Dissertation

Molecular profiling of tumor DNA for treatment selection in patients with advanced cancer

submitted by

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for the Academic Degree of

Doctor of Medical Science
(Dr. scient. med.)

at the

Medical University of Graz

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2021

Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the Guidelines of the Medical University of Graz on Good Scientific Practice.

Graz, September 2021 *Jakob Riedl eh*

Disclosures

Parts of this thesis have been published in the following article:

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Profiling of circulating tumor DNA and tumor tissue for treatment selection in patients with advanced and refractory carcinoma: A prospective, two-stage phase II individualized cancer treatment trial. Therapeutic Advances in Medical Oncology. 2021; Volume 13

Access link: https://journals.sagepub.com/doi/full/10.1177/1758835920987658

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Acknowledgements:

At this point I would like to thank all the people who made this work possible by supporting me throughout my study and beyond.

First and foremost, I want to express my warmest thanks to my supervisor Professor Armin Gerger who has been an exceptional mentor and motivator from the very first day of my research efforts. Thank you, Armin for your great support, generosity, and vision.

I would further like to express my deepest gratitude to Priv. Doz. Florian Posch and Priv. Doz. Michael Stotz for being such outstanding colleagues and teachers to me. Thank you, Florian, and Michael for your invaluable guidance and mentoring in research and the clinic through the time of my study and residency.

I would further like to specially thank Samantha Hasenleithner and Prof. Ellen Heitzer for spending great effort, time, and expertise in support of my dissertation project. My thanks also go to my dissertation committee Prof. Speicher and Prof. Hoefler, as well as to Prof. Berghold, Gudrun Pregartner and all others who have contributed to this study. Without each of you this cooperation project would not have been possible.

I would further like to thank Prof. Herbert Stöger for giving me the chance to start training in oncology, as well as Prof. Philipp J. Jost, current head of the Division of Oncology for offering me the opportunity to contribute to the advancement of the molecular tumor board and dive deeper into the field of precision oncology.

Finally, and most importantly I want to express my deepest gratitude, appreciation and love to my girlfriend Hanna, my family, and friends. It is the greatest privilege to have you around me.

The author received support by the Medical University of Graz through the Doctoral School Molecular Medicine and Inflammation.

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List of Abbrevations:

cfDNA - cell free DNA

CRC - colorectal cancer

CT - computer tomography

CTC - circulating tumor cells

CTCAE - Common Terminology Criteria for Adverse Events

ctDNA - circulating tumor DNA

CUP - cancer of unknown primary

ECOG - Eastern Cooperative oncology Group

EMA - European Medicines Agency

ESCAT - ESMO Scale for Clinical Actionability of molecular Targets

ESMO - European Society for Medical Oncology

FDA - Food and Drug Administration

FISH - fluorescence in situ hybridization

ICT - Individualized Cancer Treatment

i.e. - id est

IQR - interquartile range

MRI - magnetic resonance imaging

MP - molecular profiling

MTB - molecular tumor board

NGS - next generation sequencing

NSCLC - non small cell lung cancer

ORR - objective response rate

OS - overall survival

PD - progressive disease

PD-1 - programmed death receptor 1

PFS - progression free survival

RECIST - Response Evaluation Criteria in Solid Tumors

SAE - serious adverse event
SCNA - somatic copy number alteration
SD - stable disease
TKI - tyrosine kinase inhibitor
sWGS - shallow whole genome sequencing
WES - whole exome sequencing
WGS - whole genome sequencing

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Abstract

Introduction

Precision oncology incorporates the concept of molecular profiling-based treatment allocation. To date precision oncology trials have focused on molecular profiling of tissue biopsy-derived DNA. In the Individualized Cancer Treatment (ICT) trial we aimed to evaluate the feasibility and efficacy of targeted cancer therapy selected by molecular profiling of circulating tumor DNA (ctDNA) and/or tumor tissue in patients with advanced and refractory carcinoma in a prospective setting.

Methods

A mandatory blood draw as well as an optional tissue biopsy were obtained for molecular profiling. Shallow whole-genome sequencing and a cancer hotspot panel were performed on plasma and tissue DNA. Publicly available databases were used to match the molecular profile to targeted treatments based on the annotated results, which were discussed at a molecular tumor board. The primary endpoint was the progression-free survival (PFS) ratio (PFS on molecular profiling guided therapy / PFS on the last evidence-based therapy), whereas the success of the targeted therapy was defined as a PFS ratio ≥ 1.2. Secondary endpoints were the number of patients for whom an anti-tumor drug could be defined based on the molecular profiling results, the overall survival (OS) and the overall radiographic response rate (ORR).

Results

Due to slow patient accrual and lack of clinical benefit an interim analysis was performed after inclusion of 24 patients and the study was terminated prematurely. Molecular profiling yielded informative results from 20 patients (83%), meaning that tumor-specific DNA content in plasma was above 5% and that either a somatic mutation or copy number alteration could be detected from plasma or tissue. A potential tumor-specific drug could be matched in 11 out of 24 patients (46%). Eight patients (33%) received a matched treatment of which two experienced a numerically longer PFS compared to the PFS under last evidence-based therapy line. None of the 8 treated patients met the primary endpoint of a PFS ratio ≥ 1.2.

Conclusion

This study is the first to report prospective outcome results on the efficacy of ctDNA profiling-based treatment. Despite the premature study termination and the small samples size, this study provides important insights that are critical to further advancing the implementation of precision medicine. Among other the ICT trial indicates that molecular profiling of ctDNA harbours great potential for the concept of personalized cancer treatment. Still, at present its use should be primarily limited to clinical trials evaluating new concepts of ctDNA based treatment allocation.

Zusammenfassung:

Hintergrund:

Das Konzept der Präzisionsonkologie beruht auf der Vision, medikamentöse Tumortherapie anhand des individuellen molekulargenetischen Tumorprofils eines Patienten auszuwählen. Bis dato haben Präzisionsonkologie Studien auf die Untersuchung von Tumor DNA fokussiert, welche aus Tumorgewebsproben extrahiert wird. In der „Individualized Cancer Treatment“ (ICT) Studie haben wir es uns zum Ziel gesetzt, die Durchführbarkeit und Effizienz einer auf Basis einer molekulargenetischen Untersuchung von zirkulierender Tumor DNA (ctDNA) und/oder Gewebsbiopsie ausgewählten molekular zielgerichteten Therapie bei Patienten mit fortgeschrittener und therapierefraktärer Tumorerkrankung zu untersuchen.

Methoden

Zur Erstellung eines molekularen Tumorprofils wurde bei allen Patienten eine Blutabnahme zur Analyse von ctDNA durchgeführt und den Patienten eine optionale Tumorbiopsie zur molekulargenetischen Untersuchung des Tumorgewebes angeboten. Nach Vorliegen der Sequenzierungsergebnisse wurde für jeden Patienten ein individueller molekulargenetischer Bericht erstellt, dieser im Rahmen eines molekularen Tumorboards diskutiert und anhand des molekularen Profils eine Therapieentscheidung getroffen. Primärer Endpunkt der Studie war das Progressionsfreie Überleben (PFS) unter der zielgerichteten Therapie, verglichen mit dem PFS der letzten evidenzbasierten Therapielinie, wobei das Erreichen einer PFS Ratio > 1.2 als Erfolg festgelegt wurde. Die sekundären Endpunkte waren der Anteil an Patienten, für welche, passend zum molekularen Profil, eine zielgerichtete Therapie definiert werden konnte, die radiologische Ansprechrate, sowie das Gesamtüberleben.

Ergebnisse

Auf Grund langsamer Rekrutierung, sowie fehlenden klinischen Benefits in der Interimsanalyse, musste die Studie nach Einschluss von 24 Patienten beendet werden. Bei 20 Patienten (83%) konnte mittels molekulargenetischer Untersuchung von ctDNA oder Tumorgewebe DNA ein konklusives molekulares Profil erstellt werden und zumindest eine somatische Mutation oder Kopienzahlveränderung nachgewiesen werden. Bei 11 Patienten (46%) wurde anhand des molekularen Profils eine zielgerichtete Therapie festgelegt.

Schlussendlich haben 8 Patienten (33%) die zielgerichtete Therapie erhalten und bei zwei davon konnte ein numerisch längeres PFS gegenüber der letzten evidenzbasierten Therapie erreicht werden. Hinsichtlich des primären Endpunkts der Studie konnte bei keinem der acht therapierten Patienten das vorab definierte Ziel einer PFS Ratio >1.2 erreicht werden.

Schlussfolgerung:

In der ICT Studie wurden erstmals Ergebnisse zur Therapieeffizienz auf Basis einer molekulargenetischen Untersuchung von ctDNA ausgewählter molekular zielgerichteter Therapie präsentiert. Trotz des vorzeitigen Studienabbruchs und der kleinen Fallzahl liefert diese Studie wichtige Erkenntnisse, die für den weiteren Fortschritt im Bereich der Präzisionsonkologie entscheidend sind. Unter anderem zeigt die ICT Studie, dass die molekulargenetische Untersuchung von ctDNA großes Potenzial für das Konzept der personalisierten Tumortherapie hat, jedoch zum jetzigen Zeitpunkt primär im Rahmen klinischer Studien zum Einsatz kommen sollte.

Introduction

Epidemiology of cancer

Representing the second leading cause of death cancer is one of the major public health burdens globally. In 2020 an estimated number of 19,3 million new cancer cases occurred worldwide with female breast cancer being the most common cancer entity followed by lung, colorectal and prostate cancer (1). Despite increasing knowledge about risk factors of carcinogenesis and evolving preventative measures such as no-smoking campaigns the prevalence of cancer has constantly risen over recent years. For instance, in Austria the estimated cancer prevalence rose from approximately 200.000 cancer cases in 2000 to approximately 350.000 cases in 2018 which reflects a relative increase of 75% (2). While the number of new cancer diagnoses per year has been recently stable or slightly declining in Western countries such as Europe and the US (3), the rising cancer prevalence can be mainly explained by declining cancer lethality due to advanced cancer screening measures which often result in the diagnosis of early stage cancer and as a consequence of new treatment options leading to prolonged survival rates. Despite these advances, in 2020 approximately 10 million cancer related deaths occurred worldwide, of which Europe accounted for 20% and 20.000 cases were observed in Austria (1). These data underline the importance of developing novel therapeutic agents and innovative treatment concepts. Precision oncology which incorporates the vision of personalized cancer therapy selected by molecular profiling of tumor DNA might have the potential to become a future landmark of cancer therapy.

Evolution of cancer treatment:

The first historical report of cancer treatment dates back to the remote antiquity around 3000 b.c. in Egypt. Called the Edwin Smyth Papyrus after its European finder, this manuscript contains a copy of an Egyptian textbook on trauma surgery including the description of eight cases of female breast tumors and ulcers that were removed by cauterization (4). Hippocrates who is widely considered as the “father of human medicine” was first to provide more detailed and scientific studies on cancer. In contrast to the prior understanding that cancer was based on supernatural events he recognized cancer as a biological disease that evolved due to a misbalance of the four body humors blood, phlegm, black and yellow bile. In his writings he described different types of tumors such as cancers of the skin, breast and cervix and distinguished between hard cancers, ulcerated cancers and deep invisible tumors which he considered as incurable. As the growth of cancer reminded him of a moving crab, he established the word “carcinoma” referring to the ancient Greek word of crab, which was later translated to the Latin word “cancer” by the Roman physician Celsus. The main approaches of treating cancer at that time were the application of superficial lotions and tinctures, cautery, as well as radical surgical therapy with knifes (4-6). The theories on cancer origins postulated by Hippocrates and further supported and refined by the Roman physician Galen remained widely accepted for centuries until the beginning of the renaissance, when the invention of printing by Johannes Guttenberg and the formation of the first universities paved the way for scientific advances in the field of medicine. Autopsies led to a better understanding of the composition of a human’s body, its physiology, and its diseases. In the 15th Century the Suisse physician Paracelsus firstly promoted various chemicals such as mercury, lead, sulphur and other as remedies for various diseases including cancer. Paracelsus further stated that the dose of a substance defines whether it is poisonous or not, which comprises a central dogma in modern pharmacology (7). Over the next three centuries multiple new inventions such as the microscope enabled a more in-depth study of cancer origin and oncogenesis. By his theories on cancer origin postulated in “Die Cellularpathologie in ihrer Begrundung auf physiologische und pathologische Gewebelehre” (8) and “Die krankhaften Geschwulste” (9) the German pathologist Rudolf Virchow provided the scientific basis for the nowadays understanding of carcinogenesis. He stated that cancer originates from malignantly transformed cells which further divide and spread through the blood stream (10). Despite major advances in the field of microscopic pathology and surgical oncology, at that time cancer treatment still mainly relied on radical surgical tumor resection which often resulted in mutilation and was mostly ineffective in patients with advanced cancer. A prime example therefore represents the radical mastectomy introduced by William Hallsted in 1891 as the surgical treatment for breast cancer. Although this approach resulted in excellent local control rates the majority of patients experienced disease recurrence due to metastatic spread and ultimately died with disseminated disease (11). Finally, at the end of the 19th century the discovery of x-rays by Willhelm C. Röntgen and the subsequent finding of their potency to cause cell death and tumor shrinkage by Marie and Pierre Curie led to the birth of radiation therapy, which added another treatment option for cancer. Over the following decades radiotherapy was constantly advanced and today represents a central part of multimodal cancer treatment (12). The next groundbreaking revolution in oncology was made by the discovery and implementation of chemotherapy. Based on the accidental findings of the bone marrow depleting effect of nitrogen mustard which was used as a weapon in World War I, first clinical trials in patients with hematologic malignancies were made in the 1940s in the US, that showed significant temporary benefit for this new treatment approach. Because of severe toxic side effects and only short time effects on cancer growth, nitrogen mustard was soon considered as impracticable for treatment in humans (13). However, based on the detection of its cytotoxic effect the scientific fundament for a new era of medical cancer therapy was laid, which evolved rapidly over the next decades. Sidney Farber, a pathologist from Boston who turned to be a visionary pediatric hemato-oncologist, took a central part in these developments and is thus often considered as the father of modern chemotherapy. In a seminal trial of children with acute leukaemia he could demonstrate a significant clinical benefit for the treatment with the folic acid antagonist aminopterin which was the predecessor drug of methotrexate that is still commonly used in clinical practice (14, 15). Farber’s findings and his relentless effort in raising the public´s and politics attention on the importance of cancer research funding paved the way for the discovery and synthesis of multiple new chemotherapeutic agents resulting in major treatment successes such as the first cure of a patient with metastatic disease in 1956 (16). In parallel, the discovery of the structure of DNA by Watson and Crick and the development of novel molecular DNA analysis techniques such as polymerase chain reactions allowed a deeper insight in cancer biology and led to the finding that specific gene mutations play a central part in carcinogenesis (17-19). This knowledge opened the door for genome-targeted cancer treatment. Finally in the 1990s, until which medical cancer therapy mainly comprised of cytotoxic agents, the first effective targeted cancer agents were developed resulting in astonishing outcomes. A prime example therefore represents the monoclonal humanized antibody trastuzumab which revolutionized the treatment of HER2 receptor positive breast cancer (20). Over the following years multiple novel genome-targeted agents that significantly improve the outcome for several malignancies expanded the arsenal of anticancer drug therapy (21). The latest paradigm shift in medical oncology was achieved by the Nobel prize rewarded discovery of immune checkpoint inhibition which not only revolutionized the concept of immunotherapy but also led to remarkable survival outcomes in cancer entities previously considered as hardly treatable such as metastatic melanoma (22). The final step towards personalized cancer therapy has been made very recently by the first tumor agnostic approvals of cancer therapies, which means that an anticancer drug therapy can be administered based on the detection of a specific genetic alteration, irrespective of the cancer´s location or histology (23). Thus, in medical oncology lately there has been a transition away from a “one therapy fits all” concept towards a more biomarker stratified approach which might finally result in a truly individualized cancer treatment.

Precision oncology, a paradigm shift in oncology?

“Tonight, I'm launching a new Precision Medicine Initiative to bring us closer to curing diseases like cancer and diabetes — and to give all of us access to the personalized information we need to keep ourselves and our families healthier.” By making this announcement and the launching of a multi-million Dollar endorsement US president Barack Obama underlined his ambition to initiate a new era of personalized medicine (24). The concept of precision medicine incorporates the idea to use information of an individual´s clinical characteristics as well as his or her genetic profile to guide decision making for disease prevention, diagnosis and treatment (25). As part of the precision medicine paradigm precision oncology has lately received increasing attention. A systematic literature research study demonstrated that the total number of publications identified with the search term “precision oncology” showed a rapid increase since 2010. Interestingly, over time the meaning of “precision oncology” has significantly changed. From 2005 until 2010 the term “precision oncology” was mostly used for targeted cancer therapy in general, whereas from 2015 to now on the most prevalent definition for precision oncology and personalized cancer treatment has been to use omics-guided tumor profiling for the selection of targeted cancer therapy (26). According to experts from the German National Center for Tumor Diseases precision oncology implies the ability to predict which individual patient will likely respond to a specific cancer therapy based on high-resolution molecular diagnostics as well as the functional and mechanistic understanding of the patient´s tumor (27). In this thesis the current definition of precision oncology will be used, referring to the use of molecular profiling for guiding cancer treatment allocation.

Cancer – a genetic disease of great diversity and heterogeneity

Comprehensive sequencing efforts such as the Cancer Genome Project and the Cancer Genome Atlas have significantly improved our understanding of cancer evolution, its diversity and heterogeneity. Today, cancer is largely recognized as a disease that develops due to an accumulation of genetic alterations in genes that regulate key signalling pathways such as cell proliferation, cell invasion and cell death (28). As it was prominently shown for the tumorigenesis of colorectal cancer, the transition from benign to malignant tissue is a step wise process that occurs over time by the acquisition of a series of mutation that provide a selective growth advantage compared to a normal cell (29). These mutations are called driver mutations, whereas mutations that are not considered to impact tumor growth are classified as passenger mutations (30). To date, approximately 500-600 cancer driver genes have been identified, which can be roughly divided in oncogenes that confer a selective growth advantage by activation and tumor suppressor genes that promote tumor growth by mutation induced inactivation (31). Importantly, there is a strong variation regarding the frequency of genetic alterations between different tumor types. A systematic genomic analysis of more than 3000 samples from 27 different tumor types revealed that the frequency of mutations varies by more than 1000-fold across cancer types with the highest mutation rates present in melanoma and lung cancer. It was further shown that the mutational composition of the tumors shows strong variation dependent on its tissue type and location (32). Even more importantly growing evidence suggests an extensive genetic diversity between patients tumors of the same origin for instance in colorectal carcinoma or squamous cell lung cancer (33, 34), i.e. intertumor heterogeneity and even within one patient´s tumor sides, i.e. intratumor heterogeneity. Mutational intratumor heterogeneity is thought to be a consequence of genomic instability and branched tumor evolution in which different cancer cell sublclones evolve due to selection pressure within a given tumour micro-environment. Branched evolution can result in a strong variation of the mutational profile between the primary tumor and its metastases and even within one tumor lesion (32). Tumor evolution and mutational diversity is further driven by the application of antineoplastic therapy especially of targeted therapies, which commonly results in the emergence of resistant subclones. As a prime example, Misale et al. demonstrated that patients with primary KRAS wildtype colorectal cancer, who undergo treatment with anti EGFR antibodies develop secondary drug resistance by the acquisition of new onset KRAS mutations. Importantly, they could further show that KRAS mutant allels found in tissue re-biopsies could be detected in plasma months before radiologic tumor progression (35). This underlines the potential of ctDNA as an analyte for molecular profiling, which might overcome the central challenge of spatial and temporal mutational heterogeneity (36, 37).

