Diploma thesis

Circulating tumor DNA in sarcoma patients: liquid biopsy as a non-invasive method for treatment monitoring

submitted by

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to attain the academic degree

Doctor medicinae universae

(Dr. med. univ.)

at the

Medical University of Graz

conducted at the

Diagnostics & Research Institute

for Pathology and Molecular Biomedicine

under supervision of

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Graz, July 20, 2022

I hereby declare that I have written the submitted thesis independently and without any illegitimate assistance from third parties. Furthermore, I confirm to not have used any other than the declared sources for the preparation of this academic work. All used sources have been indicated as such and acknowledged by means of com- plete references in the text.

Graz, July 20, 2022 *Julia Korting eh.*

Acknowledgments

The completion of this diploma thesis could not have been possible without the expertise and the reliable and helpful support of Dr. Kashofer, my esteemed supervisor. I also thank Mrs. Thüringer for the great support related to the laboratory work.

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Abbreviations

CAGR	Compound Annual Growth Rate
cfDNA	Cell-free DNA
CT	Computer Tomography
CTC	Circulating Tumor Cells
ctDNA	Circulating tumor DNA
ddPCR	Digital droplet PCR
ESMO	European Society for Medical Oncology
EwS	Ewing Sarcoma
FDG-PET	Fluorodeoxyglucose-positron Emission Tomography
GIST	Gastrointestinal stroma Tumor
LB	Liquid Biopsy
MLS	Myxoid Liposarcoma
NGS	Next Generation Sequencing
OS	Osteosarcoma
PCR	Polymerase Chain Reaction
PD-L1	Programmed death-ligand 1
STS	Soft Tissue Sarcoma
WGS	Whole Genome Sequencing

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Zusammenfassung

Die Diplomarbeit befasst sich mit den Anwendungsmöglichkeiten der Flüssigbiopsie bei Sarkomen. Sarkome sind seltene, bösartige Tumoren mit schlechter Prognose, die vor allem junge PatientInnen trifft. Sie entstehen aus mesenchymalem Gewebe und können grob in Weichteil- und Knochentumore eingeteilt werden. Eine große Anzahl der Sarkome besitzen spezifische Translokationen. Translokationen treten auf, wenn Chromosomen in Stücke brechen und die fragmentierten Teile wieder an andere Chromosomen angehängt werden. Im Gegensatz zu anderen Tumorentitäten hat sich das Überleben in den letzten Jahren nicht verbessert, was mit der großen Heterogenität und Seltenheit der Sarkome zu tun hat. Die Diagnose wird mittels einer Nadelbiopsie gestellt und die Überwachung der Therapie findet hauptsächlich über radiologische Diagnostik statt, da wiederholte Biopsien eine zu große Belastung für die meist jungen PatientInnen darstellt. Die Flüssigbiopsie hingegen stellt eine minimal invasive Methode dar. Das Ziel dieser Arbeit ist es, herauszufinden, ob sich der Nachweis von zirkulierende Tumor DNA mittels ddPCR dazu eignet die Therapie von Sarkoma PatientInnen zu überwachen. Die analysierte Kohorte umfasste 18 StudienteilnehmerInnen mit verschiedenen Entitäten von Sarkomen. Um die genetischen Veränderungen analysieren zu können, und die Bruchpunkte, die meist in den Introns liegen, zu bestimmen, wurde ein Whole-Genome-Sequencing in Auftrag gegeben. Die Bruchpunkte der Translokationen bei den verschiedenen Tumorentitäten konnten bei 15 aus 18 Proben bestimmt werden. Dies lieferte die notwendigen Informationen für die Erstellung der benötigten Sonden für die digital-droplet PCR. Es wurde somit nach den tumorspezifischen strukturell veränderten DNA-Moleküle im Plasma gesucht, indem individuell erstellte ddPCR Ansätze verwendet wurden. Insgesamt konnte in 26% der Proben ctDNA nachgewiesen werden, darunter bei allen Ewing Sarkomen und einem Synovial Sarkom.

Die Ergebnisse zeigten, dass unser Ansatz vor allem bei Ewing Sarkomen eine erfolgversprechende Möglichkeit in der Therapieüberwachung darstellt. Bei einem Fall wurden die ddPCR Ergebnisse in der Zusammenschau mit dem klinischen Verlauf betrachtet. Hier zeigte sich, dass die ddPCR Ergebnisse mit den radiologischen bzw. klinischen Untersuchungen kongruent waren. Zu einem Zeitpunkt konnte bereits ctDNA nachgewiesen werden, als das PET-CT noch unauffällig war.

Auch viele weitere Studien unterstreichen das Potential von Flüssigbiopsien zur Therapieüberwachung, zur Erkennung von Resttumorzellen und zur Früherkennung von

Rückfällen. Damit das Potenzial voll genutzt werden kann, muss die analytische Validität und der klinische Nutzen in großen randomisierten Studien untersucht werden.

Abstract

This diploma thesis deals with the applications of liquid biopsy in sarcomas. Sarcomas are rare, malignant tumors with poor prognosis, affecting mainly young patients. They arise from mesenchymal tissue and can be broadly classified into soft tissue and bone tumors. Many sarcomas have specific translocations. Translocations occur when chromosomes break into pieces and the fragmented parts are reattached to other chromosomes. In contrast to other tumor entities, overall survival has not improved in recent years, which has to do with the great heterogeneity and rarity of sarcomas. Diagnosis is made by needle biopsy and monitoring of therapy is mainly via radiological diagnosis. This is because repeated biopsies are too much of a burden for the mostly young patients. Liquid biopsy, on the other hand, is a minimally invasive method. The aim of this work is to explore whether the detection of circulating tumor DNA by digital-droplet PCR is suitable to monitor the therapy of sarcoma patients. The analyzed cohort included 18 study participants with different entities of sarcomas. Whole-genome sequencing was used to analyze the genetic changes and determine the breakpoints, which are mostly located in the introns. The breakpoints of the translocations in the different tumor entities could be determined in 15 of 18 samples. This provided the necessary information to generate the required probes for ddPCR. Thus, the tumor-specific structurally altered DNA molecules in plasma were searched for by using individually designed ddPCR approaches. Overall, ctDNA was detected in 26% of the samples, including all Ewing Sarcomas and one Synovial Sarcoma.

The results show that our approach of using LB as a means to monitor the treatment of sarcoma patients is a promising option. In one case, the ddPCR results were considered in conjunction with the clinical course. Here, the ddPCR results were found to be congruent with the radiological and clinical examinations, respectively. At one point in time, ctDNA could already be detected when PET-CT was still unremarkable.

Many other studies also highlight the potential of liquid biopsies for therapy monitoring, detection of residual tumor cells, and early detection of relapse. For the potential to be fully exploited, the analytical validity and clinical utility need to be investigated in large, randomized clinical trials.

1 Introduction

Sarcomas are a rare type of cancer originating in mesenchymal tissues such as connective and visceral tissues and mesenchymal cells of bone. They represent a very heterogeneous group with over 100 different histological and molecular subtypes, which is due to the wide variety of tissue structures from which they can arise. (4) With an incidence of 1-3/100,000, it is a rare malignancy that affects patients at all stages of life. (5) Specific translocations can often be found in sarcomas. More than 140 gene fusions have been described. (6) Diagnosis and treatment decisions are currently based on invasive tissue biopsy and radiologic imaging. Biopsy can only analyze a portion of the tumor, representing the tumor status solely at a specific point in time. Because of the heterogeneity of sarcomas, histopathologic classification can be difficult. There are few and not sufficiently reliable ways to monitor treatment and detect early recurrence, and prediction of treatment is also imprecise.
Liquid biopsy, as a minimally invasive method, allows frequent evaluation of the tumor by analyzing circulating tumor DNA in the blood. Genomic biomarkers can be consulted to assess response and resistance to treatment. With the objectification and establishment of specific criteria for treatment response, there is a much-needed opportunity to compare the ever-growing number of therapeutic options.

The pilot study conducted for this diploma thesis investigated the association between the level of ctDNA and disease progression as a way to monitor treatment. Accurate breakpoints of translocations at the base pair level were defined using WGS to develop and validate a patient-specific ddPCR-assay. Translocation-specific altered DNA was searched for and quantified in plasma samples from sarcoma patients.

1.1 Sarcoma

1.1.1 Classification

Sarcomas are a heterogeneous group of tumors with over 100 histologic subtypes that differ in their behavior and clinical phenotype. It is a rare cancer in Europe and accounts for about 1% of cancers diagnosed.(7) Sarcomas are grouped in two main categories: soft tissue sarcomas and bone sarcomas. Soft tissue sarcomas are more frequent than bone sarcomas and count for about 84% of sarcomas diagnosed. Most frequent tumors are leiomyosarcoma (19%), liposarcoma (16%) and sarcoma not specifiable (14%).(8) In adults Ewing's Sarcoma and Chondrosarcoma are the most frequent bone related primary tumors.(9)

The distribution of different subtypes related to age is shown in Figure 1. The total number of sarcomas increases with age. Around the age of 90, the incidence is 18 per 100,000, which is still very low. Bone tumors are more common in children than in adults. On the other hand visceral sarcomas are almost nonexistent in children, with an incidence of 6 per 100,000 in adults at around 80 years of age. Figure B shows the distribution of Bone Sarcomas. Ewing sarcomas have a two-peak incidence at 15-30 years of age and a second not-so-high peak at 50-70 years of age, but the incidence always remains below 0.5 per 100,000. Osteosarcomas also occur in youth and then again after 50 years of age. Chondrosarcomas occur only in adults and their incidence increases until 65 years of age.

