www.frontiersin.org/articles/10.3389/fonc.2022.902177/full

Permission for the implementation of the data of this publication to this dissertation has been given by all authors.

Circulating tumor DNA for diagnosis, prognosis and treatment of gastrointestinal malignancies

Patrick Kirchweger[1,2,3], Helwig Wundsam[2], Holger Rumpold[1,3]

[1] Gastrointestinal Cancer Center, Linz, Austria

[2] Department of Surgery, Ordensklinikum Linz, Austria

[3] Johannes Kepler University Linz, Medical Faculty, Linz, Austria

Citation: Kirchweger P, Wundsam HV, Rumpold H. Circulating tumor DNA for diagnosis, prognosis and treatment of gastrointestinal malignancies. World J Clin Oncol. 2022 Jun 24;13(6):473-484. doi: 10.5306/wjco.v13.i6.473. PMID: 35949436; PMCID: PMC9244970.

Access link: https://www.wjgnet.com/2218-4333/full/v13/i6/473.htm

Permission for the implementation of the data of this publication to this dissertation has been given by all authors.

HONORS AND FUNDING

This PhD project was funded by:

- Vinzenzgruppe Österreich
- Oberösterreichische Krebshilfe

Sponsors were not involved in study design, interpretation, or presentation of the data.

Publications, projects, posters, and talks within these PhD studies have been presented and have been awarded the following:

Congress	**Award**
OeGHO FJT 2022	Best Poster Prize
ESMO TAT 2022	ESMO Merit Travel Grant
Austrian Congress of Surgery 2022	ACO ASSO Prize
ACO ASSO Congress 2022	Best Submitted Abstract Prize
ESMO TAT 2023	Best Poster Prize
OeGHO FJT 2023	Best Submitted Abstract Prize
German Congress of Surgery 2023	
Austrian Congress of Surgery 2023	Hans Werner Waclawiczek Prize
	Best Poster Prize
	Best Talk Prize
OeGHO Best of ASCO 2023	Innovation Award 1st Prize
MedGes OÖ Honorary Member Meeting 2023	Prof. Dr. Walter Pilgerstorfer Prize

XVI ABBREVIATIONS

AUC	area under the curve
BEAMing	beads, emulsion, amplification, magnetics
CA 19-9	carbohydrate antigen 19-9
CCND2	cyclin-D2
CDKN2A	cyclin dependent kinase inhibitor 2A
CEA	carcinoembryonic antigen
cfDNA	cell-free deoxyribonucleic acid
CRC	colorectal carcinoma
CT	computed tomography
ctDNA	circulating tumor deoxyribonucleic acid
DNA	deoxyribonucleic acid
DFS	disease-free survival
ddPCR	droplet digital polymerase chain reaction
ECOG-PS	Eastern Cooperative Oncology Group performance group
EUS-FNA	endosonographic ultrasound fine needle aspiration
FFPE	formalin-fixation and paraffin-embedding
IQR	interquartile range
KRAS	Kirsten rat sarcoma virus
LB	liquid biopsy
LOD	limit of detection
lPDAC	localized pancreatic ductal adenocarcinoma

MAF	mutant allele frequency
mCRC	metastasized colorectal cancer
mPDAC	metastasized pancreatic ductal adenocarcinoma
NGS	next generation sequencing
n.a.	not applicable
nonPD	non-progressive disease
n.r.	not reached
NRAS	neuroblastoma rat sarcoma virus
OS	overall survival
p	probability
PC	pancreatic cancer
PCR	polymerase chain reaction
PD	progressive disease
PDAC	pancreatic ductal adenocarcinoma
PFS	progressive-free survival
R	Spearman’s rho
ROC	receiver operating characteristic
TP53	tumor protein 53