Genomic tumor profiling

The identification of valid biomarkers predicting treatment response to antineoplastic therapy is critical to the concept of personalized cancer treatment (38). Historically the selection of antineoplastic therapy was mainly based on the primary tumor location and its histology. With the implementation of immunohistochemistry that can be used to detect protein expression at the cellular level such as the HER2 receptor in breast cancer (39) and fluorescence in situ hybridization (FISH) for the detection of DNA rearrangements (40), the first biomarker driven therapies were approved. Immunohistochemistry and FISH are still central tools for biomarker stratified treatment allocation in various cancer types (41). Until the late 2000s largescale genomic tumor profiling was accompanied by exorbitant costs and an immense timely effort and was thus mainly used for scientific purposes (42). However, based on major technological innovations that resulted in a considerable reduction of sequencing costs and turnaround time, today genomic tumor profiling is increasingly used in routine clinical practice for diagnostic and therapeutic purposes (43). At the early days of molecular profiling based treatment allocation, conventional sequencing techniques were used for the detection of single gene alterations such as the BRAF V600E mutation (44) or the EML4-ALK fusions (45) for which targeted therapies had already been developed. Conventional sequencing techniques can detect specific genetic alteration with high sensitivity and specificity however they are time consuming and do not enable the testing of several genetic biomarkers at the same time. With the rapidly increasing number of newly identified genetic cancer targets, single gene testing was gradually replaced by multiplexed diagnostic assay technology, which enables the genomic profiling of multiple genes at the same time (46). The recent development of next generation sequencing (NGS) has finally revolutionized the field of genome sequencing and has significantly enhanced our ability to interrogate multiple cancer-associated genomic changes within an individual patient’s tumor. NGS allows a cost and time effective simultaneous analysis of a broad spectrum of genomic alterations including single-nucleotide variants, insertions, deletions, gene fusions, copy number alterations, loss of heterozygosity and tumor mutational burden in tumor DNA samples at a sensitivity and specificity comparable with conventional sequencing techniques (47, 48). Owing to its high efficiency and the ability to produce an enormous amount of data at relatively low costs, NGS emerged as an indispensable tool for cancer genomics that has rapidly transformed cancer research. The widespread application of NGS has led to a comprehensive genomic characterization of various tumor types providing a deeper insight in oncogenesis, cancer progression and treatment resistance, further resulted in the identification of numerous new genomic cancer targets and accelerated the development of novel effective genome-targeted drugs (49-51). Thereby NGS provided the technological fundament for the implementation of largescale precision oncology trials. Currently, various approaches of NGS for cancer genomics are applied in cancer research and in the clinic. Among others these include targeted gene panel analysis, whole exome sequencing (WES), whole genome sequencing (WGS) as well as transcriptomics and epigenetics (43).

Targeted NGS

To date, in the clinical routine NGS is mostly employed for the tumor DNA analysis of targeted cancer gene panels also referred to as hotspot cancer panels which usually compromise 20-500 known cancer driver genes. Compared to WES and WGS cancer gene panel analysis can be performed with a lower amount of DNA and at lower costs. For the clinical adoption targeted panel analysis further holds the advantage of a deeper coverage in the areas of interest and a less complex interpretation of genomic results (52). Over the recent years multiple commercial sequencing platforms such as the Ion Torrent AmpliSeq cancer hot spot panel (53) or the FoundationOne CDx panel (54) were applied which are widely used in routine clinical practice.

Whole exome sequencing and whole genome sequencing

On the other hand, WES and WGS not only cover prespecified genomic areas of interest but provide comprehensive information on the cancer genome. Owing to its ability to detect novel pathogenic gene variants and to provide large amounts of genomic data, comprehensive genomic profiling is an increasingly valuable tool for research purposes. Due to its higher costs, the longer turnaround time and the need for extensive bioinformatic annotation support, in the clinical routine comprehensive genomic profiling is currently limited to selected cases in which targeted gene panel analysis has failed to identify targetable alterations (55). Importantly, in the recently published PERMED-01 trial evaluating the feasibility of real time extensive genomic profiling in patients with advanced cancer, WES did not provide significant benefit in terms of identification of actionable genetic alterations when compared to targeted NGS (56).

Transcriptomics

Although cancer genomics still represents the corner stone of molecular profiling-based treatment allocation, recently major research efforts have been invested in the analysis of tumor RNA for the identification of new potential treatment targets, that might be missed at the genomic level. In contrast to tumor DNA analysis, high throughput sequencing of RNA commonly referred to as transcriptomics provides information on the gene expression signature (57). Thereby transcriptomics represents a valuable tool in evaluating whether an oncogenic mutation on DNA level also results in an overexpression of the respective gene. Transcriptomics can be further particularly useful for the detection of oncogenic gene fusions (58). The recently presented WINTHER trial was the first precision oncology trial to evaluate not only DNA profiling but also RNA expression analysis for biomarker guided treatment matching. The study findings showed that the transcriptomic approach increased the number of actionable genetic alterations and even led to a slightly but not statistically higher clinical benefit rate compared to the genome based therapy arm (59). Despite these promising findings, several limitations of transcriptomics including technical and analytical issues such as RNA degradation and artifact alignment have to be overcome before its widespread use in clinical practice can be ensured (60).

Liquid biopsy

Limitations of genomic tissue analysis

Since the early days of microscopic pathology, analysis of formalin fixed tissue has been the cornerstone of cancer diagnosis, classification and treatment selection in solid malignancies and still represents the gold standard in this setting. Molecular profiling of tumor tissue has tremendously improved our understanding of cancer biology and has led to the identification of numerous genomic biomarkers predictive of treatment response to specific targeted drugs (61, 62). However, molecular profiling of tumor tissue poses several limitations: First and foremost, as tumor tissue can only be obtained by invasive methods such as surgical tumor resections or biopsies its availability is limited and comes along with patients' discomfort and potential procedural complications. This is particularly problematic for the concept of genome-based treatment allocation, as patients undergoing genomic tumor analysis usually have advanced cancer stage (63) and hence are often not feasible for time consuming invasive procedures. In addition, increasing evidence suggests that cancer is a highly dynamic disease that constantly adapts in response to internal and external factors such as the application of antineoplastic treatment. Over time different types of cancer sublclones evolve under selective pressure which can significantly impact the mutational tumor landscape of an individual´s patient tumor (64). This phenomenon of clonal evolution and temporal heterogeneity underlines the limitations of archival tumor tissue as an analyte for molecular profiling. To overcome this challenge several precision oncology trials have thus obtained newly biopsied tumor samples for sequencing (56). Nevertheless, a single snap-shot tissue biopsy cannot adequately reflect spatial heterogeneity including intratumor and intermetastatic variability which remains a central challenge for the concept of personalized cancer treatment. Moreover, in clinical practice re-biopsies often result in a delay of antineoplastic treatment initiation which can cause rapid tumor progression and clinical deterioration.

An overview of liquid biopsy

In the light of these limitations, over recent years the high potential of liquid biopsies as analytes to detect and monitor genetic biomarkers has become increasingly recognized. In oncology, the term liquid biopsy mostly refers to the sampling and analysis of blood-based cancer components including circulating tumor cells (CTC) (65), cell free nucleic acids such as ctDNA (66) and circulating cell-free RNA (67) and cancer cell vesicles (68), proteins (69) and metabolites (70). However, also other body fluids including urine (71), stool (72), ascites (73), pleural effusion (74) and cerebrospinal fluid (75) have been shown to contain tumor-derived genetic material relevant for diagnostic purposes. Here we will mainly focus on ctDNA, which has been most extensively studied and has also served as an analyte for molecular profiling in our study. Overall, compared to conventional tumor tissue sampling, liquid biopsy harbours the great advantage to be minimally invasive which enables a sequential monitoring of tumor evolution over time.

Origin, plasma levels and clearance of ctDNA

The presence of circulating cell free DNA (cfDNA) was firstly discovered in the late 1940s by Mandel et al. who detected non cell bound nucleic acids in human´s blood (76). Several decades later it was elucidated that the amount of cfDNA was significantly higher in patients with cancer compared to healthy controls and that tumor specific genomic alterations such as oncogenic mutations or microsatellite instability could be detected in these DNA strands (77, 78). These findings underlined the great potential of cfDNA as a novel analyte for cancer diagnostics at an early stage and triggered further research efforts. Today, we know that cfDNA originates not only from cancerous tissue but also from other sources including healthy and inflamed tissue, which poses one central challenge of cfDNA analysis (79). Tumor derived cfDNA, referred to as ctDNA is most accurately detected in plasma samples (80). The exact mechanisms by which cfDNA and ctDNA enter blood circulation has not been entirely clarified, however increasing evidence suggests a mainly passive release by apoptosis and necrosis of the respective cells. This hypothesis is supported by the lower amount of cfDNA detected in healthy individuals, in whom under physiologic conditions cell detritus including cell free nucleic acids might be cleared more efficiently by phagocytes (81). Further, levels of ctDNA in plasma show a strong correlation with tumor volume (82). Thus, it can be hypothesized that in patients with large tumor masses the vastly increased amount of apoptotic and necrotic cells could exceed the clearance capacity. Another theory suggests that ctDNA is actively secreted by living tumor cells (79), which however is contradicted by several studies that could demonstrate that plasma DNA molecules showed a fragmentation pattern hinting towards an apoptotic origin (83, 84). The clearance of cfDNA from the blood is thought to occur at the kidney, liver, and spleen at a rapid speed ranging from minutes to few hours (85, 86). This is crucial for the concept of precision oncology, as it enables a current snapshot of the ctDNA amount and composition. Furthermore, since tumor DNA is released from multiple tumor regions at the same time, studies suggest that the genomic analysis of ctDNA might provide a comprehensive view of the whole cancer mutational profile and thereby may better capture the molecular heterogeneity than a single tissue biopsy (87). Importantly, the overall quantity and the relative fraction of cfDNA derived from cancer shows a strong variation dependent on multiple factors which are only partly elucidated. For instance, Bettegowda et al. demonstrated that the detection rate of ctDNA is significantly higher in patients with metastatic disease compared to those with localized disease (88). Further, levels of ctDNA are largely influenced by cancer type as recently showed by a Chinese study evaluating the detection rate of ctDNA in more than 10.000 patients with various malignancies. They found that the detection sensitivity was highest in patients with small cell lung cancer and prostate cancer, and lowest in thyroid and renal cancer (89). Owing to this great variability and the fact that plasma ctDNA levels are often quite low, highly sensitive technical approaches for the accurate detection and analysis of ctDNA have been recently developed (36). Still, the analytical validity including its accuracy, reliability and reproducibility must be further improved before a widespread application of ctDNA analysis can be established in routine clinical practice (90).

ctDNA in the clinical setting and its role in precision oncology

Over the recent years multiple studies have demonstrated the vast potential of ctDNA as a novel diagnostic, prognostic and predictive biomarker in various fields of clinical cancer research. The great variety of potential applications of ctDNA, range from early and residual cancer detection to molecular cancer classification, treatment response monitoring and identification of molecular resistance mechanisms (36, 91). Here I will mainly focus on molecular profiling of ctDNA and its role as a novel biomarker for tailoring genome targeted therapy. The ability to assess the current genomic landscape of an individual's tumor via a minimally invasive blood draw makes ctDNA a particularly attractive tool for precision oncology. However, before its widespread use to serve as a key analyte for genomic profiling in the clinical routine can be safely guaranteed several requirements including a high sensitivity and specificity for the detection of genetic alterations found in tissue analysis must be ensured. Importantly, several largescale genomic studies have recently demonstrated a high concordance of the mutational profile from ctDNA and matched tissue in various cancer types (37). For instance, the assessment of the RAS mutational status from plasma ctDNA by OncoBEAM assay in patients with metastatic colorectal cancer resulted in an overall agreement of 93% supporting its clinical utility in this setting (92). Another study evaluated whether WES of cfDNA in patients with prostate and breast cancer would be feasible to obtain reliable results. They found a concordance rate of 88% for somatic mutations between ctDNA and matched tumor tissue. Importantly, only two thirds of the patients enrolled in this trial had sufficient levels of ctDNA to allow for mutational profiling (93). Aligning well with other studies this underlines a central limitation of genomic ctDNA analysis, that remains to be overcome (94). Novel analytical approaches for detecting very low amounts of ctDNA at a high sensitivity level might serve as a future solution (95). At present, best evidence for the use of ctDNA in the clinical setting exists for its capacity to monitor and identify resistance mutations for genome targeted therapies. A prime example for its clinical utility in this setting represents the ctDNA based detection of the EGFR T790M resistance mutation in patients with non-small cell lung cancer (NSCLC) treated with first generation EGFR tyrosine kinase inhibitors (TKI). In a retrospective study, Oxnard et al. demonstrated that patients with NSCLC and acquired EGFR-TKI resistance who were tested positive for the T790M mutation in plasma had equivalent outcomes when treated with the third generation TKI Osimertinib compared to patients tested positive by tissue analysis (96). These findings were further supported by a prospective validation study from the same group in which a high concordance rate for common EGFR mutations including the T790M resistance mutation could be shown (97). Importantly, the turnaround time for plasma analysis was significantly shorter compared to genotyping from tissue re-biopsy. Based on its high positive predictive value to detect the T790M resistance mutation and the ability to predict treatment response to third generation TKIs, the cobas EGFR mutation test was approved by the Food and Drug Administration (FDA) as a companion diagnostic to tailor EGFR targeted therapy in patients with NSCLC. This was the first clinical approval of a liquid biopsy-based biomarker test for the selection of a genome targeted therapy, thereby representing an important step towards the adoption of ctDNA as a biomarker in precision oncology. Besides its application in NSCLC, increasing evidence supports the clinical utility of ctDNA to monitor for secondary KRAS resistance mutations in colorectal cancer patients undergoing therapy with anti EGFR targeted antibodies such as panitumumab. A seminal study by Siravegna et al. demonstrated that sequential genotyping of ctDNA is a highly valuable tool for monitoring the mutational landscape of patients with colorectal cancer throughout the course of their disease, which might also hold crucial therapeutic implications. They could show that patients with primary RAS wildtype metastatic colorectal cancer who initially responded to anti EGFR therapy, develop resistance by the acquisition of different mutations including new onset KRAS mutations. Even more importantly, they found that mutated KRAS clones which emerged as a resistance mechanism decline upon withdrawal of EGFR blockade (98). Based on these groundbreaking findings a phase II trial was initiated by Cremolini et al. who could demonstrate that in patients with metastatic colorectal cancer who had previously progressed on an anti EGFR based regimen, a rechallenge with an EGFR specific antibody is feasible in case no resistance mutation is detected by upfront ctDNA analysis (99). This concept was further supported by promising findings from a preliminary report of the CHRONOS trial presented at the annual American Society of Clinical Oncology meeting in 2021 (100). Altogether, these data emphasize the great potential of ctDNA as an analyte to tailor molecular profiling-based therapy.

Landscape of genomic cancer targets and its agents

Major technical advances in the field of NGS have enabled a comprehensive genomic profiling of various cancer types and provided a deeper insight in oncogenesis. Based on these developments multiple new genetic alterations driving cancer progression could be discovered in recent years. This growing body of knowledge fuelled the identification of novel predictive biomarkers and the development of a variety of new genome targeted cancer drugs. Today, more than 80 targeted drug cancer therapies have been approved by the FDA for treatment of solid malignancies, of which approximately half are constrained to the detection of a specific predictive biomarker (101). From a pharmacological point of view, targeted cancer agents can be roughly divided in monoclonal antibodies that target specific antigens found on the cell surface such as growth factor receptors and small molecules that can interact with targets inside the tumor cell, such as by inhibition of tyrosine kinases. The cancer genome is characterized by various genetic alterations which provide a landscape of potential actionable targets. In the following section, an overview of the different types of genomic alterations and examples for their actionability will be given.

Gain of function gene alterations

Genes that promote cancer proliferation in case of upregulation are referred to as oncogenes. Oncogenes can be activated by a variety of genetic alterations including single nucleotide variants, base insertions, base deletions or gene amplifications. To date, a variety of gain of function gene mutations have been identified of which single nucleotide variants, also known as point mutations, are the most prevalent (102). Point mutations result from a base substitution at one nucleotide and are classified as missense mutations, when they result in a change of the amino acid sequence of the encoded protein or nonsense mutations when they lead to a premature termination of the protein transcription (30). Importantly, gain of function mutations may confer selective growth advantage to a cell when they occur at only one of the two gene allels. In this case we speak of a heterozygous mutation (103). There are different ways to target gain of function alterations, of which one is by inhibition of the abnormal gene product. A prominent example therefore is the successful targeting of the BRAF V600E missense mutation in melanoma patients. The BRAF V600E mutation is a driver mutation found in approximately 50% of all melanoma patients, that leads to an activation of the MAPK signalling pathway (44). As significantly improved outcomes could be achieved compared to classic chemotherapy, so far three potent small molecule inhibitors (encorafenib, vemurafenib, dabrafenib) of the mutated BRAF protein have been approved for treatment of BRAF V600E mutated melanoma either alone or in combination with MEK inhibitors (104).

Loss of function gene alterations

Genes that prevent cells from uncontrolled proliferation are classified as tumor suppressor genes. Like for gain of function alterations, a variety of genomic alterations can cause an inactivation or downregulation of tumor suppressor genes which subsequently promotes oncogenesis. However, in contrast to gain of function mutations in oncogenes, both alleles of the coding tumor suppressor genes must be affected to result in a loss or reduction of its tumor suppressive function. This is considered as the two-hit hypothesis (105). A common underlying mechanism represents the loss of heterozygosity which implies that a cell which is originally heterozygous at a specific gene locus loses its remaining functional allele for instance by deletion (106). In contrast to gain of function alterations, tumor suppressor gene mutations do not provide gene products that can be directly targeted. Hence, at present treatment options targeting genetic alterations in tumor suppressor genes such as the TP53 gene which is affected in approximately 50 % of all cancer patients are very limited (107). A novel therapeutic approach in this setting is based on the concept of synthetic lethality, which means that a defect in one gene has little or no effect on a cancer cell, however that a combined inactivation of two functionally related genes can result in cancer cell death (108). This concept of action holds great potential for expanding the universe of cancer targets, as recently demonstrated by the successful inhibition of the PARP inhibition in the setting of BRCA mutated carcinomas. Tumors harboring BRCA mutations are deficient of the homologous recombination repair process and thus rely on the activity of PARP 1 and 2 for DNA damage repair. A seminal study could show that BRCA mutated cancer cells are 1000 more sensitive to PARP inhibition than BRCA wildtype cells which prompted the initiation of several prospective trials evaluating the effect of PARP inhibitors in the clinical setting (109). Based on beneficial outcome findings three PARP inhibitors (olaparib, rucaparib and talazoparib) have been currently approved in different indications for the treatment of BRCA mutated ovarian, breast, pancreatic and prostate cancer (110).