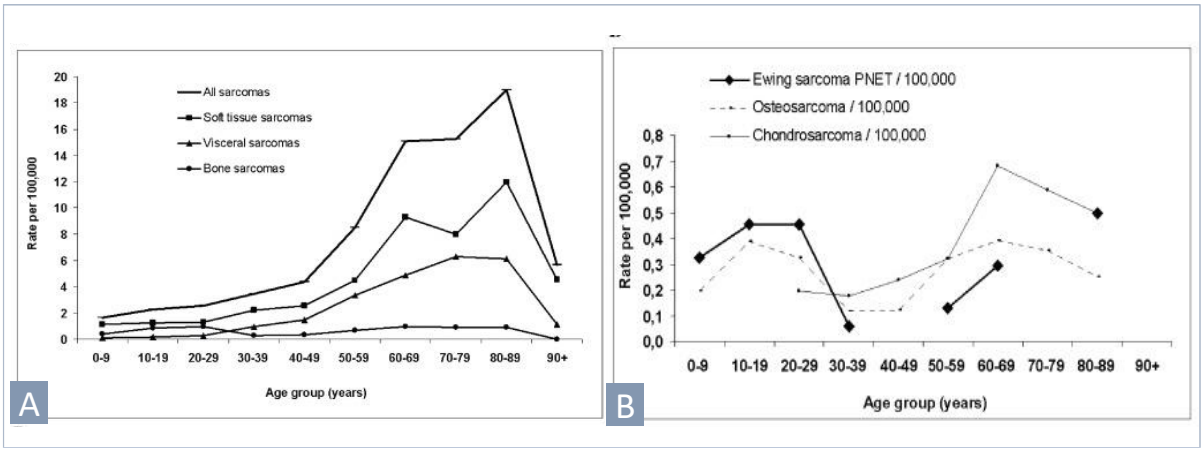


Figure 1: Distribution of sarcoma related to age
A: main subtypes of sarcoma **B:** bone sarcoma
(1)

WHO-Classification

Pathologic diagnosis of sarcomas is according to the 5th edition of the WHO classification 2020, which is summarized in Table 1. (4, 10) Sarcomas are grouped based on cell line differentiation. The group of tumors of uncertain differentiation includes all entities that do not show a clear differentiation of the mesenchymal tissue.

Adipocytic tumors	**Gastrointestinal stromal tumor (GIST)**
Well differentiated liposarcoma: lipoma-like, sclerosing, inflammatory	**Chondro-osseus tumor**
Dedifferentiated liposarcoma	Osteosarcoma, extra skeletal
Myxoid liposarcoma	**Peripheral nerve sheath tumor**
Pleomorphic liposarcoma	Malignant peripheral nerve sheath tumor
Myxoid pleomorphic liposarcoma	Melanotic malignant nerve sheath tumor
	Granular cell tumor, malignant
	Perineurioma, malignant
Fibroblastic/myofibroblastic tumors	**Tumors of uncertain differentiation**
Solitary fibrous tumor, malignant	Phosphaturic mesenchymal tumor, malignant
Fibrosarcoma	NTRK-rearranged spindle cell neoplasm
Myxofibrosarcoma	Synovial sarcoma
Low grade fibromyxoid sarcoma	Epithelioid sarcoma: proximal and classic variant
Sclerosing epithelioid fibrosarcoma	Alveolar soft part sarcoma
So-called fibrohistiocytic tumor	Clear cell sarcoma
Malignant tenosynovial giant cell tumor	Extra skeletal myxoid chondrosarcoma
Vascular tumors	Desmoplastic small round cell tumor
Epithelioid hemangioendothelioma	Rhabdoid tumor
Angiosarcoma	Perivascular epithelioid tumor, malignant
Pericytic (perivascular) tumor	Intimal sarcoma
Glomus tumor (malignant)	Ossifying fibromyxoid tumor, malignant
Smooth muscle tumors	Myoepithelial carcinoma
Inflammatory Leiomyosarcoma	Undifferentiated sarcoma
Leiomyosarcoma	Spindle cell sarcoma, undifferentiated
Skeletal muscle tumors	Pleomorphic sarcoma, undifferentiated
Embryonal rhabdomyosarcoma	Round cell sarcoma, undifferentiated
Alveolar rhabdomyosarcoma	

Pleomorphic rhabdomyosarcoma Spindle cell / sclerosing rhabdomyosarcoma Ectomesenchymoma	**Undifferentiated small round cell sarcomas of bone and soft tissues** Round cell sarcoma with EWSR1-non-ETS fusions CIC-rearranged sarcomas Sarcoma with BCOR genetic alterations

Table 1: Classification of sarcoma

The line of differentiation is determined morphologically or by immunohistochemical methods. However, histologic type alone is not sufficient to plan therapy or predict clinical course. Therefore, a malignancy grade is needed. There are two recognized classification systems: one of the FNCLCC (Férderation Nationale des Centres de Lutte Contre Le Cancer) and one of the NCI (United States National Cancer Institute). Both are based on a 3-tier system and have certain limitations. However, the FNCLCC is the most used system and is based on tumor differentiation, mitotic count, and tumor necrosis. It has been shown to correlate well with survival. (11)

From a molecular point of view, sarcomas can be divided in a simple karyotype group and a complex karyotype group. Simple genetic alterations include translocations and specific activating mutations. Most of sarcomas with simple karyotype are translocation-associated. They account for one third of all sarcomas and usually arise de novo. In some cases, these tumors have only a single defining cytogenetic abnormality that is present at onset and is maintained throughout clonal development. Most of the products of these genetic alterations encode chimeric transcription factors that dysregulate the transcription of target genes. A smaller proportion encode chimeric protein tyrosine kinases and autocrine growth factors. (12) Classical examples of this group include Ewing's sarcoma, synovial sarcoma, and alveolar rhabdomyosarcoma.

Ewing Sarcomas are characterized by the fusion between EWS and FLI1. The fusion oncoprotein EWS-FLI1 is the oncogenic activator of the tumor. Molecular markers may increase diagnostic accuracy and targeted therapy may increase survival in this group of sarcomas.

On the other hand, sarcomas with complex karyotype have versatile nonrecurrent cytogenic alterations and aneuploidy. They have a high mutational burden, but because of the diversity of molecular alterations, molecular markers and target therapy are less useful in these subsets of sarcomas. Prototypical examples include osteosarcomas, leiomyosarcomas,

myxofibrosarcomas, pleomorphic liposarcomas, and undifferentiated pleomorphic sarcomas. (6, 13, 14)

The importance of molecular genetics in the diagnosis of sarcomas has increased due to increasingly specific therapies for the different subtypes of sarcomas. FISH and PCR techniques are routinely used, and allow detection of already known gene fusions with good sensitivity and specificity. (15)

Staging

The staging of sarcomas is based on the 8th edition of the TNM classification published by the American Joint Committee of Cancer (AJCC). In contrast to the staging of carcinomas, the histopathological grade is considered in addition to tumor size, involvement of regional lymph nodes, and distant metastasis. Histopathology is divided into three grades, with grade 3 considered high-grade. The staging of sarcomas depends on the location of the tumor, which leads to differences in prognosis and treatment. For STS, the TNM distinguishes whether the tumor is localized to the trunk or extremities, retroperitoneum, head and neck, or visceral organs. For bone sarcomas, the appendicular skeleton, trunk, skull, and facial bones are considered separately from the spine and pelvis.

The size of STS is divided into 4 stages. (T1 < 5cm, T2 > 5cm, T3 > 10cm, T4 > 15cm). In bone sarcoma, the grade of tumor extension depends on the location. Any positive lymph node or distant metastasis corresponds to stage IV. (16-18)

1.1.2 Diagnostic Approach

A multidisciplinary team of pathologists, radiologists, surgeons, and oncologists is required to make a sufficiently accurate diagnosis. It is based on morphology, immunohistochemistry, and clinical aspects. If sarcoma is suspected, the patient must be referred to a specialized center. Ultrasonography can be performed as the first step in sarcoma diagnosis but should be supplemented by other imaging modalities. MRI is performed for sarcomas of the extremities, pelvis, and trunk, whereas CT is better suited to detect calcifications and is identical to MRI in the case of retroperitoneal location.

If bone sarcoma has been suspected on radiography, MRI should be performed if it has occurred in the limbs, and CT if the trunk, head, or neck are involved.

Tissue biopsy is the true gold standard for obtaining histopathologic information about the tumor, determining the subtype, and making a molecular diagnosis. Generally, multiple core needle biopsies are performed with a 14-18 G needle. Exceptions include excisional biopsy for STS when the tumor is less than 3 cm, fine needle biopsy in centers with special expertise,

and open biopsy for selected indications. The biopsy is performed by either a surgeon or a radiologist. The pathway and scar of the biopsy must be removed in the following surgery because of the high seeding tendency. The biopsy specimen is usually fixed in 4% buffered formalin, and fresh frozen tissue and tumor casts may be collected for further molecular pathology studies. In addition to the pathologic diagnosis, the malignancy grade (FNCLCC) and tumor location, depth, and size are recorded to provide prognostic information. Molecular characterization should be performed in any case when the pathological diagnosis is not beyond doubt, the pathological presentation is unusual, or when prognostic or predictive relevance is expected. It allows differentiation of various sarcoma subtypes and differentiation of malignant neoplasms from benign mimics. (19)

Nevertheless, tissue biopsy has certain limitations. It is an invasive method with the possibility of complications, it cannot be performed on every part of the body, and repeated biopsies are an excessive burden for patients. Intra- and intertumoral heterogeneity cannot always be detected.

Marginal status is assessed after surgery, and tumor tissue change can be followed if preoperative treatment was performed.

Staging always includes a chest CT. In about 10% of the cases, the sarcoma has already metastasized, with a predilection for the lung. (20) Additionally, an abdomen CT is indicated in limb myxoid sarcoma and a brain CT with alveolar soft part sarcoma, clear cell sarcoma, and angiosarcoma, due to their preferred site of metastasis. In bone sarcoma bone scintigraphy and chest radiography and CT are obligatory. A whole-body MRI, and PET Scan can be necessary and is increasingly used for staging. (21)

1.1.3 Detection of Translocations in Sarcoma

Multiplex PCR

New molecular technologies have recently revealed the genetic background of sarcoma. This has proven to be of diagnostic value in clinical practice. There are several techniques to detect translocations, fluorescence in situ hybridization (FISH) is currently the most used in routine practice. Despite their proven usefulness, FISH assays also have drawbacks, such as false-negative results in a high percentage of non-tumor cells. With the advancement of NGS, more samples with novel translocations are being detected and the need for novel methods to detect fusions has increased. A method that has a targeted enrichment strategy for library preparation for NGS is called Archer Anchored Multiplex PCR method (AMP).

It uses specific and universal primers, thus circumventing the requirement to know both fusion partners for translocation detection in advance.

RNA is extracted from tumor material, and then reverse transcription is performed to synthesize a cDNA followed by end repair, dA tailing, and ligation with Illumina or Ion Torrent molecular barcode (MBC) adapters.