Copy number alterations

Somatic copy number alterations are highly prevalent in many types of cancer and present another genomic target for cancer therapy. They are characterized by a somatic change of the chromosomal structure that result in a gain or loss of copies of focal DNA segments. A high-level gain in the copy number of a specific gene commonly referred to as gene amplification can result in an overexpression of the respective gene product. Copy number alterations can affect the activation of oncogenes and the inactivation of tumor suppressor genes. The exact mechanisms behind the origin and formation of copy number alterations are not entirely understood (111). Importantly, recurrent copy number alterations in specific cancer types could be identified for which targeted therapies were developed. A prime example therefore represents the successful treatment of ERBB2 amplified breast cancer (112). In addition, promising efficacy was recently shown for the selective MET receptor inhibitor capmatinib in patients with MET amplified NSCLC who had a gene copy number of 10 or higher (113). A rapidly emerging therapeutic field that aims to capitalize on the overexpression of amplified gene products focuses on the development of antibody drug conjugates. These consist of cytotoxic chemicals that are bound to a monoclonal antibody targeting specific proteins expressed at the cancer cell. Thereby a selective delivery of cytotoxic drugs inside the cancer cell can be attained (114). At present, four antibody drug conjugates directing against solid tumor have been approved by the FDA (trastuzumab emtansine and trastuzumab deruxtecan for metastatic HER2 positive breast cancer, enfortumab vedotin for advanced urothelial cancer and sacituzumab govitecan for metastatic triple negative breast cancer) and a plethora of new agents are currently tested within clinical trials (115).

Gene fusions and chromosomal rearrangements

Rapidly evolving technologies such as RNA sequencing and WGS have significantly improved our ability to detect and identify structural chromosomal rearrangements and gene fusions, which form by translocation, insertion, inversion or deletion of large DNA segments (116, 117). In case genetic information is lost by deletion, chromosomal rearrangements are classified as unbalanced. Chromosomal rearrangements can be simple, presenting with a single gene fusion, or complex in which at least three chromosomes are affected. Gene fusions that result in an activation of oncogenes or downregulation of tumor suppressor genes are important drivers of oncogenesis and drug resistance (118). To date, more than 10.000 distinct gene fusions have been detected in different cancer types of which the vast majority were recently identified by unbiased deep sequencing approaches. However, only a small subset are considered as pathogenic (119). These gene fusions which result in the overexpression of tumor promoting proteins or dysregulation of oncogenic enzymes such as tyrosine kinases embody attractive targets for cancer therapy. The tyrosine kinase inhibitor imatinib targeting the BCR-ABL fusion protein at the so-called Philadelphia chromosome was one of the first genome directed therapies for cancer. Its implementation led to a dramatic improvement of life expectancy in patients with chronic myeloid leukaemia, a disease which was previously accompanied by dismal prognosis (120). Another success story represents the development of tyrosine kinase inhibitors targeting ALK, MET and ROS1 kinases, which are frequently upregulated by chromosomal rearrangements in NSCLC (113, 121, 122). Based on impressive treatment response rates in basket trials two novel cancer drug agents targeting the NTRK gene fusion have been recently approved by the European Medicines Agency (EMA) and FDA for the treatment of adult and paediatric patients with NTRK fusion positive solid cancers, which represents the first tumor agnostic treatment approval by the EMA (123, 124).

Tumor mutational burden and microsatellite instability

Cancer is characterized by a high degree of genomic instability which is driven by various mechanisms including chromosomal rearrangements, loss of heterozygosity and mutations in DNA damage repair genes (125). One common form of genomic instability is microsatellite instability, which is characterized by a change in the number of repetitive oligonucleotides in microsatellite sequences. Microsatellite instability originates from loss of function mutations in genes coding for DNA mismatch repair proteins that are responsible for correcting errors in nucleotide matching. Microsatellite instability is commonly found in various types of cancer especially in colorectal adenocarcinoma, of which approximately 15% are microsatellite instable. DNA damage response deficiency leads to the accumulation of numerous mutations and is considered as a main driver of carcinogenesis (126, 127). Importantly, the number of coding mutations in the cancer genome summarized as the tumor mutational burden strongly correlates with the abundance of cancer specific neoantigens, which have been shown to induce T-cells mediated immune response (128). Based on a strong underlying mechanistical rationale, DNA mismatch repair deficiency and tumor mutational burden have been identified as predictive biomarkers for treatment response to immune checkpoint inhibitors regardless of the tumor origin or histology (127, 129). This has prompted the FDA to approve pembrolizumab, a programmed death receptor-1 (PD-1) blocking antibody for treatment of adult and pediatric patients with all types of advanced solid tumors, which either show DNA mismatch repair deficiency or a tumor mutational burden ≥10 mutations/megabase (130).

Integration of precision oncology in clinical care

The process of matching cancer drug therapy to molecular alterations detected by NGS is complex and requires multiple steps, each bearing potential pitfalls. In the following section main parts of this work-flow will be described, and potential challenges will be highlighted.

NGS

Given the decreasing costs and increasing availability of NGS there has been a recent trend towards comprehensive genomic profiling efforts including large gene panel analysis and WES/WGS. Although, these approaches supply extensive genomic data and information, its analysis and clinical interpretation becomes increasingly complex and requires bioinformatic support. Overall, DNA analysis by NGS can be broken down to three main components which are: sample preparation, DNA sequencing and data analysis (131). In line with the primary topic of this thesis I will mainly focus on NGS data analysis, whereas the process of NGS library preparation, amplification and sequencing has been discussed in more detail in the literature.

Primary data analysis

Genomic data obtained by NGS are primary summarized in FASTQ files, which contain genomic information in the form of short DNA sequences referred to as reads. In a first working step, defined as base calling each nucleotide present at a specific read position must be identified. This process is performed by software tools which additionally provide quality scores for each nucleotide, indicating the chance of a sequencing error. Next, each DNA sequence must be assigned to its genomic origin. This can be obtained by aligning each read with a reference genome (132). Various algorithms including software and hardware approaches have been developed for read alignment aiming to achieve maximal accuracy and faster execution times (133). To further improve the variant call accuracy and the downstream process, additional steps of post alignment processing are performed which include the filtering of alignment artifacts (134). The final part of primary NGS data analysis is called variant calling, which refers to the identification of somatic variants in the cancer genome and its distinction from benign germline. This can be either performed by aligning the mutational tumor profile with existing databases, or by using a non-cancerous sample from the same patient as a control (135).

Functional annotation

In a next working process, each detected somatic variant must be annotated regarding its functional and clinical significance. As pointed out previously, only a small subset of genomic alterations are considered as pathogenic, implying that they have a functional impact on a cancer cell and thus represent a potential target for cancer treatment. To facilitate the functional annotation of somatic variants, several genome databases such as My cancer genome (https://www.mycancergenome.org/), Catalog of Somatic Mutations in Cancer (COSMIC) (https://cancer.sanger.ac.uk/cosmic), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) or VarSome (https://varsome.com/) have been established, which provide compiled evidence on the frequency, function and role of genomic alterations previously detected in cancer. Despite their great value, the rapidly increasing number of curated genetic alterations provided in different genomic databases, also rises the complexity of variant annotation, which is the reason why Precision Oncology Decision Support (PODS) tools have recently gained attraction (136, 137). Another challenge of variant identification and interpretation is founded in the nomenclature of genetic alterations. Although, guidelines have recommended a consistent annotation of gene variants according to the Human Genome Organization Gene Nomenclature Committee, still various nomenclatures are used to describe the same gene variant (138). Importantly, the American Society of Clinical Oncology, and College of American Pathologists have provided a widely accepted framework to categorize gene variants into four broad categories which are: variants with strong clinical significance, variants with potential clinical significance, variants of unknown clinical significance and benign or likely benign variants (139). In general, only variants with strong or potential clinical significance are considered as potentially druggable.

Actionability assessment

After the functional annotation is completed, each detected genetic alterations must be reviewed regarding its potential therapeutic implications. Summarized as actionability assessment this process implies the overall evaluation whether a genetic alteration found in the cancer genome has an impact on treatment decision making. This can be justified by the presence of a specific genetic alteration that serves as a direct target for cancer therapy (e.g.: EML-ALK4 fusion in NSCLC) (121), by the presence of an established resistance mutation (KRAS mutation in colorectal cancer) (140), or the detection of a biomarker that predicts efficacy of a specific therapy (microsatellite instability or tumor mutational burden as tumor agnostic biomarkers for immune checkpoint inhibitor response) (127, 129). Driven by the ongoing expansion of antineoplastic drug agents and the identification of new genetic biomarkers, an increasing number of molecular aberrations have now been rendered as potentially actionable targets. These advancements have enabled the possibility of personalized cancer treatment, which sensu stricto implies the selection of cancer treatment based on the genomic tumor profile irrespective of the tumor origin and histology. Although this concept sounds appealing, the determination whether a cancer therapy can be matched to a detected genomic alteration is complex and not without ambiguity. A genetic alteration that serves as an established predictive biomarker for treatment efficacy in one cancer type, might have a different or no effect in another. Further, co-existing mutations might affect its functionality (141). Owing to the rapidly growing body of knowledge in the field of cancer genomics and the huge number of ongoing or completed clinical trials evaluating new cancer drug therapies, it is almost impossible for a single clinician to accurately assess the actionability of a genetic alteration. To facilitate this process several academic precision oncology knowledge bases have been formed (e.g. OncoKB (142)) and commercial decision support tools have been developed (e.g. NAVIFY Mutation Profiler (143)), which provide comprehensive information on the evidence of actionability of various genetic alterations found in an individual´s patient tumor. However, as different levels of evidence are considered sufficient for treatment, the actionability assessment does not follow uniform rules. To address this issue, the European Society for Medical Oncology (ESMO) Translational Research and Precision Medicine Working Group has recently provided a framework to rank genomic alterations as targets for personalized cancer treatment. The ESMO Scale for Clinical Actionability of molecular Targets (ESCAT) defines six levels of evidence for actionability of molecular targets: Tier I: established target for specific drugs as standard of care, tier II: targets that likely define a patient population that benefits from a targeted drug, tier III: target-drug matches for which a clinical benefit could be shown in other cancer types, tier IV: preclinical evidence of potential actionability, tier V: target-drug matches for which an increased treatment response rate could be shown, which however did not result in a survival benefit, Tier VI: target with no evidence of actionability (144). The postulation of this classification system has enabled a more standardized and reproducible reporting of actionability assessment. Importantly, as pointed out in more detail in the discussion section of this thesis, the ESCAT scale has been published after completion of this study and could thus not be incorporated in the treatment matching process.

Molecular tumor board

Although knowledge databases and decision support tools can aid in variant interpretation and actionability assessment, the final treatment decision in patient care must be made by clinicians. To cope with the increasing complexity of genomic data interpretation and the expanding possibilities of genome guided therapy, many cancer centers have lately established molecular tumor boards, which serve as an interdisciplinary venue to ensure optimal patient management in the era of genome guided cancer care. A molecular tumor board should be attended by experts from different specialities including the treating clinician (oncologist or clinician from other specialities involved in cancer care), an oncologist, a molecular pathologist, a human geneticist and ideally a clinical biologist and bioinformatician. Although not globally harmonized, the main tasks of molecular tumor boards are to evaluate whether a genomic tumor profiling is indicated, to interpret the genomic results and to make genome profiling-based treatment suggestions (145, 146). For accurate decision making concerning these issues, an in-depth evaluation of key information regarding the patient´s medical history and current health status, the tumors pathology and molecular characteristic, as well as a comprehensive review of genome databases, literature and other available resources is inevitable. Since the organization and implementation of a molecular tumor board is time consuming and requires a high level of expertise from different specialities, its widespread adoption especially in non-academic hospitals remains a key challenge. One potential way to overcome this hurdle might be the implementation of centrally managed virtual molecular tumor boards which provide a platform for information and knowledge exchange across multiple institutions (147, 148).

Efficacy of molecular profiling-based therapy in clinical trials

Driven by the rapidly increasing availability of NGS technologies helping to identify potential genomic treatment targets and the development of a great arsenal of targeted drug agents, multiple precision oncology trials have been conducted lately. In a pioneering study, van Hoff et al. evaluated the concept of molecular profiling-based therapy in a cohort of patients with different malignancies and advanced disease stage, who had exhausted all available treatment options. In total, 106 patients were included of which 84 showed at least one molecular alteration and finally 68 received a molecular profiling-based therapy. Twenty-seven % of the patients treated experienced a progression free survival (PFS) ratio greater than 1.3 compared to the PFS of the prior therapy line, which was prespecified as the primary endpoint of this trial (149). These promising findings have encouraged cancer centers and pharmaceutical companies worldwide to launch large-scale precision oncology research programs and further investigate the efficacy of molecular profiling-based therapy. However, clinical outcomes of precision oncology trials have so far not lived up the high expectations put into personalized cancer treatment, as recently pointed out by Tannock et al. In a meta-analysis of several prospective precision oncology trials, they could show that of 1000 patients with advanced cancer who undergo DNA sequencing for detecting potential genomic targets, only 400 have a targetable alteration, 120 receive a matched therapy and only 30 patients derive a clinical benefit from treatment. Thus, new concepts for precision oncology trials were claimed (150). One potential limitation of previous trials has been, that in most cases a single tissue biopsy was used for molecular profiling, which was often taken months or even years before study enrolment. As pointed out previously ctDNA has been shown to provide an accurate snapshot of a patient’s tumor, enabling the detection of tumor subclones from metastatic lesions. Although studies have begun to prospectively use ctDNA to funnel patients into phase I clinical trials, such trials have only recently been initiated and only preliminary outcomes have been reported yet. Ongoing precision oncology trials will be reviewed in more detail in the discussion section of this thesis.

Aims

The aim of this dissertation was to evaluate whether molecular profiling of ctDNA and/or tumor tissue could enable the matching of genetic alterations to molecular targeted therapies and improve outcomes in patients with advanced and refractory cancer. As an initial test of this concept, the Individualized Cancer Treatment (ICT) trial, a single-center prospective interventional study, was initiated to investigate the efficacy of molecular targeted cancer treatment based on the results of genomic profiling of plasma DNA and/or freshly obtained tumor tissue. To the best of our knowledge, this is the first interventional study reporting survival outcomes from ctDNA genotyping guided treatment allocation.

Methods

Preamble

Parts of the methods section were similarly published by Riedl et al (151).

Study design

The ICT trial was a prospective non-randomized open-label clinical phase II study with an optimized two stage study design according to Chen and NG (152). The study was performed at the Department of Oncology of the Medical University of Graz in cooperation with the Institute of Human Genetics, Institute of Pathology and Institute for Medical Informatics, Statistics and Documentation.

Ethic approval

This study was approved by the Ethics Committee of the Medical University of Graz (approval number 27-169 ex 14/15) and written informed consent was obtained from all patients. The study was performed in accordance with the Declaration of Helsinki.

Patient eligibility

Patients were eligible for inclusion in this trial if the following criteria were met: (1) Male/ female patients with locally advanced and/ or metastasized carcinoma for whom no further evidence-based drug therapy is established, (2) diagnosis of unresectable locally advanced and/or metastatic carcinoma with histologically confirmed disease, (3) confirmed progression after evidence-based drug therapy and no option for further evidence-based drug therapy in this indication (4) Age >18 years - ≤85, (5) Life expectancy of at least 12 weeks, (6) measurable disease according to the revised RECIST criteria version 1.1. , (7) ECOG score 0-2, (8) adequate bone marrow, liver and kidney function: laboratory cut-off levels according to the registration of registered drugs , (9) no anti-tumor therapy including radiotherapy in the past two weeks , (10) written informed consent. Patients with severe comorbidities, which were expected to significantly affect the patient`s prognosis or the ability to receive treatment were excluded from this study. The following comorbidities were defined as exclusion criteria: (1) history of significant cardiovascular disease unless the disease was well controlled. Significant cardiac disease included second/ third degree heart block, significant ischemic heart disease, poorly controlled hypertension, congestive heart failure of the New York Heart Association class II or worse (slight limitation of physical activity; comfortable at rest but ordinary activity results in fatigue, palpitation or dyspnoea), (2) history of arrhythmia that was symptomatic or required antiarrhythmic treatment, except of patients with atrial fibrillation or flutter controlled by medication, which were not excluded from participation in the trial, (3) active infection or any serious underlying medical condition that would have impaired the ability of the patient to receive protocol therapy, (4) a history of any psychiatric condition that might have impaired the patient's ability to understand or comply with the requirements of the study or to provide consent, (5) a history of hypersensitivity with anaphylactic reaction to humanized monoclonal antibodies or any of the applied drugs. Further excluded were pregnant or breast-feeding patients, as well as patients with untreated brain metastases or a history of previously diagnosed malignancies except for adequately treated non-melanoma skin cancer, in situ cancer, or other cancer from which the subject has been disease free for at least five years.

Endpoints

The primary endpoint of this trial was the PFS ratio defined as the PFS on the molecular profiling based therapy measured from date of enrolment into the trial to the date of documentation of disease progression (per revised Response Evaluation Criteria In Solid Tumors (RECIST) Criteria V1.1 or immune-related Response Criteria) or death or otherwise censored at the last radiomorphologic imaging assessment date on which the patient was reported alive without progression divided by the PFS on the last evidence based therapy in the same patient. The PFS on the last evidence-based therapy was retrospectively assessed at study inclusion by the treating oncologist from the Division of Clinical Oncology of the Medical University of Graz and was measured from the start of the last evidence-based therapy to the date of disease progression according to RECIST Criteria V1.1 or immune-related Response Criteria. The success of the molecular profiling-based therapy was defined as a PFS ratio ≥ 1.2

Secondary outcome measures were the number of patients for whom an anti-tumor drug therapy could be defined based on a molecular biologic tumor profile, the overall survival (OS) measured from the date of enrolment into the trial to the date of death or censored at the date of last follow-up, the overall radiographic treatment response rate (ORR) based on RECIST criteria 1.1 and the toxicity profile of the molecular profiling based therapy including information on frequencies and severity of adverse events according to the Common Terminology Criteria for Adverse events (CTCAE) version 4.0.

Predefined criteria for patient’s removal from trial

The following criteria for patient’s removal from the trial were defined prior start of recruitment:

1. Patient request – The patient may withdraw from the study at any time.
2. Lack of compliance – The sponsor or investigator may remove a patient from the study for lack of compliance to the study protocol.
3. Intolerable toxicity - Patients may be removed from study treatment for intolerable adverse events attributable to the therapy. Any serious adverse event deemed life threatening by the treating physician that is definitely related to the study device cause immediate cessation of treatment.
4. Radio morphologic progressive disease on molecular profiling-based therapy according to RECIST 1.1
5. Occurrence of a criterion which is clinically relevant or for other reasons that are considered necessary by the investigator.

Predefined criteria for premature termination of the trial

The following criteria for premature termination of the trail were defined prior start of recruitment:

Premature termination of the clinical trial will be considered when the risk-benefit ratio changes markedly for the patient, the use of the study medication is no longer justifiable, the sponsor believes it is necessary to terminate the clinical trial for safety reasons, when early evidence of the superiority or inferiority of a treatment group is obtained by an interim analysis or by other research results, or when the clinical trial proves to be impracticable. For the benefit of, and in the interest of the patients, the principal investigator may terminate the trial at any time when serious adverse effects or other unforeseen circumstances occur.

Study visits

Patients included in this trial had prespecified study visits. At study visit 1, patients were screened for study inclusion. First, the in- and exclusion criteria were assessed. If the patient was deemed eligible for study inclusion, complete information about the study concept and procedures were provided orally and in written form. Next a written informed consent was obtained from each patient and baseline information including patients age and sex were obtained and documented. Further a blood draw was performed for laboratory analysis including a complete blood count with a differential blood count, a serum chemistry panel and coagulation tests. In female patients with childbearing potential a serum or urine pregnancy test was performed to exclude pregnancy. To assess the current extent of disease a radio morphologic staging was organized. Radio morphologic imaging generally included a computer tomography (CT) scan of the thorax, abdomen, and pelvis, with the option to perform a magnet resonance imaging (MRI) of the abdomen instead of an abdomen CT scan and the option to perform an additional bone scan in case bone metastases were suspected. In case imaging was performed in routine clinical practice shortly before study inclusion these imaging data could be used for initial staging if a timeframe from 28 days to the start of molecular profiling-based therapy was not exceeded. Finally, a mandatory buccal swab for the detection of germline DNA variants and a blood draw for ctDNA analysis was performed. An additional tissue biopsy or resection of a metastasis for molecular tumor profiling was optional, depending on the patient's consent. If a tissue biopsy had been performed in routine clinical practice within three months prior to study entry, this tissue was considered suitable for molecular profiling. According to the study protocol the first study visit had to be within 28 days before start of molecular profiling-based therapy.

At study visit 2, a detailed medical history including assessment of concomitant medication was obtained from each patient and the PFS of the last evidence-based therapy was assessed by an Oncologist according to RECIST Criteria V1.1. Further a detailed physical examination, assessment of the Eastern Cooperative Oncology Group (ECOG) performance status, as well as an electrocardiogram was performed, to evaluate whether the patients were feasible for further treatment.

At study visit 3, the molecular profiling-based therapy was started in those patients for whom a targeted therapy could be matched according to the molecular profile. Before start of treatment study in and exclusion were again checked and a comprehensive laboratory panel was performed, in order to ensure that the patient was still feasible for therapy.

In patients who received a molecular profiling-based therapy further treatment visits were performed at least every two weeks until disease progression or death. At each treatment visit a physical examination, including assessment of vital signs and ECOG performance status as well as basic laboratory panel including a complete blood count was performed. Further adverse events associated with the study anti-cancer drug therapy were assessed and classified according to CTCAE 4.0. Every eight weeks after start of molecular profiling-based therapy a radio morphologic imaging including CT scans of the chest, abdomen, and pelvis, as well as an optional MRI scan of the abdomen and bone scans were performed and evaluated according to revised RECIST criteria version 1.1. In all patients included in the trial the survival status was checked bi-monthly via telephone calls or as part of clinical visits.

Clinical data acquisition of prior medical treatment

Information on prior medical therapy including treatment response rates and progression free survival rates of previous palliative therapy lines were retrospectively assessed from the in-house electronic healthcare database “Medocs”, the in-house chemotherapy prescription program “Oracle” as well was from handwritten progress notes from the oncological outpatient clinic at the date of study enrolment.

Molecular profiling of plasma DNA and tumor tissue

At the first study visit a mandatory blood draw for ctDNA analysis and a buccal swab for the detection of germline DNA variants were obtained in all patients enrolled in this study. An additional tissue biopsy or resection of a metastasis for molecular profiling of tumor tissue was optional, depending on the patient's consent. If a tissue biopsy had been performed in routine clinical practice within three months prior to study entry, this tissue was considered suitable for molecular profiling. In case the patient did not consent to an additional biopsy and no tissue was available, only ctDNA was used for molecular profiling.

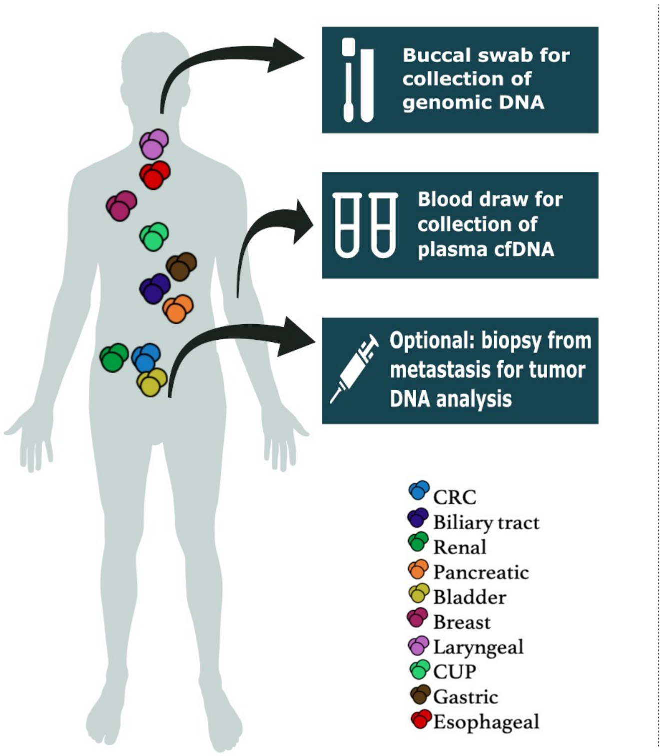


Figure 1. Sample collection and tumor DNA extraction (as published in (151)). Abbreviations: CRC – Colorectal cancer, CUP – Cancer of unknown primary

DNA isolated from tissue biopsies of metastatic lesions was subjected to library preparation using the Ion AmpliSeq Cancer Hotspot Panel v2 (Thermo Fisher) covering mutations from 50 oncogenes and tumor suppressor genes. All libraries were sequenced on the Ion Torrent platform. Plasma DNA was isolated using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) and shallow whole-genome sequencing (sWGS) libraries were prepared and sequenced using the plasma-Seq method (153).

Next, an analysis of somatic copy number alteration (SCNA) and identification of significant tumor-specific focal events was performed. Plasma-Seq requires a minimum of 5% tumor-derived DNA fragments to achieve informative copy number results. Estimation of tumor fraction from sWGS data was performed using the ichorCNA algorithm, which is a probabilistic HMM model for the estimation of tumor fraction, similar to analysis of tumor purity from bulk tumor analyses. For plasma DNA mutation analysis, library preparation was performed with plasma DNA in duplicates using either the TruSeq Cancer Amplicon Panel (Illumina) covering 48 genes which are targeted with 212 amplicons or the NEBnext Direct Cancer Hotspot Panel (New England Biolabs) covering 50 genes. To avoid false positive results from cancer hotspot panels very stringent filter criteria for the detection of ctDNA with a detection limit of ≥5% were applied. In addition, germline DNA isolated from a buccal swab was analysed to differentiate somatic mutations from germline DNA variants. Bioinformatics analyses were performed using open-source software. Briefly, FASTQ files were pre-processed to remove adapters and filtered for quality and were aligned with BWA. UMI sequences were added to the resulting BAM files with the AnnotateBamWithUmis function from the fgbio package (https://fulcrumgenomics.github.io/fgbio/). Duplicate reads were marked with the MarkDuplicates function from Picard and variant calling was performed using the MuTect2 program in tumor-only mode and VCF files were annotated with ANNOVAR. Resulting VCFs were filtered to only keep variants present in both duplicates but absent in genomic DNA with variant allele frequencies (VAF) ≥ 5%.

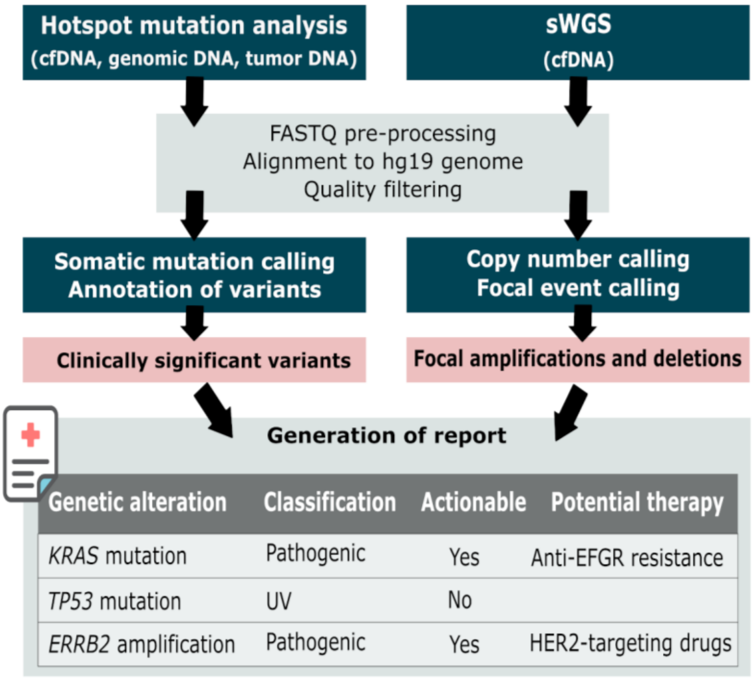


Figure 2. Data analysis and generation of patient reports (as published in (151)).

Abbreviations: cfDNA – cell free DNA, sWGS – shallow whole genome sequencing

Preparation of molecular report and treatment decision making

Molecular profiling results of each patient were systematically analysed and reviewed by a human geneticist and/or a molecular pathologist. Subsequently, an individual molecular report summarizing all relevant somatic molecular alterations found in the cancer genome was prepared for each patient and shared with the clinical oncologist. Finally, publicly available databases such as My Cancer Genome, DGidb, Mining the Druggable Genome and ClViC were thoroughly searched, and a comprehensive literature research was performed in order to elucidate which genomic alterations might be matched to an available targeted treatment. All relevant information were then presented and discussed at a molecular tumor board, which was attended by a medical oncologist, a pathologist and a clinical geneticist and took place as soon as the molecular report preparation and literature review was completed. The treatment decision making was based on several key aspects, including the actionability of molecular alterations found in the cancer genome of plasma DNA, and, if available, of tumor tissue, as well as the presence of other prognostic and predictive biomarkers. These additional biomarkers consisted of established parameters already obtained in routine clinical practice, such as hormone receptor status, HER-2/neu-status, RAS mutation status, EGFR mutation status, ALK rearrangement and PD-1 expression. Further, information on previously performed antineoplastic treatment including data on treatment response rates and persisting toxicity, the comorbidity profile and latest ECOG performance status of the patient as well as the availability and toxicity profile of suitable targeted drugs were taken into consideration. All potentially applicable tumor-specific drugs were pre-specified and are listed in **Table 1**. If a molecular alteration was deemed actionable by a targeted therapy, the drug with the strongest evidence for a gene target-indication was selected. If two or more targetable molecular alterations were identified which allowed for a promising combination treatment, also a combination therapy was considered. If no consent could be achieved within the molecular tumor board, the medical oncologist made the final treatment decision. In case a molecular targeted therapy could be matched the treatment start had to be scheduled within 28 days after the first study visit. If a treatment was started after this 28-day time frame, a note to file was generated. All patients who received treatment with a matched molecular targeted agent had biweekly treatment visits according to a pre-specified follow-up plan. In case patients were still considered suitable for antineoplastic treatment after progression on the molecular profiling-based therapy a salvage line of palliative antineoplastic treatment was offered according to the treating physician's choice.

Table 1. Possible tumor-specific drugs

Drug name		
Abiraterone Acetate	Epirubicin Hydrochloride	Panitumumab
Ado-Trastuzumab Emtansine	Enzalutamide	Pazopanib Hydrochloride
Afatinib Dimaleate	Epirubicin Hydrochloride	Pegaspargase
Anastrozole	Eribulin Mesylate	Peginterferon Alfa-2b
Arsenic Trioxide	Erlotinib Hydrochloride	Pemetrexed Disodium
Asparaginase Erwinia chrysanthemi	Etoposide	Pomalidomide
Axitinib	Everolimus	Ponatinib Hydrochloride
Azacitidine	Exemestane	Prednisone
Bendamustine Hydrochloride	Fulvestrant	Regorafenib
Bevacizumab	Fludarabine Phosphate	Rituximab
Bleomycin	Fluorouracil	Ruxolitinib Phosphate
Bortezomib	Fulvestrant	Sorafenib Tosylate
Bosutinib	Gefitinib	Sunitinib Malate

Brentuximab Vedotin	Gemcitabine Hydrochloride	Tamoxifen Citrate
Cabazitaxel	Goserelin Acetate	Temozolomide
Capecitabine	Ibritumomab Tiuxetan	Temsirolimus
Carboplatin	Ibrutinib	Thalidomide
Cetuximab	Ifosfamide	Topotecan Hydrochloride
Chlorambucil	Imatinib Mesylate	Toremifene
Cisplatin	Interferon Alfa-2b recominant	Trametinib
Clofarabine	Ipilimumab	Trastuzumab
Crizotinib	Irinotecan Hydrochloride	Vemurafenib
Cyclophosphamide	Lapatinib Ditosylate	Vinblastine Sulfate
Cytarabine	Lenalidomide	Vincristine Sulfate
Cytarabine, Liposomal	Letrozole	Vinorelbine Tartrate
Cyclophosphamide	Leucovorin Calcium	Vismodegib
Dabrafenib	Leuprolide Acetate	Ziv-Aflibercept
Dacarbazine	Mercaptopurine	
Dactinomycin	Methotrexate	
Dasatinib	Mitomycin C	
Daunorubicin Hydrochloride	Nilotinib	
Decitabine	Obinutuzumab	
Degarelix	Ofatumumab	
Docetaxel	Oxaliplatin	

Non study treatment administration

If no molecular targeted therapy could be matched to a patient or the patient was not eligible for the planned treatment, this patient was excluded from the primary endpoint analysis. In this case further medical treatment was performed according to treating physician`s choice and in case antineoplastic therapy was considered treatment decisions were based on outcome reports of phase II trials, retrospective cohort studies or case reports. All patients enrolled in the ICT trial were evaluated for the secondary endpoint analysis i.e. the number of patients for whom a molecular profiling-based therapy could be defined and the overall survival measured from date of study enrolment until death or censored alive at the last day of follow up.

Samples size calculation and statistical analyses

All statistical analyses, including the definition of primary and secondary endpoints as well as sample size calculation and outcome analysis were performed in close cooperation with the Institute for Medical Informatics, Statistics and Documentation, Medial University of Graz and were partly published in Riedl et al (151).

A success for the molecular profiling-based therapy was defined as a PFS ratio greater than 1.2. For the determination of the sample size the optimal two-stage design of Chen and Ng was used. Based on the pilot study from Von Hoff et al. (154) a success rate of 25% was assumed for the molecular profiling-based therapy. Considering a success rate of $p1=0.25$ and $p0=0.1$ at a significance level of $\alpha=5\%$ and a power of $1-\beta=90\%$, it was planned to include 24 (21-28) in the first stage of the trial. If the number of successes would have been >2 with 21-24 or >3 with 25-28 patients, the second stage would have been opened and a total of 57-64 patients would have been included in the study. The plan was to reject the null hypothesis (≤ 10% of this patient population would have a PFS ratio of ≥ 1.2) if the number of successes would have been >9 with 57-61 patients or >10 with 62-64 patients. Data were descriptively summarized using mean and standard deviation, median and interquartile range (1st and 3rd quartile, IQR) or range (minimum and maximum), or absolute and relative frequencies. Survival outcomes were presented with the Kaplan-Meier curve. R version 3.6.1. was used for all analyses.

Results

Parts of this section were similarly published in Riedl et al. (151).

Cohort description

Between July 2015 and March 2018, a total number of 24 patients were enrolled in this study. Due to slow patient accrual and lack of treatment response in patients undergoing molecular-profiling-based therapy, enrolment was then stopped by the principal investigator of the study and a premature outcome analysis was performed. At study enrolment, median age of the study population was 59.5 years (IQR 53.8-67.0) and 11 patients were female (46%). All patients had an ECOG performance status of 0 or 1 and were heavily pre-treated with a median number of 2.0 (IQR 2.0-3.2) previous palliative treatment lines. The most common cancer types were gastrointestinal carcinomas with five colorectal cancers, two gastric cancers, two pancreatic cancers and one biliary tract and oesophageal cancer, respectively. Further baseline characteristics of the screening and treatment cohort are summarized in **Table 2**. As outlined in the following, the study was terminated after the enrolment of 24 patients and no further patients were recruited. Thus, this thesis comprises the final outcome analysis of the ICT trial.

Table 2. Baseline characteristics of the study cohort (as published in (151)).