Fragments are subjected to two consecutive rounds of PCR amplification. Two sets of gene-specific primers are used. In the first round GSP1 and in the second round a nested GSP2 pool located 3' downstream of GSP1.At the end of the two PCR-steps the target-enriched library can be sequenced. Breakpoint sequences located in the introns can thus be detected. (22)

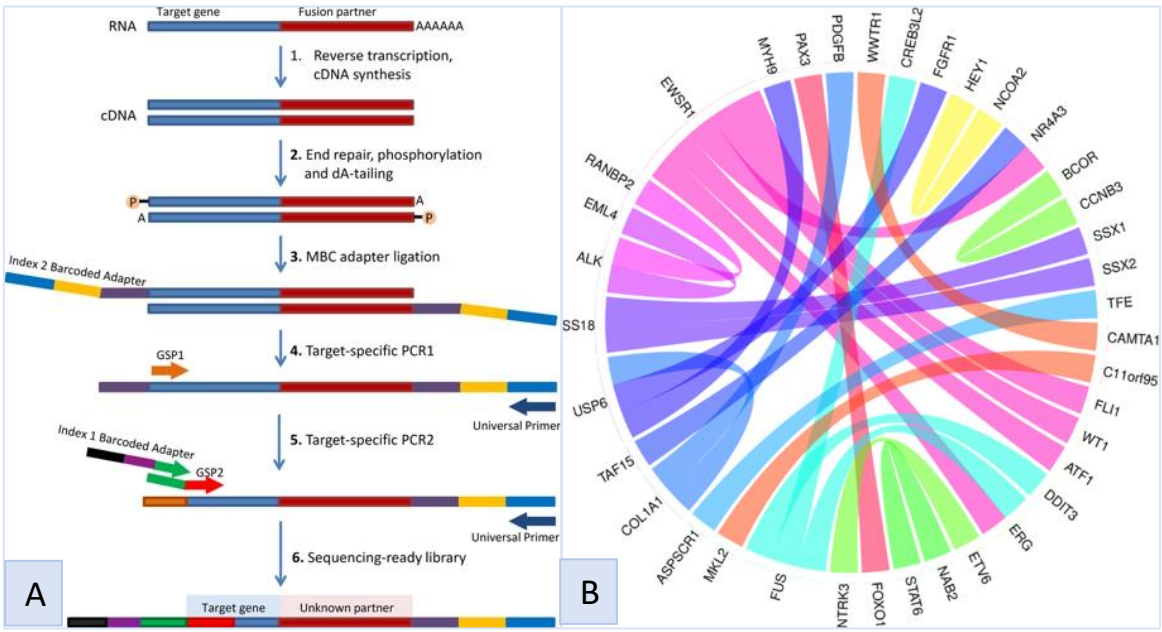


Figure 2: Multiplex PCR
A: Schematic representation of the Archer Anchored Multiplex PCR (AMP™) workflow (2)
B: Chord diagram displaying the 25 fusion gene variants (3)

Digital-droplet PCR:

Samples are portioning into multiple parallel quantitative PCR-reactions.

Droplet-based digital PCR uses aqueous droplets dispersed in oil, enabling the compartmentalization in theoretically unlimited separated PCR reactions. In every droplet is a single molecule. Some droplets contain the mutated sequence (positive), and some do not (negative). Absolute quantification and detection of rare alleles is possible, and high sensitivity is achieved by separating the reactions. Allele frequencies as low as 0.01% can be quantified with ddPCR, but multiplexing capacity is limited and mutations must be known in advance. (23) Thus, it requires individual assays. Figure 2 shows a 2D representation of a ddPCR result as an example. Each dot represents a droplet containing either wild type DNA,

mutant DNA, both, or no DNA. Channel 1 fluorescence (reference probe) plotted against channel 2 fluorescence (wild type).

Shown in gray are the double-negative results, orange the double-positive, green the HEX-positive, i.e. the wild type, and blue the FAM-positive, i.e. the droplets containing a single piece of mutant DNA.

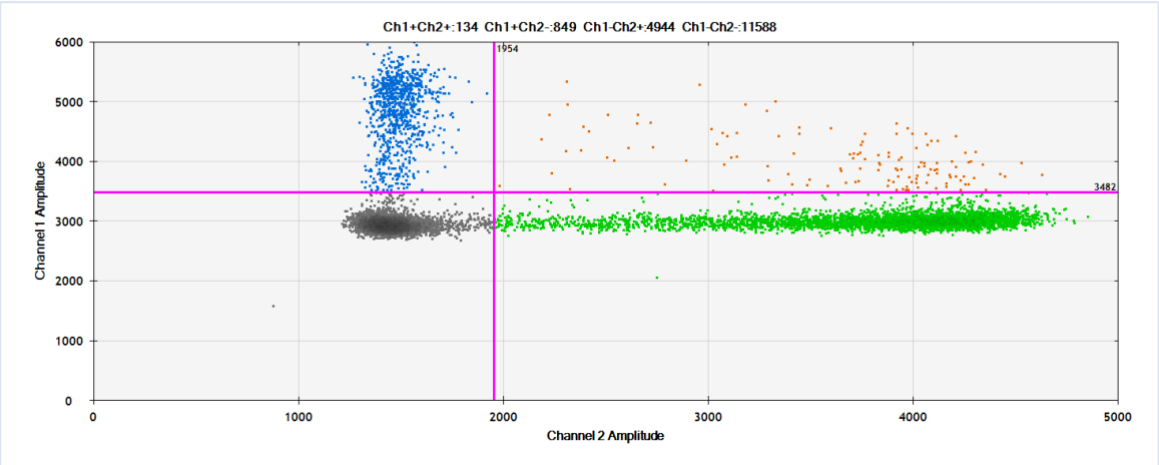


Figure 3: 2D plot of ddPCR

Whole Genome Sequencing (NGS)

WGS enables high-resolution, base-by-base representation of the entire genome. New structural variants and chromosomal aberration can be identified. In recent years, the price has decreased significantly, making WGS attractive for clinical implementation.

Genetic analysis of the tumor can confirm the diagnosis and it provides the necessary information of the exact breakpoints, mostly located in the introns, needed for the generation of a ddPCR assay and thus for quantification of ctDNA during progression. One difficulty is the large amounts of data that are generated. Another challenge is that 1-3µg of DNA is required for a PCR-free library and in addition, sufficient tumor load must be present. A heterozygous mutation in a sample with 50% tumor material means that only every 4th read covers the breakpoint.

Even if you have an assay with very high sensitivity, the accuracy of the method increases with a higher level of ctDNA. This is because the prerequisite for detection is the presence of the mutant DNA of interest in the sample taken.

A larger sequencing breadth results in higher costs but gives an overview and the possibility to find previously unknown changes. A large sequencing depth is accompanied by a smaller

breath as shown in Figure 3. A balance must be found depending on the question, previous knowledge, and the financial possibilities.

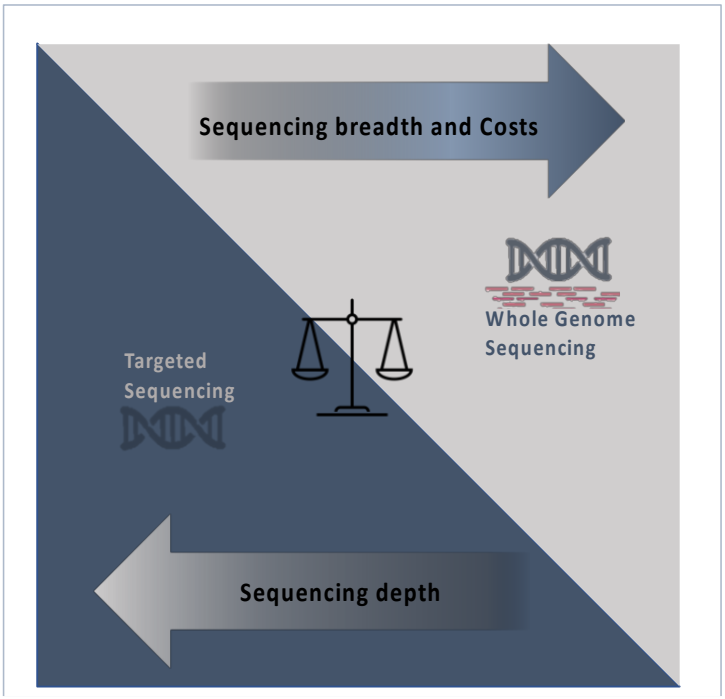


Figure 4: Balance of breadth, depth, and costs

1.1.4 Prognosis

Progression of sarcoma is related to tumor volume and location, tumor grade, histologic subtype, and increasing age, as well as the presence of metastases at diagnosis.

Most STS metastasize to the lungs, but extrapulmonary metastases may also occur in MLS. The 5-year survival rate for soft tissue sarcomas is 81% in a localized stage, 57% in a regional stage, and 16% if distant metastases have occurred. (24)

Overall survival is related to metastatic status: 50-60% in localized disease and 22% in distant metastases. (25) 20-25% of patients have detectable metastases at initial presentation. (22). Subcutaneous tumors have a fivefold lower risk, and intramuscular tumors have twice the risk of metastasis as extra compartmental tumors. (20)

Extremity and trunk tumors have a better prognosis than retroperitoneal tumors. This is because surgery is more difficult, and the lesions are already large at the time of diagnosis. The subtype is also an important prognostic factor; for example, synovial sarcomas, rhabdomyosarcomas, and Ewing's sarcomas are generally associated with a high rate of metastasis, whereas dermatofibrosarcoma protuberans rarely metastasizes. (26)

1.1.5 Therapeutic intervention

Treatment of bone sarcomas and STS requires a referral center with surgeons specially trained in these diseases, including performing a biopsy of a sarcoma-suspected lesion. Studies have shown that treatment by a specialized multidisciplinary team improves the patient survival rate. (27)

The mainstay of therapy is wide, margin free surgical resection of the primary tumor. It is mostly supplemented with neoadjuvant/adjuvant chemotherapy. A study has shown that there is no statistically significant difference in oncologic outcome between limb salvage and amputation when wide, margin-free resection has been achieved. (28)

The first-line chemotherapy for bone sarcoma is doxorubicin combined with cisplatin, methotrexate and ifosfamide. The modality of systemic therapy depends on the subtype. EwS and OS are frequently sensitive for chemotherapy. To lower the risk of systemic disease progression neoadjuvant and adjuvant chemotherapy is applied. (21)

In STS chemotherapy is based on anthracycline possibly combined with ifosfamide.