Data represent medians [1st-3rd quartile] for continuous data and absolute frequencies (%) for categorical data. *One patient was incorrectly treated with a molecular targeted agent, as a tissue biopsy prior to three months of the treatment start was used for molecular profiling. This patient was consequently excluded from the treatment cohort. Abbreviations: ECOG – Eastern Cooperation Oncology Group performance status, CUP – Cancer of unknown primary

Variables	**Overall cohort (n=24)**	**Treatment cohort (n=8)***
Female gender	11 (46%)	5 (62.5%)
Age at baseline (years)	59.5 [53.8-67.0]	56.0 [51.5-63.5]
ECOG performance score at baseline		
0	11 (46%)	2 (25%)

Variables	**Overall cohort (n=24)**	**Treatment cohort (n=8)***
1	13 (54%)	6 (75%)
Tumor location	/	/
Colon cancer	5 (25%)	4 (50%)
Pancreatic cancer	4 (16.7%)	2 (25%)
Gastric cancer	3 (12.5%)	0 (0%)
Biliary tract cancer	3 (12.5%)	1 (12.5%)
Rectal cancer	2 (8.4%)	0 (0%)
CUP	2 (8.4%)	0 (0%)
Esophageal cancer	1 (4.2%)	0 (0%)
Breast cancer	1 (4.2%)	0 (0%)
Bladder cancer	1 (4.2%)	0 (0%)
Kidney cancer	1 (4.2%)	1 (12.5%)
Laryngeal cancer	1 (4.2%)	0 (0%)
Previous lines of palliative chemotherapy	2.0 [2.0-3.2]	2.5 [2.0-5.0]
Metastasis present	24 (100%)	8 (100%)
Number of metastatic organs		
1	5 (20.8%)	1 (12.5%)
2	11 (45.8%)	5 (62.5%)
3	8 (33.3%)	2 (25%)
Liver metastasis present	13 (54.2%)	5 (62.5%)
Lung metastasis present	13 (54.2%)	4 (50%)
Time from date of tumor diagnosis to study enrollment (days)	674 [499, 1253]	715 [655, 1019]
Time from progression under last palliative chemotherapy until study enrollment (days)	10.0 [5.5, 19.5]	16.0 [7.8, 20.2)]

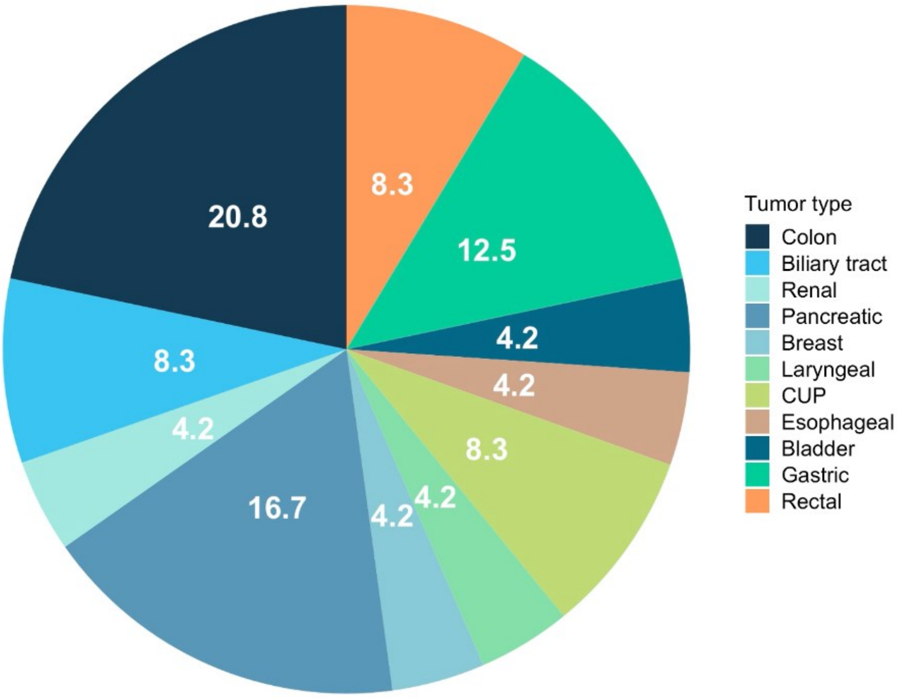


Figure 3. Cohort composition by primary tumor entity (n=24) (as published in (151)). Abbreviations: CUP - Cancer of unknown primary

Informative results from molecular profiling of ctDNA and tissue

As described in detail in the methods section a mandatory blood draw was obtained from all patients (n=24) for molecular profiling of plasma DNA. In 17 patients (71%) we were able to detect ctDNA from plasma and to obtain informative molecular profiling results therefrom. Each of these 17 cases showed at least one molecular alteration including a somatic mutation, a focal SCNA or both. The median tumor-specific fraction calculated from sWGS with ichorCNA in plasma was 22.7% (IQR 5.2-40.3) and was highest in patients with colorectal cancer. In 7 patients (29%) no ctDNA could be detected from plasma, which was likely attributed to the limit of detection of plasma-Seq, which requires a minimum of 5% tumor-derived DNA fragments to achieve informative copy number results. Furthermore, in order to avoid false positive findings from molecular profiling very stringent filter criteria for the detection of ctDNA were applied when using the cancer gene hotspot panels (limit of detection 5%).

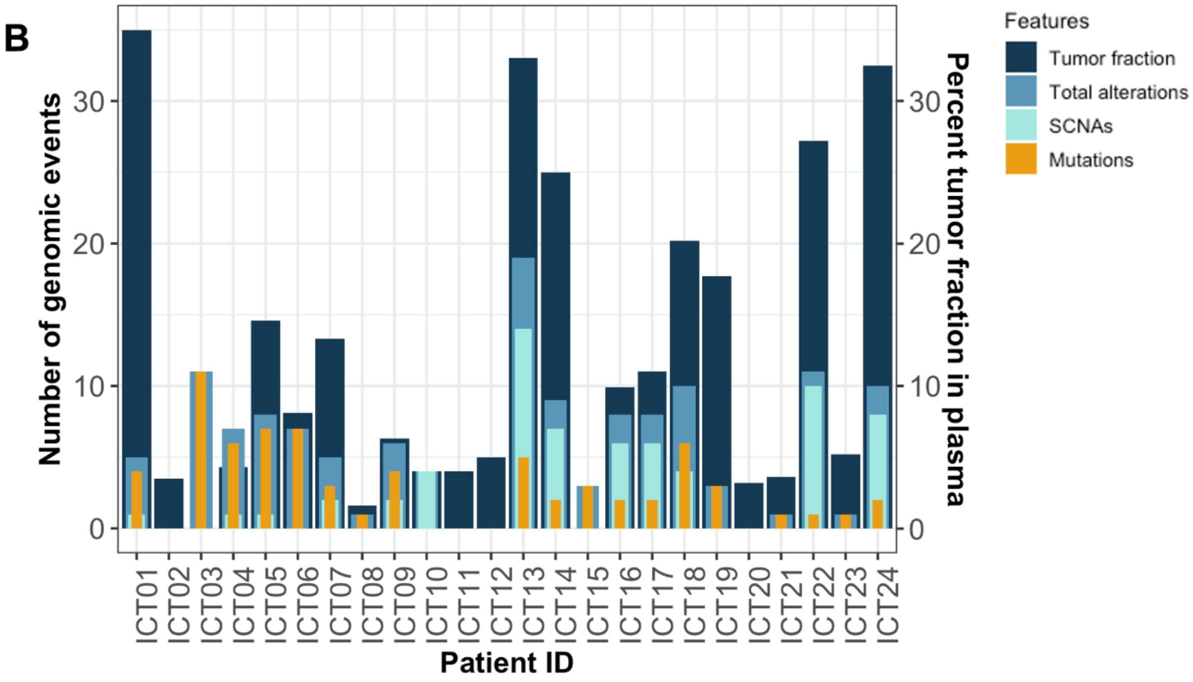


Figure 4. Molecular composition of the study cohort (as published in(151))

Bar plot representing total tumor fractions in plasma estimated from ichorCNA, number of alterations detected combined from plasma and tissue, SCNAs detected in plasma and mutations per patient detected combined from plasma and tissue results.

Eleven patients (46%) also consented to an additional tissue biopsy, of which 9 (38%) yielded informative molecular profiling results. In one case, no malignant tissue (ICT11) could be obtained and in another patient, no mutation could be identified (ICT20) by molecular profiling. In four patients (ICT2, ICT11, ICT12, ICT20) neither tissue biopsy nor ctDNA yielded informative molecular profiling results. Thus overall, a conclusive molecular profile either from plasma, tumor or both could be obtained from 20/24 patients (83%). Median time from blood collection for ctDNA analysis and tissue biopsy to finalization of the molecular report was 12 days (IQR 9-14) and 24 days (IQR 18.5 – 29), respectively.

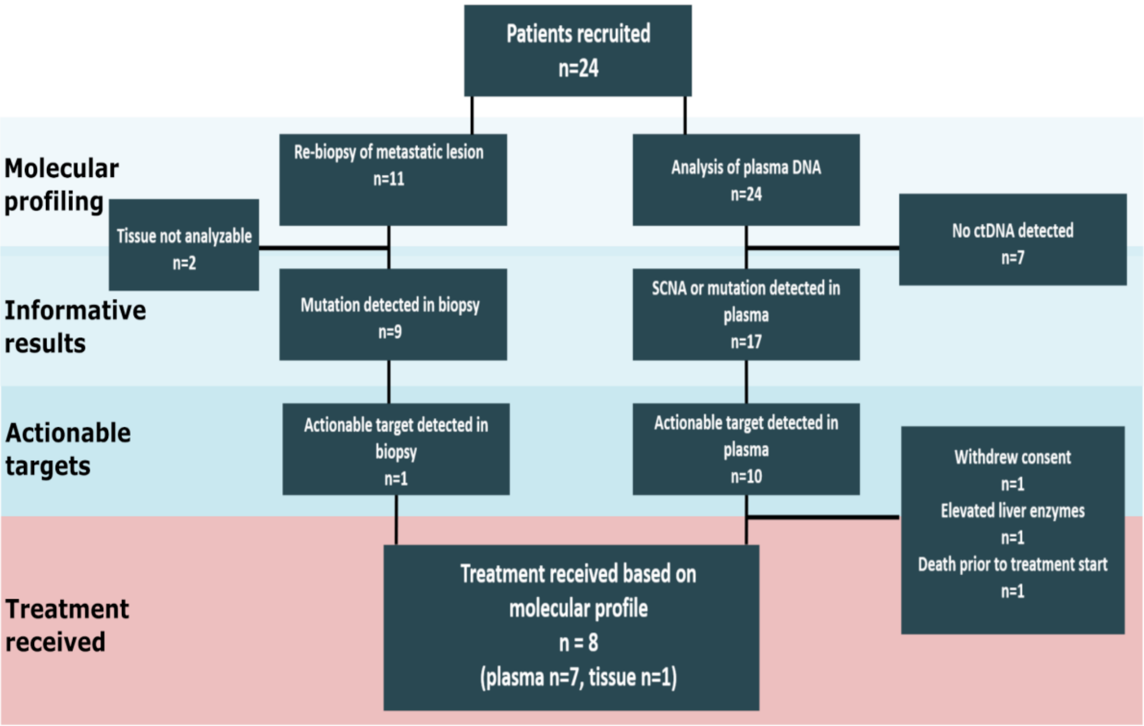


Figure 5. Flowchart of the srudy cohort (as published in (151))

Concordance of variants from tumor tissue and plasma

To evaluate the concordance of molecular profiling results from tumor tissue and plasma DNA, only mutations which were covered by both gene panels used for tissue and plasma analysis were considered. Somatic copy number alterations were not considered for concordance analysis as they were very only available from plasma DNA analysis. Of the nine patients with informative molecular profiles from the tumor tissue, ctDNA with tumor fraction above the detection limit of >5% could be detected in six (67%) of the respective plasma samples, whereas in the other three patients (ICT08, ICT13 and ICT15), no tumor-specific mutation could be identified in plasma due to a low tumor fraction. Overall, the concordance between mutations from tissue detected in plasma and from plasma detected in tissue was rather low at 77% and 53%, respectively. In two patients (ICT09 and ICT13), the same mutations detected in tissue were only observed in one duplicate of plasma DNA and therefore were not called by the algorithm. On the contrary, in one patient with a gastric adenocarcinoma (ICT09), an NRAS mutation was identified in plasma, which was not found in the corresponding tissue. Likewise, in patient ICT19 who had an adenocarcinoma of the colon, a CDKN2A mutation was identified in plasma in addition to a TP53 and a PIK3CA mutation known from the biopsy. In patients ICT18 and ICT16, the same TP53 and SMAD4 mutations were identified in plasma and metastatic tissue, respectively. However, in patient ICT18, plasma DNA analysis further revealed a KRAS mutation that was originally identified in the primary tumor but missed in the metastatic biopsy. Vice versa, in patient ICT05, three out of four mutations identified in the biopsy (APC, KRAS, and TP53) were also detected in plasma, but the SMAD4 mutation could not be detected. A particularly interesting case was ICT06, in which the TP53 mutation detected in the biopsy material could not be identified in plasma, but five completely different mutations were found (APC, KDR, PTEN, RET, SMO).

Table 3. Molecular profiling results from plasma and tissue (as published in(151))

Patient	**Tumor entity**	**Informative results from plasma**	**Molecular aberrations from plasma**	**Informative results from tissue**	**Molecular aberrations from tissue**	**Mutation panel**
ICT01	Colon	Yes	*Amplification:* FLT3 *Mutations:* KRAS c.147C>G, p.Gly12Cys, STK11, c.147C>G, p.Tyr49Ter, TP53 c.892G>T, p.Glu298Ter	NA	NA	TruSeq Cancer Amplicon Panel
ICT02	Biliary tract	No	NA	NA	NA	NA
ICT03	Colon	NA	NA	Yes	*Mutations:* KRAS p.G12S, TP53 p.R273C, PBX1 p.R234Q, AFF3 p.V748L, APCc.2748_2749del, p.T916fs, RECQL4 p.A939V, TCL1A p.G8R, PALB2 p.R566H, CIC	AmpliSeq Cancer Hotspot Panel v2

					p.A1259T, MYH9 p.LEEEQ1738Q, USP9X p.R2304H	
ICT04	Renal	Yes	*Amplifications:* CDK13 *Mutations:* PIK3CA c.265C>A, p.Leu89Ile, FGFR3 c.2174+30T>A, KDR c.3613G>A p.Glu1205Lys, MET c.3082+13T>C, PTEN c.777delC p.His259Glnfs*7, STK11 c.203A>T p.Asp68Val	NA	NA	TruSeq Cancer Amplicon Panel
ICT05	Colon	Yes	*Amplifications:* FLT3 *Mutations*: APC c.3917delA p.Ile1307*, KRAS c.436G>A p.Ala146Thr, TP53 c.817C>T p.Arg273Cys	Yes	*Mutations*: APC c.3917delA:p.E1306fs ,KRAS c.G436A, p.A146T,TP53 c.C817T, p.R273C,SMAD4 c.G344C, p.C115S	TruSeq Cancer Amplicon Panel (plasma); AmpliSeq Cancer Hotspot Panel v2 (tissue)
ICT06	Pancreatic	Yes	*Mutations*: KDR c.3991G>T, p.Glu1331Ter , APC c.3782C>G, p.Thr1261Ser , SMO c.1000G>T, p.Val334Phe	Yes	*Mutations*: IDH2 c.G516T, p.R172S , TP53 c.G524A, p.R175H	TruSeq Cancer Amplicon Panel (plasma); AmpliSeq Cancer Hotspot Panel v2 (tissue)

			, RET c.2385C>A, p.Ser795Arg , PTEN c.65A>G, p.Asp22Gly			
ICT07	Biliary tract	Yes	*Mutations*: PTEN, CDKN2A deletions; BRCA2 c.A144C: p.E48D, ERBB4 c.G404A: p.G135E, SMO c.G1598A: p.S533N	NA	NA	TruSeq Cancer Amplicon Panel
ICT08	Breast	No	NA	Yes	*Mutations*: TP53 c.868delC, p.R290fs	TruSeq Cancer Amplicon Panel (plasma); AmpliSeq Cancer Hotspot Panel v2 (tissue)
ICT09	Gastric	Yes	*Amplifications*: KRAS, MED29 *Mutations*: NRAS c.37G>T, p.Gly13Cys	Yes	*Mutations*: APC c.C3329G, p.S1110X , APC c.G3949C, p.E1317Q , MET c.A1124G, p.N375S	TruSeq Cancer Amplicon Panel (plasma); AmpliSeq Cancer Hotspot Panel v2 (tissue)
ICT10	Gastric	Yes	*Amplifications*: DOK5, ZNF217, TSHZ2, ERG	NA	NA	TruSeq Cancer Amplicon Panel
ICT11	Laryngeal	No	NA	NA	NA	TruSeq Cancer Amplicon Panel
ICT12	CUP	No	NA	NA	NA	TruSeq Cancer Amplicon Panel

ICT13	Rectal	Yes	*Amplifications*: PTK7, GJB7, SMIM8, C6orf163, C6orf164, ANKRD46, YWHAZ, MLLT6, CASC3, CDC6, CWC25, ERBB2, FBXL20, SUPT4H1	Yes	*Mutations*: PIK3CA c.A3140G, p.H1047R, FGFR3 c.T1156C, p.F386L, APC c.3887A, p.A1296E, JAK3 c.G2164A, p.V722I, TP53 c.C528A, p.C176X	TruSeq Cancer Amplicon Panel (plasma); AmpliSeq Cancer Hotspot Panel v2 (tissue)
ICT14	Colon	Yes	*Amplifications*: ERBB2, GRB7, STARD3, CDC6, PDXK, CSTB, RRP1, AGPAT *Mutations*: PIK3CA c.3140A>G, p.His1047Arg, TP53 c.700T>A, p.Tyr234Asn	NA	NA	TruSeq Cancer Amplicon Panel
ICT15	Pancreatic	No	NA	Yes	*Mutations*: CDKN2A c.C247T, pH83Y, KRAS c.G35T, p.G12V, TP53 c.C832A,p.P278T	TruSeq Cancer Amplicon Panel (plasma); AmpliSeq Cancer Hotspot Panel v2 (tissue)
ICT16	Gastric	Yes	*Amplifications*: ARPC1A, MYC, TSSK6, NDUFA13, ATP13A1, ZNF101 *Mutations*: TP53 c.514G>T, p.Val172Phe	Yes	*Mutations*: TP53 c.G514T, p.V172F	TruSeq Cancer Amplicon Panel (plasma); AmpliSeq Cancer Hotspot Panel v2 (tissue)

ICT17	Pancreatic	Yes	*Amplifications*: EPHA3(partial), TSSK1B,YTHDC2,CDO1,PGGT1B,MYC,YES1 *Mutations*: CDKN2A deletion; KRAS c.35G>A, p.G12D, TP53 c.559+2delT	NA	NA	NEBnext Direct Cancer Hotspot Panel
ICT18	Rectal	Yes	*Amplifications*: RIPK1, TNK1 *Mutations*: TP53, MAP2K4 deletions; KRAS c.35G>C, p.G12A, SMAD4 c.1572G>C, p.W524C, APC c.3805_3806del, p.I1269fs, APC c.3921_3925del, p. I307fs	Yes	*Mutations*: KRAS c.35G>C, p.G12A, SMAD4 c.G1572C, p.W524C	NEBnext Direct Cancer Hotspot Panel (plasma); AmpliSeq Cancer Hotspot Panel v2 (tissue)
ICT19	Colon	Yes	*Mutations*: PIK3CA c.T1258C, p.C420R, CDKN2A, c.C238A, p.P80T, TP53 c.C637T, p.R213X	Yes	*Mutations*: PIK3CA c.T1258C, p.C420R , TP53 c.C637T, p.R213X	NEBnext Direct Cancer Hotspot Panel (plasma); AmpliSeq Cancer Hotspot Panel v2 (tissue)
ICT20	CUP	No	NA	No	None detected	NEBnext Direct Cancer Hotspot Panel (plasma); AmpliSeq Cancer Hotspot Panel v2 (tissue)

ICT21	Biliary tract	Yes	*Mutations*: PIK3CA c.G2064T, p.L688F	NA	NA	NEBnext Direct Cancer Hotspot Panel
ICT22	Esophageal	Yes	*Amplifications*: FGFR4, CDK6, ARPC1A, LMTK2, SMURF1, MTDH, YWHAZ, TRIB1, MYC, CCNE1 *Mutations*: TMPRSS2-ERG_small,TMPRSS2 deletions; TP53 c.G438A, p.Trp146*	NA	NA	NEBnext Direct Cancer Hotspot Panel
ICT23	Bladder	Yes	*Mutations*: TP53 c.C637T, p.R213*	NA	NA	NEBnext Direct Cancer Hotspot Panel
ICT24	Pancreatic	Yes	*Amplifications:* PHA5,KDR,KIT,PDGFRA,BCR,YESP,EP300 *Mutations:* CDKN2A deletion; KRAS c.G34C, p.G12R, TP53 c.C637T, p.R213*	NA	NA	NEBnext Direct Cancer Hotspot Panel

Molecular alterations in ctDNA and tumor tissue

Overall, most somatic mutations detected from both plasma and tissue analyses were missense mutations. In selected cases also frameshift and nonsense mutations, as well as deletions were observed. The most affected genes in both plasma and tumor tissue were TP53, KRAS, PIK3CA and APC (**Figure 6 and 7**). In detail, TP53 was affected in 62% and 80%, KRAS in 31% and 40%, PIK3CA in 31% and 20% and APC in 19% and 40% in plasma and tissue DNA, respectively. Further mutations were detected in the genes KDR, PTEN, STK11, BRCA2, CDKN2A, NRAS, RET, SMAD4 and SMO in plasma (**Figure 6**) and in SMAD4, AFF3, CDKN2A, CIC, FGFR3, IDH2, JAK3, MET, MYH9, PALB2, PBX1, RECQL4, TCL1 and USP9X in tissue (**Figure 7**).