Radiation therapy is used for positive margins (if R 0 is not feasible), in high-grade STS and in high-risk patients to decrease the risk of local recurrences. (21)

For GIST, therapy is most developed and well-studied. Patients with advanced GIST benefit from targeted therapy. Imatinib as a tyrosine kinase inhibitor is widely used, but secondary resistance is observed. (29) Immunotherapy is another promising opportunity in sarcoma e.g., PD-L1 in Ewing Sarcoma, but clinical trials are still ongoing. (21, 30) (31)

Follow-up of sarcoma patients is an important part of management due to their high rates of relapses. Surveillance aims to detect metastasis or local recurrences as early as possible, in a grade where treatment is still possible and effective. Follow up of sarcoma patients is basically based on imaging modalities. There has been tremendous progress toward early detection by FDG/PET-CT, but it still involves radiation exposure and residual molecular disease cannot be detected.(32)

Due to lack of evidence, there is no generally accepted follow-up strategy. But surveillance should be done for at least 10 years and tumor grade, size, and the site should also be regarded to determine the frequency of follow-up. The ESMO-Guidelines recommend a frequency of 3-4 times/year in high-grade tumors for the first 3 years after surgery.

Physical examination should always be done. Additionally, an MRI is helpful to detect local relapses and a chest X-Ray or CT-Scan can detect lung metastasis. In Ewing Sarcoma an additional isotope-bone scan can be considered.(31, 33)

1.2 Liquid biopsy

Liquid biopsy describes the analysis of liquid samples for the detection of tumor material. In most cases, blood is used as matrix, but urine, ascites or pleural effusions are also suitable as analysis material. (34)

Liquid biopsy enables tumor profiling and provides information on tumor burden, subtype, and developmental dynamics. It also reflects tumor heterogeneity and provides real-time information. Tumor-derived markers that can be detected include circulating tumor cells, circulating extracellular nucleic acids, circulating extracellular vesicles, nucleosomes, various glycoproteins, antigens, and tumor-produced platelets. Circulating extracellular nucleic acids include cell-free DNA, mRNA, and microRNA. (34, 35)

1.2.1 Development of liquid biopsy

Ashworth suspected circulating tumor cells (CTC) in the blood as early as 1869, and Mandel and Metais were finally the first to demonstrate circulating cell-free DNA (cfDNA) in 1948 (36). However, these results were ignored for a long time because nothing was known about the biological function of circulating nucleic acids. In 1977, Leon et al. took up the discovery, and by radioimmunoassay demonstrated that cancer patients had higher levels of cfDNA than healthy individuals. He identified cfDNA as a possible marker for relapse. (37)

The first reliable and standardized assays were developed for the detection of circulating cell-free trophoblast DNA. These non-invasive prenatal tests (NIPT) aim to detect aneuploidies such as trisomy 21, 18, 13. However, it is still a screening test with false positive and false negative results.(38)

With the improvement of analytical methods and bioinformatic advancement, completely new possibilities opened up. The isolation, quantification and sequencing of tumor material became possible. Bioinformatics enables the handling, evaluation, and processing of large amounts of data. Knowledge of circulating nucleic acids increased as sequencing capabilities improved. Prices for NGS have dropped rapidly in recent years, making sequencing affordable as part of liquid biopsy.

In addition, the development of artificial intelligence in bioinformatics and multiparametric analysis are facilitating analysis.

In its report, Technavio predicts the global liquid biopsy market to grow at a CAGR (Compound Annual Growth Rate) of $4.02 billion, representing a CAGR of 38% for the forecast years from 2020 to 2024. (39)

1.2.1.1 *Cell-free DNA*

Cell-free DNA can be released by various mechanisms. This includes cellular apoptosis, necrosis, phagocytosis, but it can also be released by active secretion. In cases of cell damage e.g. through ischemia, trauma, infection or inflammation a higher level of cfDNA is expected. (40) In healthy individuals cfDNA originates mostly from hemopoietic cells because of their high turnover and their localization in the vascularity.

Cell-free DNA consists of fragments of double-stranded DNA with a length between 150 and 200 base pairs. Typically, they have 166 base pairs: one time wrapped around nucleosome (147bp) + linker DNA. It seems that ctDNA is a little shorter. (41)

The clearance of cfDNA is not yet fully understood. The half-life varies from 16 minutes to 2.5 hours, depending on whether or not it is protected by protein complexes or membrane vesicles. (42) In this way, information about the molecular characteristics of the tumor can be obtained in real time. The cfDNA obtained from different tumor cells provides a lot of information about tumor burden as well as sub clonal mutations and intratumorally heterogeneity. (35)

The advantages of cell-free DNA are ease of storage and transport without compromising quality. (43-46) CfDNA circulates in plasma in small amounts. There are 10ng of DNA in 1 ml of plasma from a cancer patient, which is equivalent to 1,500 diploid genome equivalents. (47)

To further develop Liquid Biopsy and implement it as an integral clinical part, more research is needed to learn further about the biology, origin, and release of cfDNA and ctDNA.

1.2.2 Liquid biopsy in sarcoma: Clinical implementation

There are many areas in the handling of sarcoma patients where Liquid Biopsy may produce clinical benefit. The advance in high-through-put next generation sequencing lowered cost of Whole Genome sequencing enormously. WGS is more widely available and makes it accessible in practical medicine and enables by generating an individual genetic profile, the advancement of personalized medicine. With the development of personalized treatment, it becomes important to assess individual response and resistance to therapy, in addition to detecting relapses. Even though the development in imaging techniques is enormous, it exposes the patient to ionizing radiation.(32) Liquid Biopsy may have utility in different stages of sarcoma management. This includes diagnosis, molecular profiling, treatment monitoring, detection of residual disease, and detection of mutations that lead to treatment

resistance. It is important to consider the relevance of the information provided by a liquid biopsy at different time points during the disease to understand the clinical utility.

CtDNA analysis can be considered as a tool for quantitative analysis and genomic analysis. The different assays have different strengths and weaknesses (see Figure 5) therefore the choice of assay must be adapted to the question.

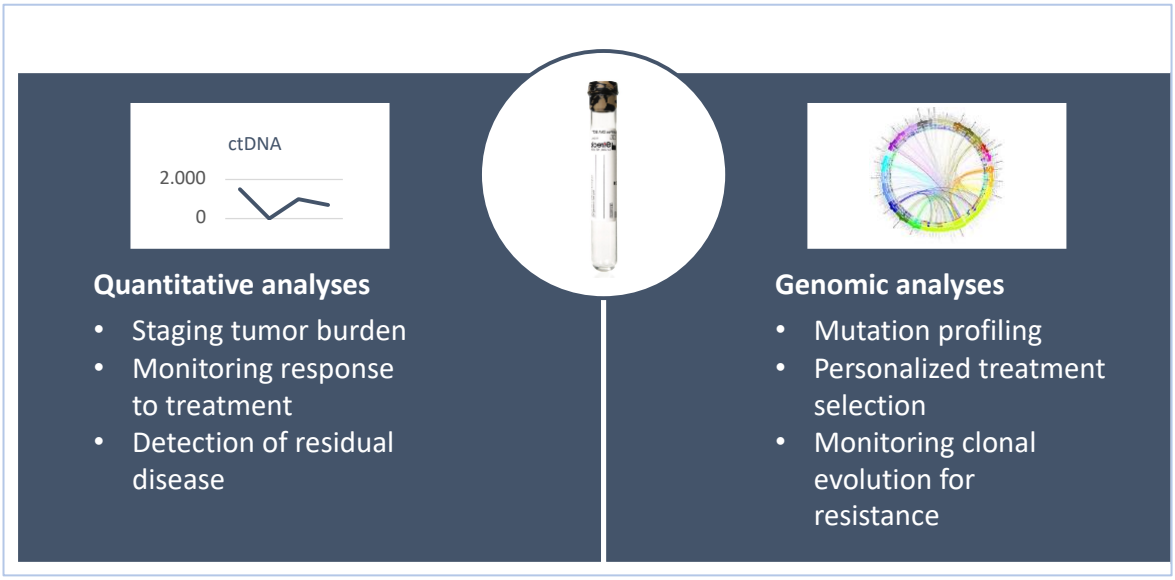


Figure 5: Quantitative and genomic analyses

1.2.2.1 Potential diagnostic/ therapeutic approaches

Liquid Biopsy has utility at different stages of therapy monitoring. An overview of the different applications of liquid biopsy in the treatment of sarcoma patients is shown in Figure 6.

***Diagnosis**: localized cancer*

Due to heterogeneity and the presence of many subtypes, classification can be very difficult; therefore, molecular profiling is playing an increasingly important role in classification of sarcomas.(10) Sensitive and specific detection of mutations may decreases the time to the right diagnosis and speed up the time to appropriate therapy. Currently diagnosis is made by analyzing tumor material gained by tissue biopsy. But this is associated with procedural complications in one of sixth.(48) In addition, sometimes it is unfeasible, or it is difficult to gain sufficient material in quantity and quality.

Curative-intent treatment: Surgery/ Chemotherapy

Minimal residual disease and recurrences monitoring

Quantification of ctDNA may help in deciding the aggressiveness of treatment. If ctDNA remains detectable after surgery in sano with curative intent, it indicates the presence of minimal residual disease. These high-risk patients can be stratified to receive adjuvant chemotherapy. If no ctDNA can be detected, the risk of recurrence is lower, and patients can be spared overtreatment and the associated discomfort and comorbidities.

Treatment selection

Personalized medicine is playing an increasingly important role in oncology. The goal is the adaption of medical treatment to individual, molecular characteristics. That means every tumor is assigned to the appropriate therapy. Currently tissue biopsy is the gold standard to obtain DNA from the tumor. Molecular profiling led to a stratification of treatment. But tissue biopsy cannot map the intertumoral heterogeneity and thereby resulting in a not optimal therapy selection.

Monitoring disease burden

Currently treatment monitoring of sarcoma patients is performed through imaging, including metabolic methods like PET-CT. If the change in tumor burden is large enough to be visible on imaging, the therapy can be adjusted. This leads to a limited accuracy, and it is associated with exposure to ionic radiation. Whereas LB means nothing more than a blood draw for the patient, which can be done anywhere and with little effort. Due to the short half-life of cfDNA it provides real-time information about tumor burden and may identify changes earlier.

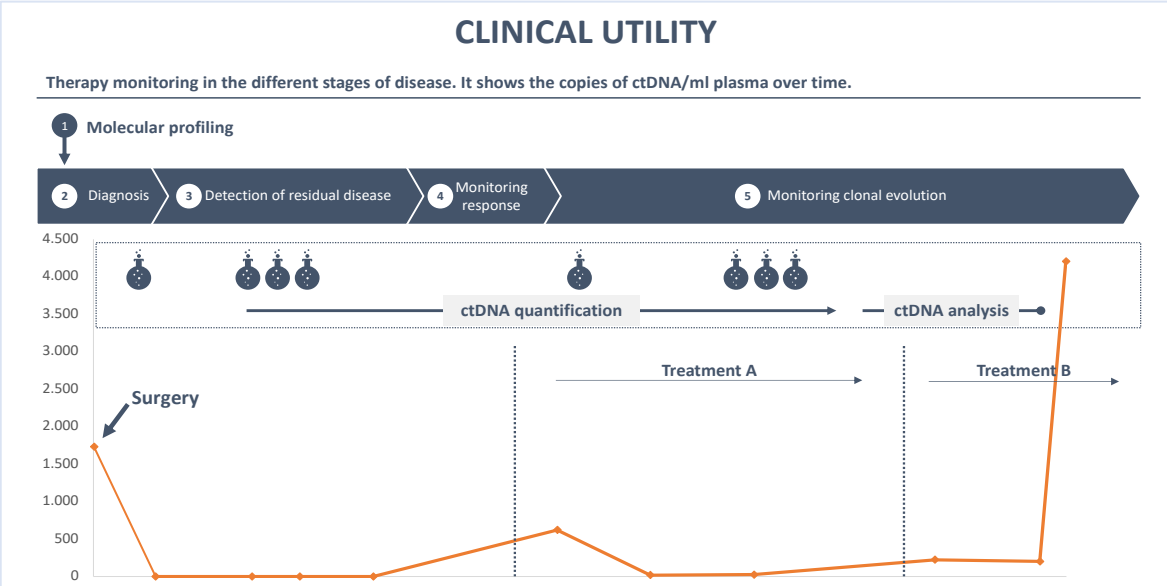


Figure 6 : Therapy monitoring at different time points

1.2.3 Comparison of liquid-based to tissue-based Biopsy

Table 3 shows the difference between liquid biopsy and tissue biopsy. Tissue biopsies can only be performed when the location of the tumor is known and accessible for collection. Tissue biopsy is an established procedure and is currently the gold standard. Liquid biopsies are well suited for screening, identification of mutations and their changes even in metastasis. Although both biopsies have their strengths, it is important to know how they differ.

	Advantages	**Disadvantages**
Tissue Biopsy	• Gold standard: clinically validated, basis for diagnosis, staging, and treatment selection • Pathological and histological information • Evaluation of non-DNA biomarkers	• Invasive procedure: Pain and possible complications • Not always feasible, depending on the site • Time intensive, not every physician has the required competence • Information only about a small part of the whole tumor • Monitoring treatment response not possible
Liquid Biopsy	• Easy sampling, regardless of site • Rapid procedure • Panoramic view of heterogeneity, even with metastasis • Real-time monitoring: response/resistance to drugs, recurrences • Enables personalized therapy • In follow-up phase: Blood collection is possible even from primary care physicians in rural areas	• Not yet standardized procedure for sarcomas: lack of standards for sampling and analysis • Cannot assess non-DNA biomarkers

Table 2: Comparison of Liquid Biopsy and Tissue Biopsy

2 Material and Methods

2.1 Patient enrollment

All patients with suspected mesenchymal tumors are recruited through the Department of Orthopedics and Orthopedic Surgery, the Department of Oncology as well as through the Department of Pediatric Haemato-Oncology of the LKH University Hospital Graz. Patients with benign and malignant mesenchymal tumors are included, with focus on sarcomas.

2.2 Ethics approval and consent to participate

Local approval was obtained of all clinical material in this study according to standard clinical practice. Special informed consent forms were obtained for children and adults. Ethics approval was obtained from the Institute of Cell Biology, Histology and Embryology of the Medical University of Graz. The study was approved by the Ethics Committee of the Medical University of Graz (EK 28-397 ex 15/16).

Tumor tissue collection was performed either in the outpatient clinics of the University Hospital or during the surgical procedure to confirm the diagnosis. (49)

2.3 Blood sampling collection and plasma processing

Peripheral blood was drawn in Streck Cell-Free DNA BCT® tubes (Cat-Nr: 218997). Plasma was extracted by two centrifugation steps. In the first step at 500g an in the second step at 1600G, each for 10 min. Samples were stored at -80°C in 1.5mL plasma aliquots. Qiagen QIAmp Circulating Nucleic Acid Kit (Cat-Nr: 55114) was used to obtain cfDNA from 1 to 5mL of Plasma. DNA was measured using ThermoFischer Quant-iT™ PicoGreen™ dsDNA Assay Kits and dsDNA Reagents (Cat-Nr: P11496)

2.4 Tumor tissue processing

Formalin fixed, paraffin-embedded blocks of tumor tissue were obtained and sliced in 10-µm sections by the Diagnostic and Research Institute of Pathology Graz. For all cases a translocation-associated sarcoma was diagnosed by an experienced tumor pathologist in advance.

2.5 Whole Genome Sequencing

Using DNA from the obtained FFPE sections, whole genome sequencing was performed in collaboration with the Research Center for Molecular Medicine of the Austrian Academy of Sciences in Vienna. WGS was performed using the standard Illumina workflow on the NovaSeq 6000 system with a S4 flow cell. The NovaSeq 6000 system uses patterned flow cell technology. Billions of nano wells are distributed across both surfaces of the patterned flow cell, resulting in control of cluster size, and spacing and enabling accurate resolution of high-density flow cells. The library was prepared using Illumina® DNA PCR-Free Prep, Tagmentation (Cat. Nr: 20041794). It uses On-Based Tagmentation: a combination of adapter ligation and fragmentation (our DNA was already fragmented). Quantification and dilution for sequencing is done with a Qubit Quantification. (higher data output, more sequencing reads, faster run times).

RTA3 software was used to provide real-time run quality metrics. The data obtained were stored as InterOp files containing tile, cycle, and read-level metrics.

Reads were aligned to hg38 reference genome and calling of sequence variants was performed by SVABA. (50) The exact breakpoints were determined manually by inspection for the mismatch in the bases.

With the knowledge of the specific breakpoints, primers spanning the breakpoints were developed. The software used was Primer 3 plus. Using the designed primers, PCR was performed on all plasma samples. Agarose gel electrophoresis was performed to check the PCR products for amplification of the desired DNA segments.

2.6 Digital droplet PCR

Bio-Rad's QX200 ddPCR System (Bio-Rad Laboratories, Hercules, CA) was used for absolute quantification of circulating tumor DNA. The assay was performed in duplicates and in the case of sufficient DNA of a sample in triplicates. A sequence from the chromosome section 2p14 primer was used as a reference. First droplets were generated by the QX200 Droplet Generator and then the droplets were transferred in a thermal cycler for PCR (C1000 Touch^{TM}Thermal Cycler). After amplification droplets were placed in the QX200 Droplet Reader. Droplets are classified in four groups: double negative (gray), FAM positive (blue), HEX positive (green) and double positive (orange). For correct quantification threshold was set manually to ensure a correct classification. Data were analyzed using QuantaSoft™ Software. Concentration is calculated by counting the positive

and negative droplets and modeled by Poisson distribution. The result is indicated in copies of target per volume analyzed. (51)

Concentration = $-\ln(\frac{N_{neg}}{N}) / V_{droplet}$ (51)

2.7 Bioinformatics

The analysis of the WGS data was first done fully automated following five steps:

1. alignment to the human reference genome 2. filtering of PCR artifacts 3. filtering of mutations and recalculation of quality scores 4. calculation of mutations and 5. annotation of mutations. The alignment was cleaned of PCR duplicates, considering that PCR duplicates carry equal start and end positions, as well as equal sequence. This was calculated using Picard, a program based on SAMtools and its open file format SAM/BAM. Due to the heuristic approach of alignment, positions that are close to insertions or deletions are often considered false-positive mutations because their exact assignment at these positions is difficult. Multiple alignment of reads close to known insertions and deletions can minimize these errors. Various filters were applied to distinguish high quality mutations from technical artifacts, with potential artifacts not removed from subsequent analysis steps to ensure high sensitivity and to still be able to check structural variants for possible false negative mutations. These steps were done by Genome Analysis Toolkit. SVaba was used to identify the breakpoints in the WGS data. The exact breakpoints of the translocations were finally determined manually and primers for both breakpoints were created using Primer3plus software. (52)

3 Results

3.1 Workflow

The aim of this study is to demonstrate that the detection of gene fusions in the blood of sarcoma patients can be used for therapy monitoring. For this purpose, a WGS was performed to be able to determine the individual breakpoints of translocations, which are often located in introns, and thus develop an individual assay for ddPCR. This allows quantification of tumor DNA in the blood of patients.