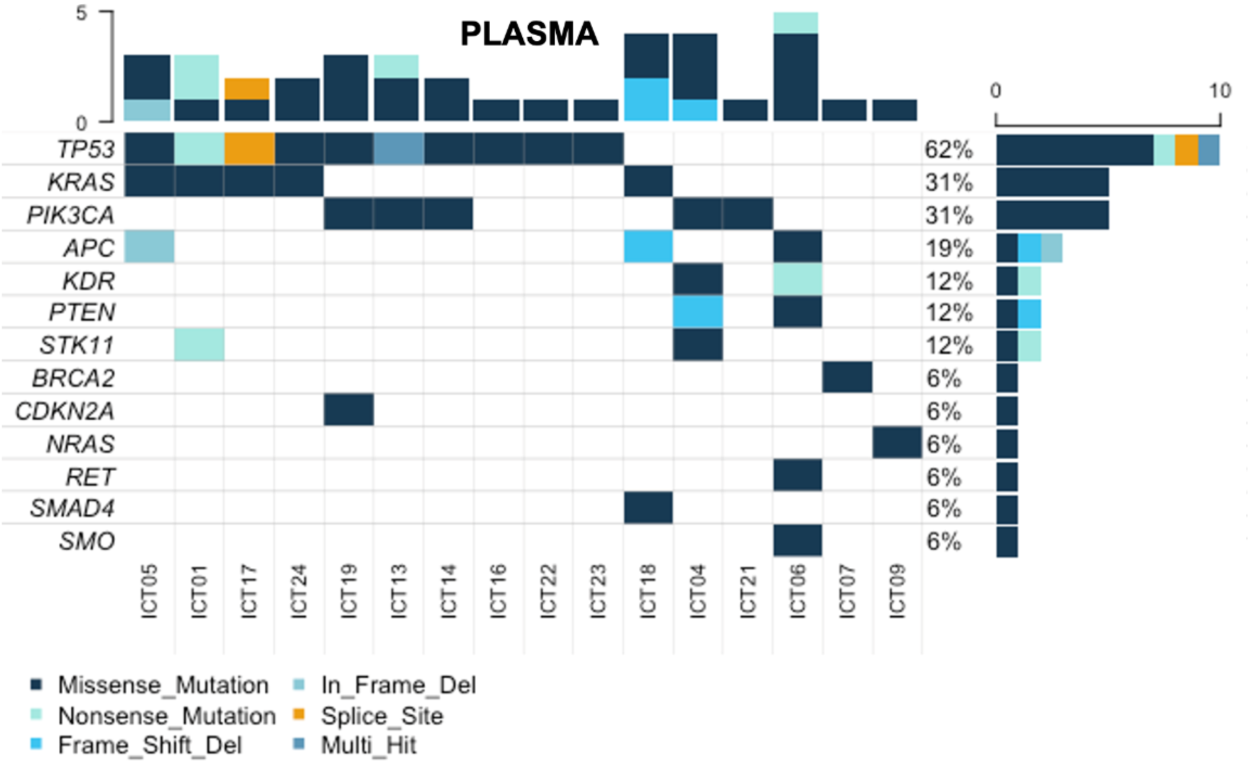


Figure 6. Oncoplot of most frequently detected mutations from plasma DNA (as published in (151))

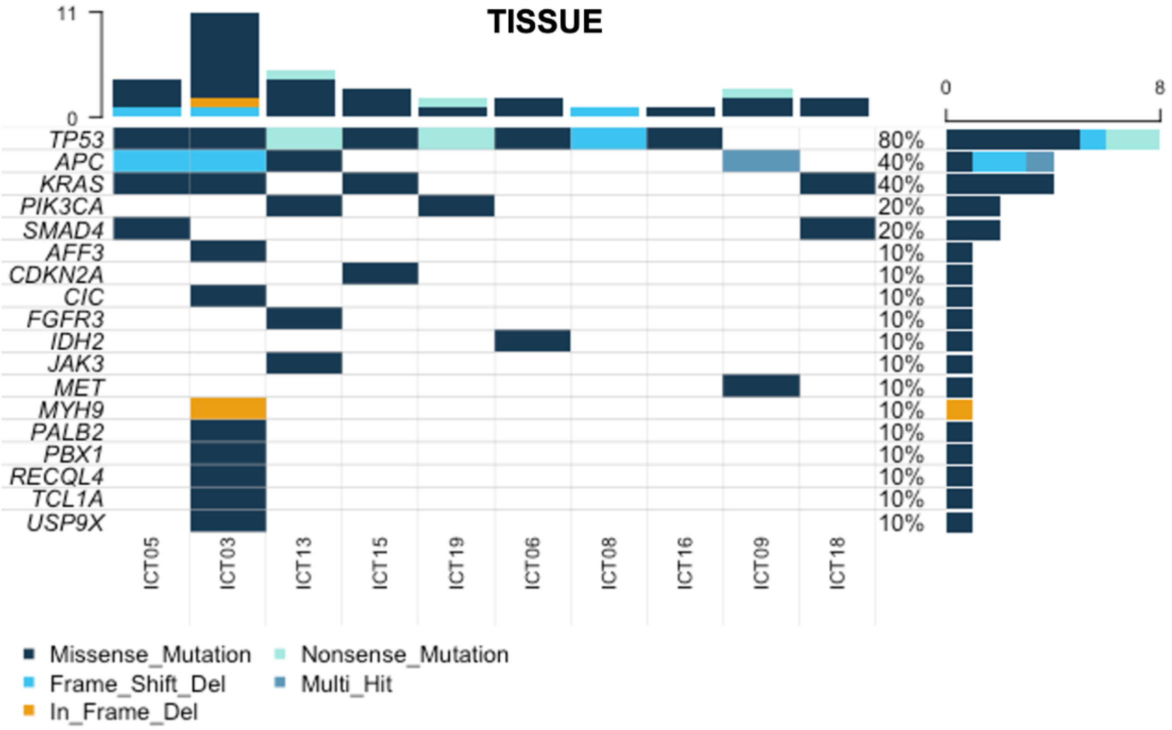


Figure 7. Oncoplot of most frequently detected mutations from tissue DNA (as published in (151)

In plasma, higher average variant allele frequencies generally correlated with the presence of higher overall tumor fractions. Still, there were several outliers, such as patient ICT01, who had a very high tumor fraction in plasma, but low average variant allele frequencies. To determine the relationship between variant allele frequencies derived from somatic copy number alteration or from somatic mutations a Lin's concordance correlation coefficient was calculated (**Figure 8**). The correlation index of 0.6 indicates a potentially relevant difference in mutation-derived vs. SCNA-derived variant allele frequencies.

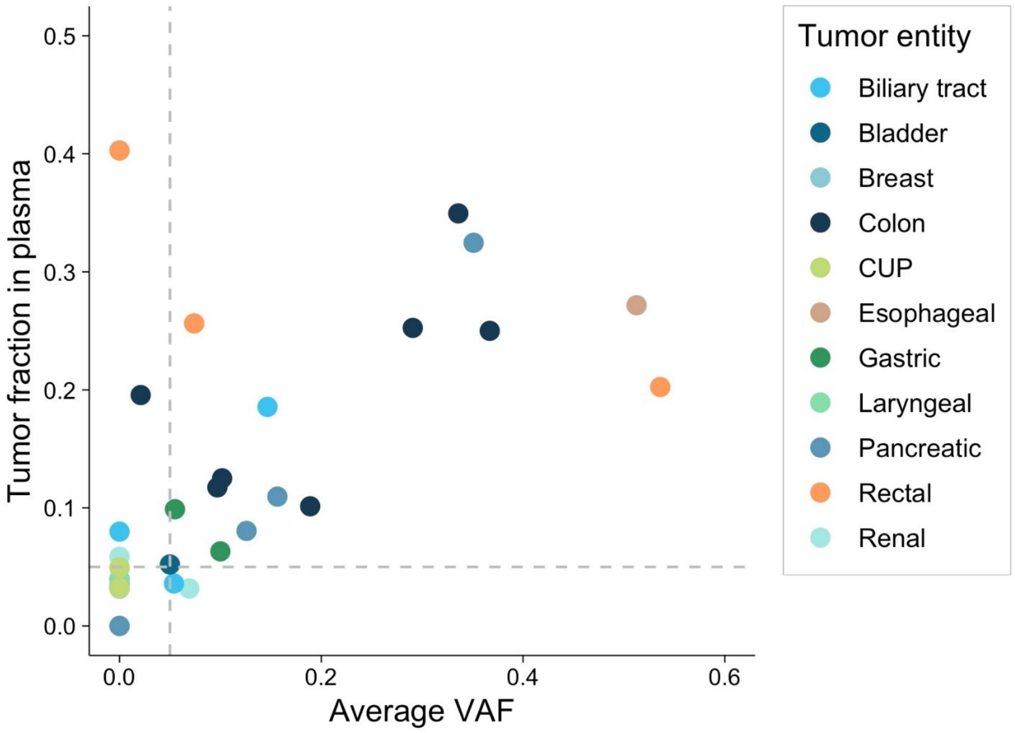


Figure 8. Correlation of tumor fraction in plasma DNA across tumor entities (as published in (151))

Tumor fractions were estimated from plasma DNA using the ichorCNA algorithm. Variant allele frequencies (VAF) from all detected mutations in plasma were averaged across each patient. The dashed line represents the algorithm's detection limit of 3%. Each patient is plotted according to tumor entity.

When looking at the molecular profiling results from plasma DNA, a strong variation in the overall number of molecular alterations as well as in respect to the relative number of somatic mutations or somatic copy number alterations was found. Interestingly, the relative proportion of molecular alterations was rarely balanced between mutations and SCNAs, but were mostly comprised of either SCNAs or mutations, varying from patient to patient (**Figure 4**). For example, in 10 patients, mutations comprised ≥80% of the alterations detected whereas in 7 cases, SCNAs were more frequently detected than mutations (**Table 4**). Importantly, each molecular profile of the 20 patients with available molecular profiles from tissue and/or plasma DNA was unique demonstrating the concept of "molecular snowflakes".

Table 4. Composition and actionability assessment of molecular profiling results from plasma and tissue DNA (as published in (151))

Abbreviations: SCNAs–somatic copy number alterations

Patient	Tumor type	Reported mutations	Reported SCNAs	Total reported alterations	Actionable mutations	Actionable SCNAs	Total actionable	Percent actionability
ICT01	Colon	4	1	5	0	1	1	20%
ICT03	Colon	11	0	11	0	0	0	0%
ICT04	Renal	6	1	7	2	1	3	43%
ICT05	Colon	7	1	8	0	1	1	12.5%
ICT06	Pancreatic	7	0	7	0	0	0	0%
ICT07	Biliary tract	3	2	5	0	0	0	0%
ICT08	Breast	1	0	1	0	0	0	0%
ICT09	Gastric	4	2	6	0	0	0	0%
ICT10	Gastric	0	4	4	0	0	0	0%
ICT13	Rectal	5	14	19	0	1	1	5.3%
ICT14	Colon	2	7	9	0	1	1	11.1%
ICT15	Pancreatic	3	0	3	1	0	1	33.3%
ICT16	Gastric	2	6	8	0	1	1	12.5%
ICT17	Pancreatic	2	6	8	0	5	5	62.5%
ICT18	Rectal	6	4	10	0	0	0	0%

ICT19	Colon	3	0	3	1	0	1	33.3%
ICT21	Biliary tract	1	0	1	0	0	0	0%
ICT22	Esophageal	1	10	11	0	2	2	18.2%
ICT23	Bladder	1	0	1	0	0	0	0%
ICT24	Pancreatic	2	8	10	0	3	3	30%
total		**71**	**66**	**137**	**4**	**16**	**20**	**14.6%**

Actionability of detected targets

Overall, informative molecular patient profiles could be achieved in 20 patients of which 10 were derived from plasma only, seven from plasma and tissue and three from tissue only. In eleven cases molecular alterations were deemed as druggable by the molecular tumorboard which accounted for 55% of the patients with informative results and 46% of the entire recruited cohort. The majority of druggable molecular targets were detected from plasma, as at least one actionable target could be identified in 10 patients (91%) according to analysis of plasma DNA and in only one patient according to tissue biopsy. In nine out of eleven cases the druggable alterations consisted of focal SCNAs, whereas in 2 cases mutations were considered an actionable target (**Table 5**). The overall percent actionability from the molecular profiling results calculated by dividing the number of actionable alterations by the total number of detected alterations was 14.6% across the entire cohort (**Figure 9**)

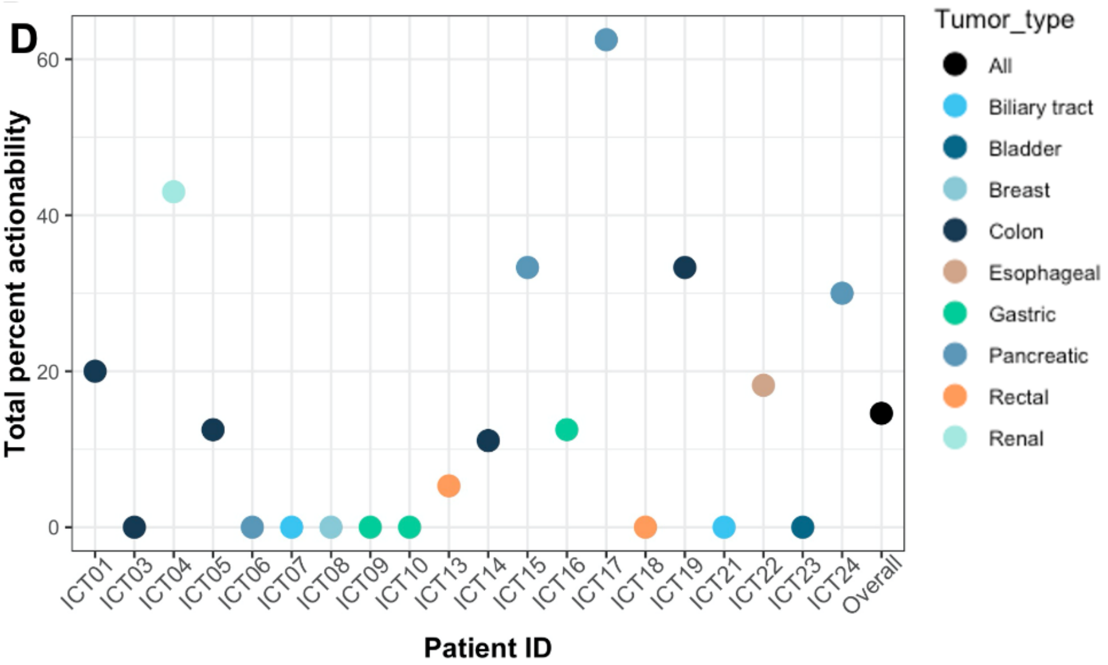


Figure 9. Overall percent actionability calculated for the 20 informative cases per profile combined from plasma and tissue results (as published in (151))

Treatment matching

The next step of the treatment matching process consisted of a mining of various cancer gene databases and an extensive literature research for potential actionability of molecular targets found in the 20 cases with informative molecular profiling results, which was then summarized in a molecular report. Each report consisting of technically and clinically annotated molecular profiling results from ctDNA and/or tumor tissue was finally discussed at a molecular tumorboard and a treatment recommendation was made based on the evidence level of target actionability.

Overall, a molecular profiling-based therapy was recommended in eleven out of 20 patients with informative molecular profiling results (55%) and in 46% of the overall study cohort. Three of these patients (27%) for which a matched therapy was recommended based on the molecular profiling results were subsequently not eligible for treatment, either due to death (ICT16), withdrawal of consent (ICT14) or inadequate liver function (ICT22). Consequently, a total number of eight patients (33% of total cohort) were treated with a matched drug in accordance with the molecular profiling results. In two patients with colorectal cancer (ICT01 and ICT05), a focal amplification of chromosome 13q12 harboring the FLT3 gene was detected and a targeted therapy with the multityrosine kinase inhibitor sorafenib was matched. In three patients (ICT04, ICT07, and ICT19) a molecular profiling-based therapy with a mechanistic target of Rapamycin (mTOR) inhibitor was matched based on alterations in the PTEN and PIK3CA genes, respectively. Two patients (ICT13, ICT14) showed amplifications in the ERBB2 gene which were targeted with the combination of trastuzumab and lapatinib and one patient (ICT24) received molecular profiling-based treatment with nintedanib for receptor tyrosine kinase (RTK) targets such as PDGFR and FGFR. (**Table 5**) The median time from study enrollment until treatment start was 34 days (IQR 28.5 – 37.3) and only one of the eight treated patients receive treatment within the prespecified timeframe of 28 days.