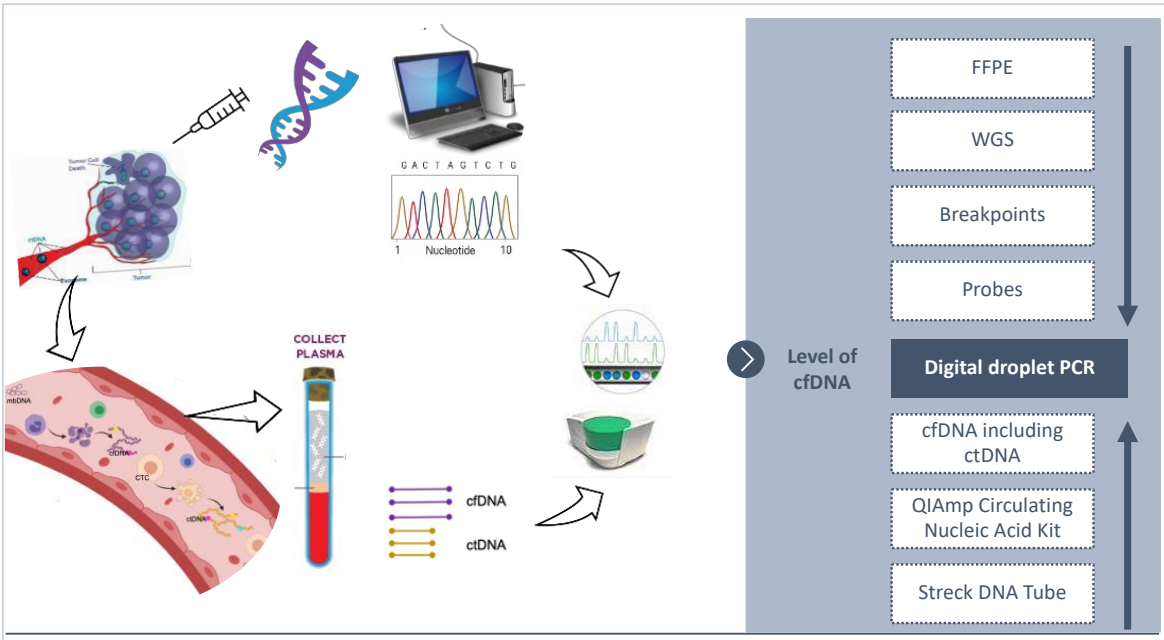


Figure 7: illustration of workflow

3.2 Cohort

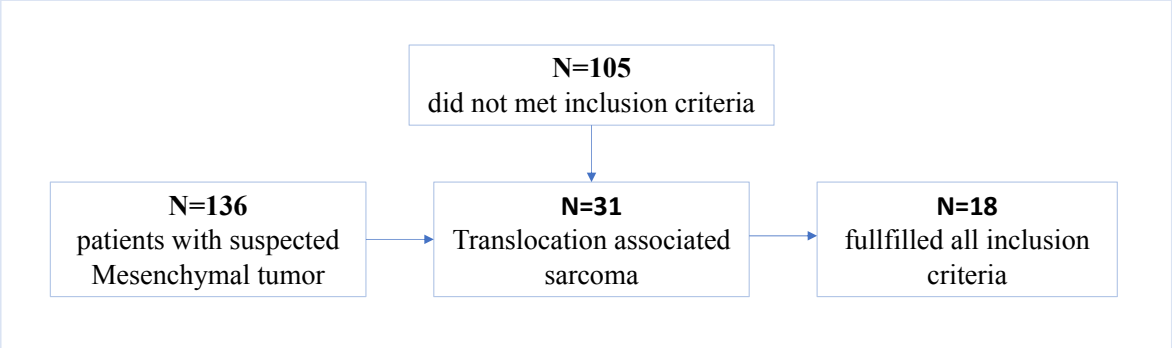


Figure 8: Inclusion criteria

In general, only patients with suspected benign and malignant mesenchymal tumors were included in this study. However, in addition also patients suspected of recurrence of previously confirmed diagnosis were included. In total, 136 patients with suspected mesenchymal tumor were included in the study, of which 31 patients had a confirmed diagnosis of translocation-associated sarcoma. At the end, 18 patients were recruited with a total of 87 blood samples.

3.2.1 Samples

The largest proportion of subjects (N=6) suffered from Ewing sarcoma followed by synovial sarcoma (N=4) and myxoid liposarcoma (N=3). A total of 87 LB samples were examined. The distribution is shown in Table 3.

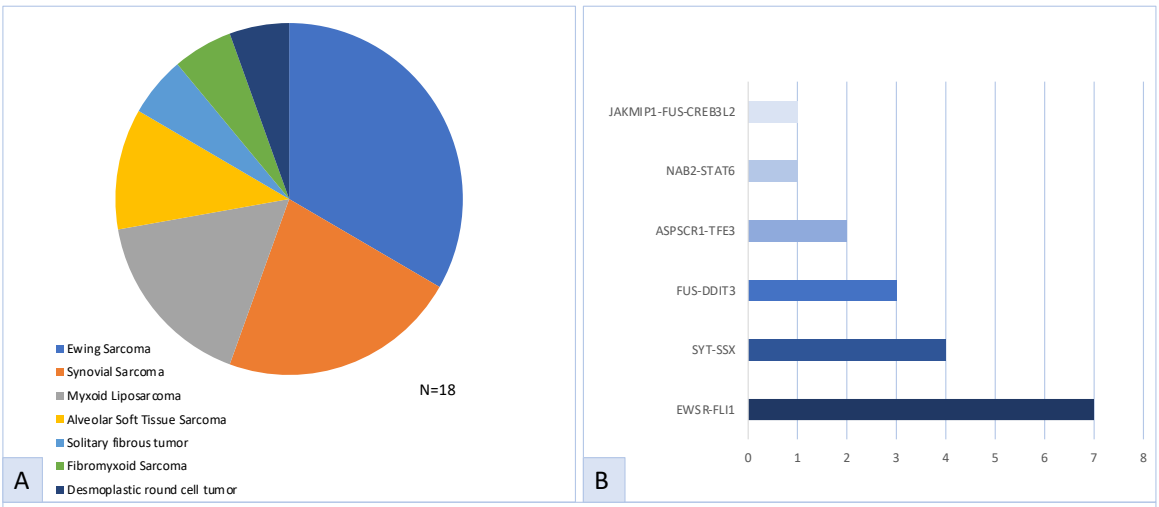


Figure 9: Distribution of A: subtypes **B:** translocations

Patient ID	Tissue	Archer Fusion Panel	Disease	LB Samples
S69	Bone	EWSR1-FLI1	Ewing Sarcoma	6
S97	Lung	EWSR1-FLI1	Ewing Sarcoma	5
S101	Soft Tissue	EWSR1-FLI1	Ewing Sarcoma	7
S113	Soft Tissue	SYT-SSX	Synovial Sarcoma	1
S121	Soft Tissue	EWSR1-FLI1	Ewing Sarcoma	11
S142	Soft Tissue	FUS-DDIT3	Myxoid Liposarcoma	3
S19	Soft Tissue	FUS-DDIT3	Myxoid Liposarcoma	5
S1	n.a.	EWSR1-FLI1	Ewing Sarcoma	8
S8	CNS	ASPCR1-TFE3	Alveolar Soft Tissue Sarcoma	2
S17	Peritoneum	EWSR1-WT1	Desmoplastic round cell tumor	2
S18	Soft Tissue	NAB2-STAT6	Solitary fibrous tumor	10
S48	Soft Tissue	FUS-DDIT3	Myxoid Liposarcoma	5
S31	n.a.	JAKMIP1-FUS-CREB3L2	Fibro myxoid Sarcoma	
S49	Soft Tissue	ASPSCR1-TFE3	Alveolar soft tissue Sarcoma	4
S70	Soft Tissue	EWSR1-FLI1	Ewing Sarcoma	8
S56	n.a.	SYT-SSX	Synovial Sarcoma	2
S57	n.a.	SYT-SSX	Synovial Sarcoma	1
S39	n.a.	SYT-SSX	Synovial Sarcoma	7
				87

Table 3: Overview of LB samples

3.3 Whole genome sequencing

A total of 15 bone and soft tissue tumor samples were subjected to WGS. Mean read length was 151bp. A median coverage of 41X was obtained with a range from 24X to 67X. Bases ≥ 30X was achieved in more than 67 % and in 8 samples over 80% of the bases were covered 30X. The general statistics of the WGS are summarized in table 4.

	Sample ID	**Tissue**	**Sex**	**% Aligned**	**Insert Size**	**% Dups**	**TiTV ratio (known)**	**TiTV ratio (novel)**	**Median Coverage**	**Bases ≥ 1X**	**Bases ≥ 10X**	**Bases ≥ 20X**	**Bases ≥ 30X**
S97	*A1638_16*	Lung	MALE	100%	176 bp	10.2%	1.9	2.7	41.0X	94%	93%	90%	81%
S101	*A2067_6*	Soft Tissue	MALE	100%	164 bp	11.7%	3.6	6.5	49.0X	94%	93%	92%	87%
S69	*A274 _16*	Bone	MALE	100%	145 bp	11.4%	2.3	3.6	27.0X	94%	91%	78%	41%
S113	*A3206_3*	Soft Tissue	MALE	100%	174 bp	11.1%	1.7	1.9	67.0X	94%	93%	93%	91%
S121	*A4157_21*	Soft Tissue	FEMALE	100%	158 bp	11.5%	1.9	3.2	36.0X	93%	92%	90%	73%
S142	*A7228_3*	Soft Tissue	MALE	100%	181 bp	10.8%	1.7	1.5	64.0X	94%	93%	93%	91%
S19	*K6768_3*	Soft Tissue	MALE	100%	142 bp	13.2%	3.1	5.1	24.0X	94%	90%	66%	27%
S1	*K6844_4*	unknown	FEMALE	100%	157 bp	11.0%	4.0	7.5	36.0X	93%	92%	90%	73%
S8	*K6908_6*	CNS	MALE	100%	163 bp	12.4%	3.0	6.5	46.0X	94%	93%	92%	87%
S17	*K7274_30*	Peritonem	MALE	100%	163 bp	11.4%	2.6	4.5	51.0X	94%	93%	92%	88%
S18	*K7523_5*	Soft Tissue	MALE	100%	169 bp	11.7%	1.9	3.0	56.0X	94%	93%	92%	87%
S48	*K7910_6*	Soft Tissue	FEMALE	100%	150 bp	10.9%	1.8	2.4	24.0X	93%	92%	73%	28%
S31	*K8251_30*	unknown	FEMALE	100%	149 bp	10.8%	2.3	4.9	27.0X	93%	91%	75%	40%
S49	*K8800_6*	Soft Tissue	MALE	100%	150 bp	13.9%	2.5	4.6	25.0X	94%	91%	71%	35%
S70	*K9820_24*	Soft Tissue	FEMALE	100%	162 bp	10.8%	1.7	1.5	43.0X	94%	93%	91%	82%

Table 4: general statistics of WGS

3.3.1 Detection of Breakpoints

We used SvABA, an efficient and accurate SV detection method to identify the breakpoints in the WGS data. In this way, for the translocations determined in advance for each patient, the exact breakpoints could be determined. Figure 10 shows an example of an EWSR-FLI1 translocation. The upper image shows the EWSR gene on chromosome 11 and the lower image shows the FLI 1 gene on chromosome 22. The breakpoint region is highlighted in blue.

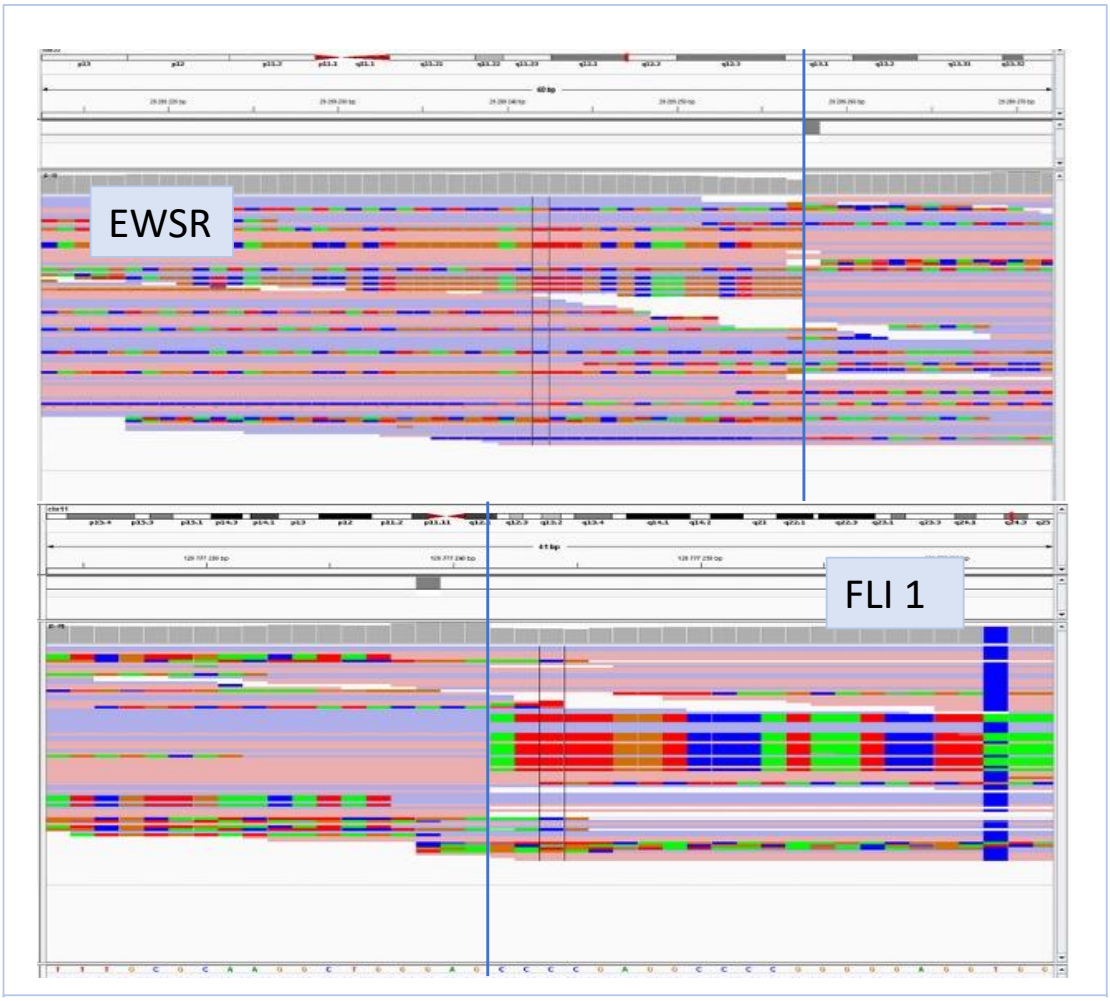


Figure 10: EWSR-FLI1 translocation:
breakpoint region is highlighted in blue

Table 5 gives an overview of the samples for which a breakpoint could be determined and the exact location of those breakpoints. In three cases, the breakpoint has already been determined using SureSelect (Agilent). This is a target enrichment solution for Next Generation Sequencing. In another three cases manual breakpoint determination is still pending.

			Breakpoint 1	**Breakpoint 2**
Ewing Sarcoma *EWSR-FLI1*	S69	Breakpoint determinable	G [chr22:29289258[	]chr11:128777239] A
	S97	Breakpoint determinable	]chr22:29292655]T	G [chr11:128795030[
	S101	Breakpoint determinable	C [chr22:29287795[	]chr11:128786094] G
	S121	Breakpoint determinable	G [chr22:29288035	]chr111128796041] A
	S1	Breakpoint determinable	]chr22:29288203]G	T [chr11:128806738[
	S70	Breakpoint determinable	C [chr22:29287723[	]chr11:128797267] T
Myxoid Liposarcoma *FUS-DDIT3*	S142	Breakpoint determinable	[chr16:31184800[A	[chr12:57519178[T
	S19	Breakpoint determinable	[chr16:31188174[T	[chr12:57519699[T
	S48	Breakpoint determinable	[chr16:31184465[C	[chr12:57518887[A
Fibromyoxid Sarcoma *JAKMIP1-FUS-CREB3L2*	S31	Breakpoint determinable	C]chr16:31185136]	C]chr7:137908373]
Desmopl. Round Cell Tumor *EWSR1-WT1*	S17	Breakpoint determinable	C[chr22:29287273[	G]chr11:32395098]
Solitary Fibrous Tumor *NAB2-STAT6*	S18	Breakpoint determinable	A[chr12:57099028]	T]chr12:57094571]
Synoovial Sarcoma *SS18-SSX1*	S56	SureSelect		
	S57	SureSelect		
	S39	SureSelect		
	S113	Manual breakpoint determination necessary		
Alveolar Soft Tissue Sarcoma *ASPSCR1-TFE3*	S8	Manual breakpoint determination necessary		
	S49	Manual breakpoint determination necessary		

Table 5: Breakpoints at base-pair level

3.3.2 Development of ddPCR Assays

The primers used in this study, were designed based on the breakpoint sequences that were identified with WGS. Primers were designed by extending artificial breakpoint reference sequences derived from the NGS reads up and down 200 bases, as appropriate. Criteria for primer selection were melting temperature, length, base composition, and GC content. We ensured that primers did not show significant complementary between the 3'-ends to avoid primer-dimers. Primer oligos were ordered from Eurofins. Table 7 shows the list of primers used.

```
Template masking not selected
No mispriming library specified
No internal oligo mishyb library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%  any_th  3'_th hairpin seq
LEFT PRIMER          2   21   59.15   47.62    0.00   0.00    0.00 TGTGGGGTTGTTAAGGTCAGT
RIGHT PRIMER       113   20   58.86   50.00    0.95   0.00    0.00 TTGAAACAGGGCCTCACTCT
INTERNAL OLIGO      52   20   59.96   50.00    4.53   0.00   31.94 TTTAAACCACAGAGTGCGCC
SEQUENCE SIZE: 151
INCLUDED REGION SIZE: 151

PRODUCT SIZE: 112, PAIR ANY_TH COMPL: 8.02, PAIR 3'_TH COMPL: 0.00
TARGETS (start len
    1 GTGTGGGGTTGTTAAGGTCAGTAAAAGCATAACCAGGTCATCTGGCAGAACTTTAAACCA
       >>>>>>>>>>>>>>>>>>>>                              ^^^^^^^^^
   61 CAGAGTGCGCCACTTCACTCCAGCCTGAGCGACAGAGTGAGGCCCTGTTTCAAAATAAAA
      ^^^^^*^^^^^                      <<<<<<<<<<<<<<<<<<<<
  121 ATATTAAACAGAGGCTTAACATGCCTCTTTG
```

Results for A1638-16:

* target
>>> left primer
<<< right primer
^^^ internal oligo

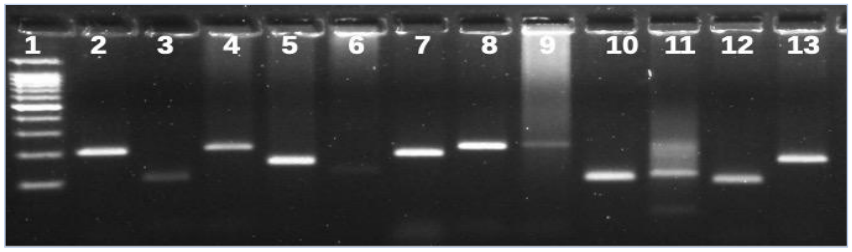
Figure 11: Sequence of one WGS read covering the breakpoint

Oligoname	Sequence 5`-> 3’	Tm (°C)	GC %
S70_rev	TGACTGATAGGGAGGCCAAA (20)	57,3	50
S70_fwd	CGTGAATCCAAGACCACAGAC (21)	59,8	52,4
S31_rev	CGGCGGTGGTGGTTACAA (18)	58,2	61,1
S31_fwd	GAGGGGCTGTGGGTCTGA (18)	60,5	66
S48_rev	TCACACCTGTAATCCCAGCA (20)	57,3	50
S48_fwd	CAGTGAGCCGAGATTGTGC (19)	58,8	57,9
S18_rev	CACTAAGCCCCTGACCTACC (20)	61,4	60
S18_fwd	TTCACAGCTTTCTTTGCCCC (20)	57,3	50
S17_rev	TGAGTTGCTAAGAGAGAAAACCA (23)	57,1	39,1
S17_fwd	CATAACATGCCACTGGTCCC (20)	59,4	55
S1_rev	TGATGGTACTGAGGCTGTGG (20)	59,4	55
S1_fwd	AGTTCTTCTGTATGGAGAGAGGT (23)	58,9	43,5
S19_rev	AGTCCCTGCCTCTTTAACCC (20)	59,4	55
S19_fwd	CCGAGGGCCTTTAGTGACAT (20)	59,4	55
S142_rev	TCCTCCACTCAAAACCCTTCA (21)	57,9	47,6
S142_fwd	GCCAGGCAATGAAGGACAAA (20)	57,3	50
S121_rev	GTGTTTTGGTTACCTCTCTCCA (22)	58,4	45,5
S121_fwd	CCCCTGCCAAGTATCTACCT (20)	59,4	55
S101_rev	TCTCAAGTGATCCTCCTGCC (20)	59,4	55
S101_fwd	CTGATGCCCAAGTGCCAAAA (20)	57,3	55
S97_rev	TTGAAACAGGGCCTCACTCT (20)	57,3	50
S97_fwd	TGTGGGGTTGTTAAGGTCAGT (21)	57,9	47,6
S69_rev	CAGTAGGAAGTGAGCCCATAAT (22)	58,4	45,5
S69_fwd	TCGAAGAAACGGAGGGCG (18)	58,2	61,1

Table 6: List of Primers

3.3.2.1 Amplicon Verification

Primers were used to amplify the targeted regions. Lengths of PCR products were visualized by agarose gel electrophoresis to ensure that the products corresponded to the sequence sought. In all but one case, the PCR products analyzed corresponded to the length of the sought gene regions.



PCR analysis: with the primers corresponding to the determined breakpoints

1: 100bp **2:** S69 **3:** S97 **4:** S101 **5:** S121
6: S142 **7:** S19 **8:** S1 **9:** S17 **10:** S18
11: S48 **12:** S31 **13:** S70

Figure 12: Results of the PCR analysis

3.4 Digital Droplet PCR

3.4.1 Gradient PCR

Gradient PCR was performed for each ddPCR assay to determine the temperature with the highest specificity. One result is shown in Figure 13 as an example. In this case, the highest specificity was achieved at 55.8°C.