Table 5. Patients matched to molecular profiling-based treatment (as published in (151))

Abbreviations: MTB – molecular tumorboard, NA – not available, SD – stable disease, PD – progressive disease

Patient ID	**Druggable target(s) identified**	**Absolute copy number**	**Profiling source (i.e. plasma or tissue) used to make decision**	**Matched treatment**	**Best treatment response**
ICT01	FLT1, FLT3 amplification	FLT1: 17.25 FLT3: 17.25	Plasma	Sorafenib	PD
ICT04	PTEN mutation, PIK3CA mutation	NA	Plasma	Everolimus	SD
ICT05	FLT3 amplification	FLT3: 7.94	Plasma	Sorafenib	PD
ICT07	PTEN deletion	PTEN: -1.87	Plasma	Temsirolimus	PD
ICT13	ERBB2 amplification	ERBB2: 3.79	Plasma	Trastuzumab + Lapatinib	PD
ICT14	ERBB2 amplification	ERBB2: 4.41	Plasma	Trastuzumab + Lapatinib	Not given (withdrew consent)
ICT15	RET amplification	RET:	Tissue	Sunitinib	PD
ICT16	MYC amplification	MYC: 8.23	Plasma	Death prior to MTB	Not given (death)
ICT19	PIK3CA mutation	NA	Plasma	Everolimus	PD
ICT22	FGFR4 and CDK6 amplification	FGFR4: 7.89 CDK6: 5.85	Plasma	Nintedanib or Palbociclib	Not given (elevated liver transaminases)
ICT24	KDR, KIT, PDGFRA amplification	PDGFRA: 3.09	Plasma	Nintedanib (in combination with nab-paclitaxel)	PD

Outcome analysis

Due to slow patient enrolment and lack of clinical benefit observed at a first interim analysis, the study had to be stopped prematurely by the principal investigator. Although two patients (ICT07 and ICT15) experienced a numerically longer PFS with the molecular profiling-based treatment compared to the last evidence-based treatment, none of the eight treated patients reached the prespecified margin of a PFS ratio ≥ 1.2 for the MP-guided therapy compared to the last evidence-based treatment line (**Figure 10**).

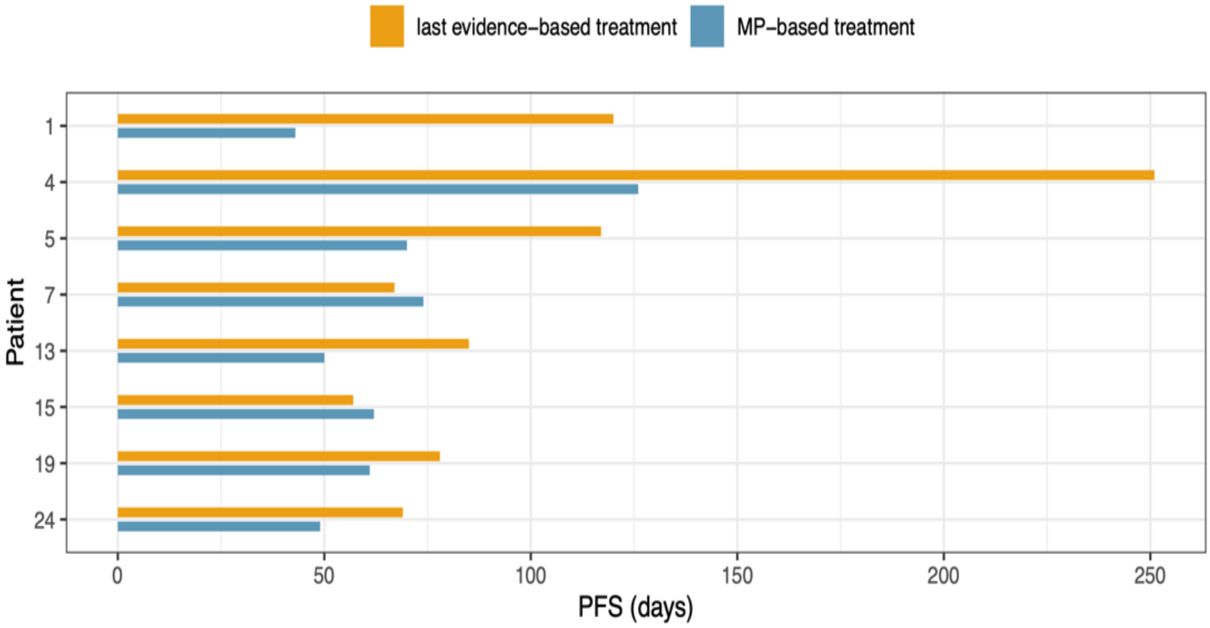


Figure 10. Swimmers plot presenting individual progression free survival data of MP-based treatment and the last evidence-based treatment line for each patient (as published in (151))

Abbreviations: MP – molecular profiling

The median PFS with the molecular profiling-based treatment was 61.5 days (IQR 49.8-71.0) compared to 81.5 days (IQR 68.5-117.8) with the last evidence-based treatment line, resulting in a median PFS ratio of 0.7 (IQR 0.6-0.9).

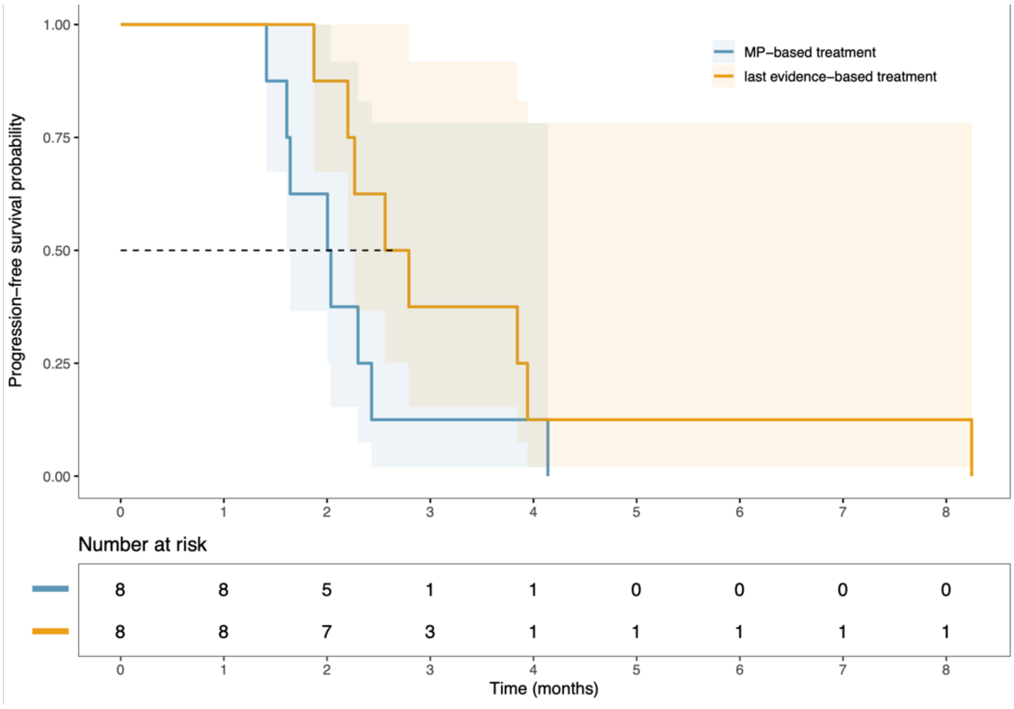


Figure 11. Kaplan-Meier curve comparing PFS from the MP-based treatment with the last evidence-based treatment line. (as published in (151))

Abbreviations: MP – molecular profiling

The objective response rate according to RECIST criteria defined as a composite of complete or partial tumor remission with the molecular profiling-based therapy was 0% with five patients experiencing primary disease progression and with one patient achieving stable disease as bes treatment response. In two patients who received treatment, radiologic imaging was not performed due to prior clinical progression.

The median overall survival (OS) from date of study enrolment up to date of death or censoring was 207 days (IQR 126-292) and 112 days (IQR 60-164) in patients who did and did not receive molecular profiling-based treatment, respectively (**Figure 12**). No treatment-related deaths or treatment related serious adverse events were observed. **(Table 6**).

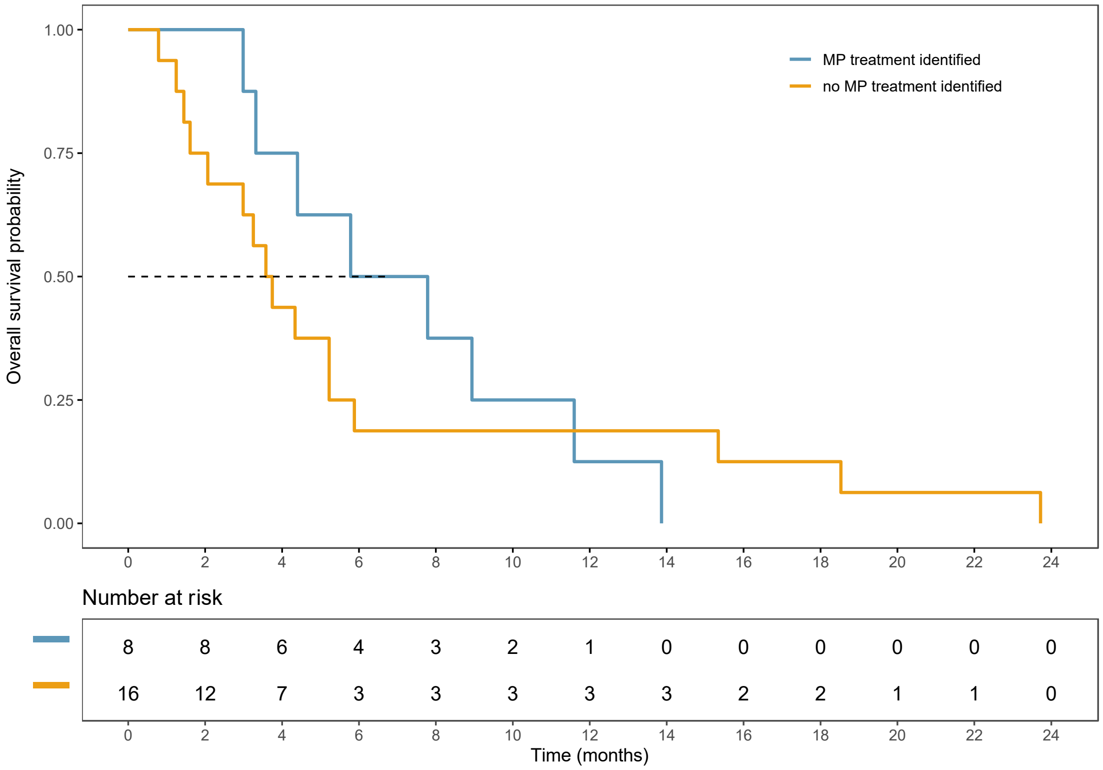


Figure 12. Kaplan-Meier curve for overall survival of patients by type of therapy (as published in (151))

Abbreviations: MP – molecular profiling

Table 6. Individual data on patient demographics, adverse events and survival outcomes.

Abbreviations: ECOG - Eastern Cooperation Oncology Group performance status, SAE – serious adverse events, PFS – progression-free survival, MP – molecular profiling, OS – overall survival, CUP – cancer of unknown primary, n.a. – not applicable

Patient ID	Age	Sex	Diagnosis	Prior palliative treatment lines	ECOG score at inclusion	Received matched therapy	Serious Adverse Event*	SAE related to treatment	PFS of MP-guided treatment (months)	OS (months)
ICT01	47	F	Colon cancer	3	0	Yes	Yes	No	1.4	3.3
ICT02	66	M	Biliary tract cancer	2	0	No***	n.a.	n.a.	n.a.	3.6
ICT03	67	M	Colon cancer	3	1	No	n.a.	n.a.	n.a.	15.3
ICT04	48	M	Renal cell cancer	1	0	Yes	Yes	No	4.1	7.8
ICT05	73	F	Colon cancer	6	1	Yes	n.a.	n.a.	2.3	13.9
ICT06	42	F	Pancreatic cancer	2	1	No	n.a.	n.a.	n.a.	3.7
ICT07	58	F	Biliary tract cancer	2	0	Yes	Yes	No	2.4	4.4
ICT08	40	F	Breast cancer	3	0	No	Yes	No	n.a.	23.7
ICT09	73	M	Gastric cancer	2	1	No	n.a.	n.a.	n.a.	4.3
ICT10	72	M	Gastric cancer	2	0	No	n.a.	n.a.	n.a.	5.2

ICT11	59	M	Laryngeal cancer	2	1	No	n.a.	n.a.	n.a.	5.9
ICT12	47	F	CUP	2	1	No	Yes	No	n.a.	3.3
ICT13	38	F	Rectal cancer	5	1	Yes	No	No	1.6	8.9
ICT14	69	M	Colon cancer	9	1	No	n.a.	n.a.	n.a	1.2
ICT15	65	M	Pancreatic cancer	2	0	Yes	Yes(2x)	No(2x)	2.0	5.8
ICT16	57	F	Gastric cancer	3	0	No	n.a.	n.a.	n.a.	0.8
ICT17	66	F	Pancreatic cancer	2	1	No	n.a.	n.a.	n.a.	1.4
ICT18	67	M	Rectal cancer	4	1	No	No	n.a.	n.a.	1.6
ICT19	54	F	Colon cancer	5	0	Yes	No	n.a.	2.0	11.6
ICT20	56	M	CUP	2	0	No	n.a.	n.a.	n.a.	5.2
ICT21	60	F	Biliary tract cancer	2	1	No	n.a.	n.a.	n.a.	18.5
ICT22	53	M	Esophageal Cancer	4	1	No	n.a.	n.a.	n.a.	2.1
ICT23	65	M	Bladder cancer	3	1	No	n.a.	n.a.	n.a.	3.0
ICT24	63	M	Pancreatic cancer	2	0	Yes	No	n.a.	1.6	3.0

Discussion

Parts of the discussion section were similarly published in Riedl et al (151).

The ICT trial represents the first prospective trial reporting outcome results on the efficacy of ctDNA and optional tissue DNA profiling-based treatment in heavily pretreated metastatic cancer patients. Overall, a conclusive molecular profile could be achieved in 20/24 (83%) of the patients screened and in almost 50% (11/24 patients), a potentially actionable molecular alteration could be identified. In total, eight patients (33%) were treated with a molecular targeted agent but based on the predefined criterium of a PFS ratio of ≥1.2, none of these derived a clinical benefit therefrom. As patient accrual was slower than expected and no clinical benefit could be demonstrated in the patients treated according to their molecular profile, the study was terminated after an interim analysis was performed. Within the limitations of this trial, these findings do not support the routine use of ctDNA genomic profiling based molecular targeted treatment outside clinical trials.

The concept of matching anticancer therapy to each patient´s individual molecular tumor profile irrespective of the primary tumor´s origin is very appealing and might represent a future landmark in cancer treatment. However, several major hurdles have to be overcome before its widespread adoption in routine clinical practice can be ensured. At present, the overall molecular screening efficiency is rather low with only a small fraction of cancer patients benefiting from genomic tumor profiling. In a recently published cross sectional study, retrospective data from more than 500.000 thousand metastatic cancer patients were assessed to evaluate the effectiveness of genomic profiling and genome driven therapy in real world practice. Although the number of patients with cancer eligible for genome guided therapy has steadily increased over recent years, in 2018 still only 8% of all metastatic cancer patients were potential candidates for FDA approved genome guided therapy and only 5% were estimated to derive a clinical benefit therefrom (155).

In studies evaluating the feasibility and efficacy of cancer genome guided therapy outside their clinical indication treatment benefit rates are even lower. To assess the detection rate of actionable genomic targets and the efficacy of genome guided therapy the ProfiLER trial, a French prospective multicenter study enrolled 2597 patients with solid or hematologic advanced cancer noneligible to curative treatment. In each patient molecular tumor profiling by targeted NGS covering 69 cancer hotspot genes was performed. Overall, an actionable molecular target could be identified in 27% of patients, however only 6% received a genome guided therapy and the overall treatment response rate was less than 1%. Importantly, this study had several limitations. For instance, different types of tissue including archival tumor samples were used for molecular profiling. In addition, the median time from study enrollment until discussion at the molecular tumor board was 86 days, which is very long and might partially explain the very high dropout rate of patients with potentially actionable targets who did not receive the recommended therapy (156). Despite the limitations, the overall findings of the ProfiLER trial are largely in line with other studies evaluating the feasibility of tissue based genomic profiling. The MOSCATO trial another large-scale French prospective multicenter study which performed more extensive genomic profiling including array comparative genomic hybridization, NGS of tumor DNA and RNA sequencing showed comparable results. Altogether 1035 patients with advanced cancer were enrolled, in 411 patients an actionable alteration was identified, 199 patients received a genome guided therapy, and 22 patients had an objective treatment response. Although, the primary endpoint of an improved PFS ratio was reached in 33% of the patients who received genome guided therapy, only 7% of the overall screening population derived a clinical benefit from tissue genome profiling (157). Consequently, Tannock et al. postulated that response rates observed in phase 1 trials in which drugs are not selected based on molecular profiling are markedly higher and thus concluded that the off-label use of molecularly targeted agents should be discouraged outside clinical trials (150, 158). In addition, the SHIVA trial, so far, the only trial evaluating the efficacy of genome matched targeted agents in a randomized controlled setting did not meet its primary endpoint of improved PFS compared to treatment at investigator´s choice. In this study an actionable genetic alteration could be identified in 293 out of 741 patients (40%), of which 195 patients (26%) were randomly assigned to either receive a matched molecular targeted agent to one of ten prespecified regimens or to treatment at physician´s choice. The median PFS was 2.3 months in the experimental group and 2.1 months in the control group (hazard ratio 0·88, 95% CI 0·65–1·19, p=0·41) (159).

One central limitation of genomic profiling-based treatment allocation is founded in the heterogeneity and complexity of cancer biology. Albeit strong efforts in cancer genome sequencing have generated an improved understanding of key oncogenic pathways, the functional and clinical interpretation of genomic results remains a major challenge for clinicians (160). Although many potentially druggable oncogenic alterations such as EGFR, BRAF or ERBB2 mutations can be found across various cancer types, clinical activity of molecular treatments targeting those alterations varies greatly depending on tumor location. Naming a prominent example is the inhibition of the BRAF mutation V600E by small molecule tyrosine kinase inhibitors such as vemurafenib which has been shown to have a strong antitumor effect in patients with metastatic melanoma and NSCLC harboring this driver mutation, but not in patients with BRAFV600E mutated metastatic colorectal cancer (161-163). Prahallad et al. could demonstrate that the unresponsiveness of BRAF inhibition in metastatic colorectal cancer is caused by a rapid feedback upregulation of EFGR leading to a sustained proliferation signal (164). Based on these findings the recently published BEACON trial was conducted which proofed the clinical efficacy of a BRAF inhibitor combined with an EGFR antibody in patients with BRAF mutated colorectal cancer (165). This example greatly illustrates the complexity of inter tumor heterogeneity and underlines the challenge of genomic data interpretation and genome guided treatment allocation.

In our study this phenomenon was further greatly exemplified in the two CRC patients ICT01 and ICT05 harboring FLT3 gene amplifications. FLT3 is an established driver gene in hematological malignancies such as acute myeloid leukemia that can be effectively targeted by the multi tyrosine kinase inhibitor sorafenib (166). A case study of a patient with metastatic colon cancer who was found to have FLT3 amplification and who had exhausted all established treatments reported partial response to sorafenib (35). Therefore, this alteration was deemed as potentially actionable by the molecular tumor board and both patients received sorafenib, which unfortunately did not result in treatment response. Based on our knowledge today, this is not surprising. Inspired by the findings from the ICT trial we subsequently characterized the chromosome 13q12 amplification to elucidate the candidate driver gene of carcinogenesis (167). We found that FLT3 does not demonstrate an oncogenic role in colorectal cancer but rather that an amplification of POLR1D is the likely driving event in these cases. Another example for the rapidly evolving understanding of cancer biology achieved through the systematic sequencing of tumor genomes and the subsequent development of novel targeted agents is patient ICT15, who had a pancreatic adenocarcinoma and was found to have a RET amplification. Based on promising response rates observed in a study that has evaluated the role of multi-kinase inhibitors in patients with NSCLC harboring RET rearrangements this patient was treated with the multi-kinase inhibitor sunitinib (168). Most recently remarkable response rates could be demonstrated for the highly selective RET kinase inhibitor selpercatinib in RET fusion positive NSCLC, which was not available at the time the patient in question was treated (169). These examples just greatly illustrate the emerging opportunities of personalized cancer treatment, but also underline potential challenges of genome guided treatment allocation, such as the commonly limited access to novel therapy agents.

The concept of personalized cancer therapy is further challenged by spatial and temporal intratumor heterogeneity. In a seminal study, Gerlinger and colleagues pointed out that cancer cells from different cancer sites and even within one cancer lesion have great variability of their genomic mutational profile. In contrast to trunc mutations such as the Hippel Lindau gene mutation in renal cell carcinoma which arise early in carcinogenesis and are consistently expressed on the majority of cancer cells, branch mutations occur over time and are thus mainly responsible for intratumoral heterogeneity (64). Although most branch mutations are thought to be passenger mutations that do not accelerate cancer growth, other acquired mutations might impact carcinogenesis and might play a key role in resistance mechanisms to targeted therapy. These findings paired with the knowledge that cancer cells dynamically adapt to cancer treatment and evolve over time raise the question whether the genomic profile obtained from a single biopsy of one cancer lesion is representative and should be used for the selection of targeted antineoplastic agents (98). Liquid biopsy offers the inherent advantage of non-invasive cancer genotyping and thus enables the real time tracking of tumor dynamics. Recent studies further suggest a strong concordance of ctDNA based genomic profiling with tumor tissue sequencing in patients with metastatic cancer (92, 93). We thus tried to address the issue of intratumor heterogeneity by obtaining a liquid biopsy for ctDNA analysis and an additional optional tissue biopsy prior treatment start for genomic profiling. The ICT trial represents the first prospective study evaluating this novel concept. Overall, only 11 patients volunteered for a re-biopsy of a metastatic lesions, whereas all patients were willing to donate blood samples. This reflects a typical clinical setting in which recently obtained tissue is commonly not available for genomic profiling and patients are not willed to face the potential risks of additional biopsies. Particularly, in the setting of very advanced cancer stage tissue biopsies must not result in a delay of antineoplastic therapy. Therefore, in the ICT trial most treatment decisions made by the molecular tumorboard were based on profiling results obtained from ctDNA. Unfortunately, in 7 patients no informative profiling results could be achieved from ctDNA which is likely attributed to the limit of detection of the plasma-Seq method, which requires a minimum of 5% tumor-derived DNA fragments to yield informative copy number results (170). Furthermore, very stringent filter criteria were applied for mutation analysis of ctDNA using the cancer hotspot panels. To avoid false positives, only variants with a VAF ⩾5% were considered.

For the limited cases in which molecular profiling results from ctDNA and tissue biopsy samples were available, the concordance of mutation analysis between plasma DNA and tissue was evaluated. The observed concordance rate of 77% of tissue-derived variants also detected in plasma in the ICT trial was lower than rates described previously. For instance, Odegaard et al. who evaluated the clinical feasibility of a ctDNA sequencing test for comprehensive tumor genotyping reported a positive concordance rate for the detection of seven selected genomic biomarkers (EGFR, ALK, ROS1, RET, BRAF, MET, and KRAS) in NSCLC between ctDNA and tissue based genotyping ranging from 92% to 100% (171). Another study by Adalsteinsson et al. demonstrated an 88% concordance rate of clonal somatic mutations between ctDNA and matched tumor biopsies in 41 patients with breast or prostate cancer using whole exome sequencing (93). Importantly, in the ICT trial different panels and sequencing platforms had to be used to analyze tissue and plasma due to limited availability which according to a recent study by a Stetson et al. is a major contributor to assay discordance (172). In addition, as pointed out previously very stringent criteria using a detection cut-off of ⩾5% for the mutational hotspot panel analyses from ctDNA were applied which might have resulted in a potential underreporting of mutations in ctDNA. Since the completion of this trial, novel NGS panels employing UMI technology with a much broader gene coverage and well-established bioinformatics workflows were established, which now enable a ctDNA detection rate down to 0.1% VAF and may thus lead to the detection of a higher number of potentially actionable genomic alterations in future studies, even at low VAFs (173).

Although our study had to be terminated after an interim evaluation due to lack of clinical benefit, this study provides important considerations for future study designs evaluating the concept of personalized cancer treatment. On the one hand our sequencing results indicate that molecular profiling of ctDNA is feasible and holds great potential as an add on or alternative to tissue genotyping for future precision oncology trials. In 70% (17/24) of the patients included in the ICT trial a conclusive molecular profile could be obtained from ctDNA analysis and in

40% a potential actionable target was identified. In contrast only 11 patients consented to an additional biopsy of which 2 did not yield informative results. The median time until finalization of the molecular report was half as long for ctDNA analysis compared to tissue genotyping. Finally, in 7 out of 8 cases molecular profiling-based treatment allocations were based on ctDNA results. These findings are largely in line with a preliminary report of the TARGET trial which will be discussed in more detail below (174). On the other hand, the lack of clinical benefit observed in our trial underlines that several key aspects in terms of methodology and patient selection should be reconsidered for future trials evaluating the concept of molecular profiling-based treatment allocation:

First, the timing of molecular tumor profiling and personalized cancer treatment might significantly impact its feasibility and efficacy. In line with previous precision oncology trials, patients with very advanced disease who had already exhausted all evidence-based therapy lines were enrolled in the ICT trial. Due to the emergence of several resistant clones under the selective pressure of different antineoplastic treatments, advanced tumors can be extensively heterogeneous which might limit the efficacy of a single drug targeting one mutation or functional pathway. Thus, it can be hypothesized that personalized cancer treatment might be more effective in earlier treatment lines either as monotherapy or in combination with established treatments such as chemotherapy, albeit toxicity might be a limiting factor in this context.

Second, an accurate selection of patients who might benefit from molecular tumor profiling is essential. Despite stringent inclusion criteria of an ECOG performance status of 0 or 1 and an expected survival of at least three months two out of 12 patients with matched therapies were unable to receive the recommended treatment due to rapid clinical deterioration (ICT22) or death (ICT16). Considering the high costs, molecular profiling should be only performed in patients to be considered fit enough for potential antineoplastic therapy. For this purpose, a thorough evaluation of the general health status of a patient including a laboratory assessment of adequate organ function including liver, renal and bone marrow function is required.

Third, due to a rapid tumor evolution in the setting of advanced cancer the turn-around time of the whole treatment matching process from biospecimen sampling over NGS data interpretation and treatment selection must be limited to a minimum amount of time. Otherwise, patients may no longer be suitable for the selected treatment. In the present study the median time frame from study enrolment until start of molecular profiling-based treatment was 34 days, which was significantly shorter compared with other trials evaluating the concept of molecular profiling-based treatment matching. For instance, in the recently published French PERMED-01 trial the median time from patient inclusion until discussion at the molecular tumor board was 58 days (56), which resulted in a very high dropout rate. Although the timeframe of 5 weeks in our trial was relatively short, it might still be to long for a relevant proportion of patients. One central challenge was founded in the limited immediate access to drugs due to reimbursement issues, which in several cases resulted in a delay of treatment start. This is a common problem in precision oncology trials, which remains to be overcome. Ideally, contracts with various companies should be made prior trial start ensuring an immediate access to novel antineoplastic agents.

Finally, the actionability assessment and treatment matching process in the ICT trial did not follow uniform rules but was rather made individually. Since the completion of the trial different classification systems such as the ESCAT framework for the clinical actionability assessment of molecular targets were published which now enables a more standardized classification of biomarker-drug interactions and genome guided treatment recommendations. To ensure quality control and reproducibility future precision oncology trials should therefore provide a level of evidence for each matched therapy according to ESCAT or OncoKB criteria (142, 144).

Currently several precision oncology trials evaluating new concepts of molecular profiling-based treatment matching are ongoing. Recently, preliminary reports from three promising trials have been presented (175): The British TARGET (Tumour characterization to Guide Experimental Targeted therapy) study evaluates the clinical utility of comprehensive genomic profiling of ctDNA to match patients with various advanced cancers to different phase I clinical trials. For molecular profiling a large panel covering both somatic mutations and copy number alterations across 641 cancer associated genes is used. In a preliminary report of the first 100 patients included a concordance rate of 74.5% for detected mutations in patients with both tumor tissue and ctDNA analysed was observed, using a VAF threshold of 2.5% for mutation analysis of ctDNA. The mean time from blood draw until finalization of the molecular report was 33 days. Overall, alterations considered actionable were detected in 41 patients, of whom 11 patients matched to a clinical trial and received the study treatment. The objective response rate and disease control rate was 4/11 and 11/11 respectively. The other 30 patients with an actionable alteration either received a non-matched therapy or no further therapy mainly due to limited trial availability or clinical deterioration (174). The I-PREDICT (Profile-Related Evidence Determining Individualized Cancer Therapy) study, a US American cross institutional prospective trial investigates the concept of combination therapies selected based on tumor DNA sequencing including tissue genomic profiling and if available immunohistochemistry of programmed death ligand 1 (PD-L1), TMB, MSI status and NGS of ctDNA. For NGS analysis different gene panels by Foundation Medicine covering 236 to 405 gene are used. At a first stage of this trial 149 patients with refractory metastatic cancers were enrolled, of whom 66 patients did not receive further treatment due to clinical deterioration and 10 patients were administered a non-matched therapy. The remaining 73 patients (49%) had actionable alterations and were treated with at least one matched therapy according to the molecular profile results. The median time from study enrolment until treatment initiation was less than one month, however molecular profiling already performed in routine practice prior study inclusion was also considered for treatment matching. Patients undergoing matched therapy received a median of two drugs ranging from monotherapy to five drug treatment combinations. Overall, 17 patients experienced an objective response, and 21 patients achieved a disease control of ≥ 6 months. The matching score defined as the proportion of detected molecular alterations targeted by administered drugs emerged as a significant predictor of treatment response and longer PFS. Serious adverse events were observed in 19% of the patients with molecular matched therapies (176). The multinational WINTHER trial investigates the clinical efficacy of molecular profiling-based therapy selected according to tumor DNA sequencing covering 236 genes or RNA expression analysis of fresh tumor tissue biopsy in relation to normal adjacent tissue. Overall, 303 patients were enrolled and in 253 patients a successful biopsy of tumor and normal tissue could be achieved. In 158 patients an actionable alteration was identified either by DNA or RNA analysis and 107 patients received the recommended therapy. In 69 patients the matched therapy was selected according to DNA sequencing results and in 38 patients according to transcriptomic profiling. The overall disease control rate was 26.2% and the objective response rate was 11.2%. In 22.4% of all treated patients the PFS on the matched therapy was ≥ 1.5 times longer than the PFS on the patient`s last previous therapy which was not enough to meet the primary study endpoint. In line with results from the I-PREDICT study a higher matching score was associated with better outcome.

Importantly although not reaching statistical significance the disease control rate was slightly higher in patients treated according to transcriptomic results which underlines the high potential of this novel approach as an add on to conventional DNA sequencing (59). To sum up, these three trials have demonstrated the feasibility of different innovative approaches of molecular profiling-based treatment matching that will significantly aid in further improving the concept of personalized cancer therapy. Nevertheless, at present the overall screening efficiency and clinical efficacy of molecular profiling-based therapy is still too low to consider it a standard of care in routine clinical practice.

Limitations

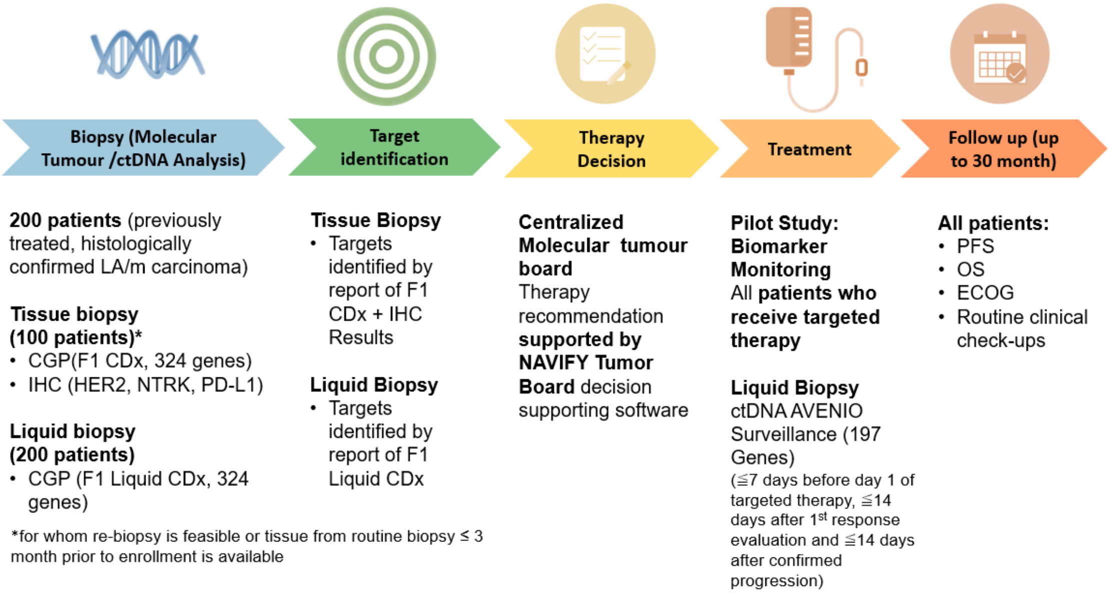
Finally, several limitations of this study have to be discussed. First and foremost, the premature study termination and small sample size of the study limits its validity. Unfortunately, patient accrual was lower than expected. Second, the majority of patients screened and treated within this trial had gastrointestinal cancers harbouring the risk of potential selection bias. Third, no predefined treatment algorithm was used in the ICT trial, which as pointed out previously hinders its reproducibility. However, given the rapid advances in the field of oncology we aimed to preserve the maximum flexibility possible within a prospective trial. Fourth, different gene panels had to be used for mutational analysis due to limited availability. Finally, this study was performed at a single academic centre and did not have a randomized control group.

Conclusion

In summary, the ICT trial represents the first prospective trial that has evaluated the concept of personalized cancer treatment based on results from molecular profiling of ctDNA and/or tumor tissue. Despite the premature study termination and negative outcomes this study provides several key insights that are critical to further advancing the successful implementation of precision oncology. Importantly, sequencing results from the ICT trial indicate that molecular profiling of ctDNA harbours great potential for the concept of personalized cancer therapy. Still, the modest efficacy of genome guided therapy observed in this study underlines that its widespread application is not ready yet for routine clinical practice and that further studies evaluating new innovative approaches of molecular profiling-based treatment allocation are desperately needed.

Outlook

Based on the insights from the ICT trial our study group has planned a large-scale follow up project in the form of a prospective interventional multi-centre phase II trial, which is planned for initiation in autumn 2021. The aim of the SOUND study is to evaluate the efficacy of comprehensive genomic tumour profiling from liquid and/or tissue biopsy in patients with advanced or metastatic carcinoma. To overcome the challenges and pitfalls faced in the ICT trial several major adjustments have been made regarding the study workflow. Importantly, the SOUND study is fully funded and will be curated by a fulltime project manager ensuring adequate patient accrual and high-level data management and monitoring. Overall, 200 patients with previously treated and histologically confirmed advanced cancer from five cancer centres in Austria are planned for recruitment. At study inclusion a mandatory blood draw for ctDNA analysis and an optional tissue biopsy will be performed in each patient. To ensure uniformity and validity molecular profiling will be highly standardized. Comprehensive genomic profiling of ctDNA will be performed with the FoundationOne Liquid CDx panel which covers 324 genes. In all patients with tumor tissue biopsy available, additional comprehensive genomic profiling of tumor DNA using the FoundationOne CDX panel covering the same 324 genes and immunohistochemistry on HER-2, PD-L1 and NTRK will be performed. Molecular profiling results of ctDNA and/or tissue DNA will be summarized within a standardized molecular report provided by Foundation Medicine. Alongside other patient`s relevant medical data each molecular report will be evaluated at a centralized molecular tumor board. The molecular tumor board will take place virtually via the NAVIFY Tumor Board Software on a weekly basis and consist of experts in clinical oncology, pathology and medical genetics from each participating center. To optimize the treatment matching process the molecular tumor board will receive further assistance by the NAVIFY Tumor board decision support software that provides evidence-based information on the actionability of molecular targets. The primary outcome measure of the SOUND trial is the proportion of patients with a PFS on molecular profiling matched therapy > 1.3 than the PFS on the last evidence-based therapy line. In all patients who receive a matched therapy, additional liquid biopsies will be obtained prior treatment start, at the first radio morphologic response assessment and at disease progression for monitoring of genomic cancer evolution. All patients enrolled will be followed up to 30 months.



200 patients (previously treated, histologically confirmed LA/m carcinoma)

Tissue biopsy (100 patients)*
- CGP(F1 CDx, 324 genes)
- IHC (HER2, NTRK, PD-L1)

Liquid biopsy (200 patients)
- CGP (F1 Liquid CDx, 324 genes)

*for whom re-biopsy is feasible or tissue from routine biopsy ≤ 3 month prior to enrollment is available

Tissue Biopsy
- Targets identified by report of F1 CDx + IHC Results

Liquid Biopsy
- Targets identified by report of F1 Liquid CDx

Centralized Molecular tumour board
Therapy recommendation **supported by NAVIFY Tumor Board** decision supporting software

Pilot Study: Biomarker Monitoring
All **patients who receive targeted therapy**

Liquid Biopsy
ctDNA AVENIO Surveillance (197 Genes)
(≦7 days before day 1 of targeted therapy, ≦14 days after 1st response evaluation and ≦14 days after confirmed progression)

All patients:
- PFS
- OS
- ECOG
- Routine clinical check-ups

Figure 13. Workflow of the SOUND study

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