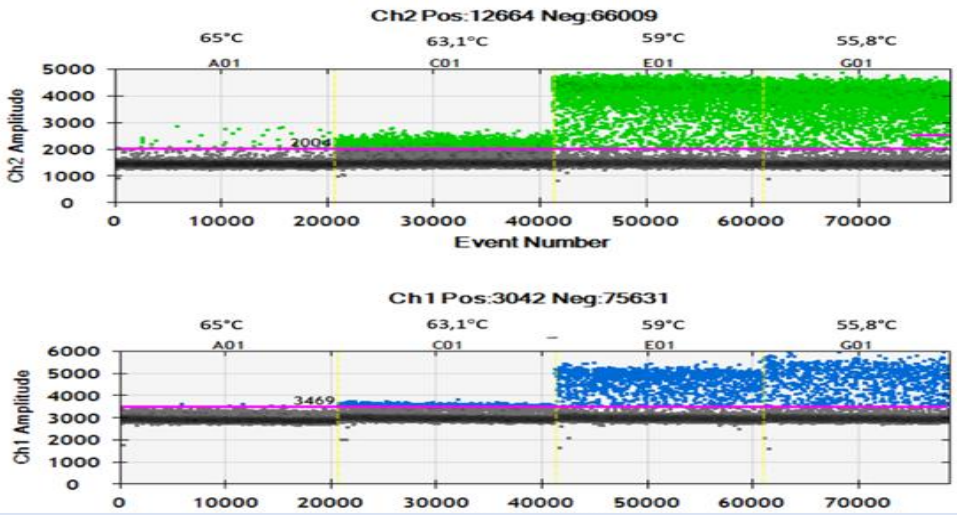


Figure 13: Temperature gradient experiment
green: reference gene:2p14, blue: translocated sample

3.4.2 Cohort results

CtDNA was detectable using individual ddPCR approaches in a total of 7 patients across 23 samples, means in 26.4%. (Median: 3,5 samples per patient). CtDNA was detected in all patients with Ewing sarcoma, as well as in one patient with synovial sarcoma.

Tissue	**Archer**	**Disease**	**LB**	**Results**	
			samples	Negative	positive
Bone	EWSR1-FLI1	Ewing Sarcoma	6	1	5
Lung	EWSR1-FLI1	Ewing Sarcoma	5	4	1
Soft Tissue	EWSR1-FLI1	Ewing Sarcoma	7	5	2
Soft Tissue	SYT-SSX	Synovial Sarcoma	1		
Soft Tissue	EWSR1-FLI1	Ewing Sarcoma	11	4	7
Soft Tissue	FUS-DDIT3	Myxoid Liposarcoma	3	-	-
Soft Tissue	FUS-DDIT3	Myxoid Liposarcoma	5	5	0
unknown	EWSR1-FLI1	Ewing Sarcoma	8	7	1
CNS	ASPCR1-TFE3	Alveolar Soft Tissue Sarcoma	2	-	-
Peritoneum	EWSR1-WT1	Desmoplastic round cell tumor	2	2	0
Soft Tissue	NAB2-STAT6	Solitary fibrous tumor	10	10	0
Soft Tissue	FUS-DDIT3	Myxoid Liposarcoma	5	5	0
unknown	JAKMIP1-FUS-CREB3L2	Fibro myxoid Sarcoma			
Soft Tissue	ASPSCR1-TFE3	Alveolar soft tissue Sarcoma	4	-	-
Soft Tissue	EWSR1-FLI1	Ewing Sarcoma	8	2	6
unknown	SYT-SSX	Synovial Sarcoma	2	2	0
unknown	SYT-SSX	Synovial Sarcoma	1	0	1
unknown	SYT-SSX	Synovial Sarcoma	7	7	0
			87	***54***	***23***

Table 7: Overview of cohort results

Figure 14 shows the individual patient results in copies/ml. To arrive at this, the qubit concentration was multiplied by the elution volume of the ddCPR and divided by the isolated volume. There was a wide range of results, which were between 0.69 to 4203 copies per ml.

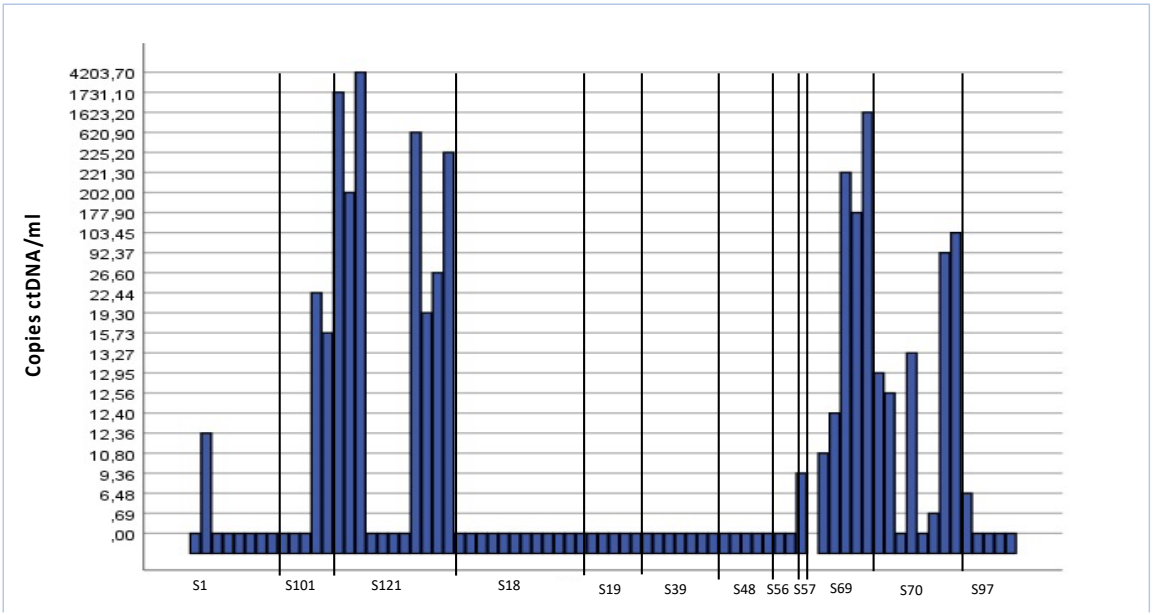


Figure 14: Individual results of the ddPCRs: in copies of ctDNA/ml

3.4.3 Evolution of copies ctDNA over time

Figure 15 shows the changing level of ctDNA from the 4 patients with Ewing sarcomas during the course of the disease. Clinical data were collected for only one patient.

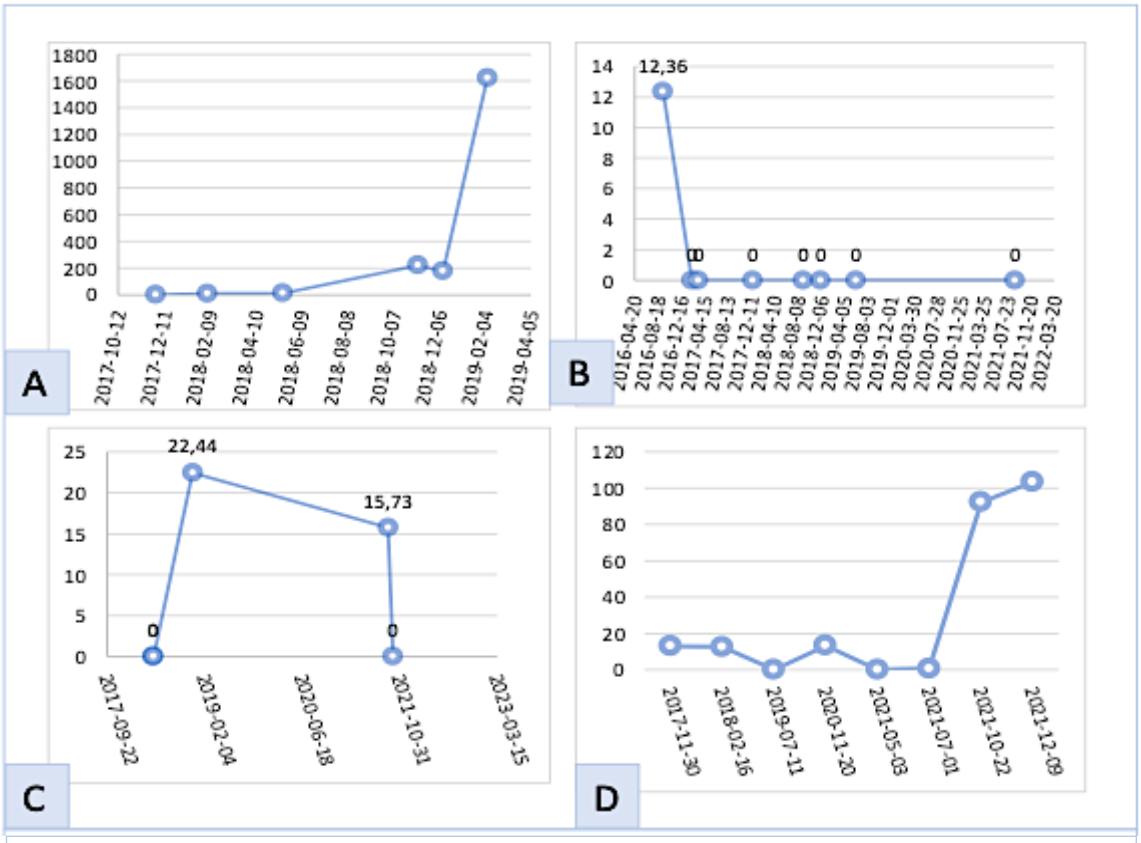


Figure 15: Disease and Treatment Monitoring of:
A: S69 B: S1 C: S101 D: S70

3.4.4 Disease and Treatment Monitoring of S121

An 18-year-old female patient was diagnosed with molecularly confirmed Ewing's sarcoma of the right thigh with involvement of the acetabulum, superior pubic ramus ossis, and soft tissues on June 12, 2018. The initial blood sample, which was taken a few days after the biopsy, showed a positive result with 1731 copies ctDNA/ml plasma. After induction chemotherapy, but before tumor resection, ctDNA molecules could already no longer be detected. Shortly after surgery, tumor DNA continued to be undetectable in the samples. During postoperative chemotherapy, the first two follow-ups remained negative. Almost half a year passed until the next liquid biopsy was performed. In this examination, 621 copies of ctDNA/ml could be detected. Radiologically, early recurrence was seen occipitally with intracranial involvement. This was followed by chemotherapy, excision of the tumor, and radiation to the metastases in the thorax. In the LB, the amount of tumor DNA had decreased significantly, but was still positive. In August 2020, PET-CT was unremarkable, but the ddPCR result still showed detectable tumor DNA, albeit at a low level. Maintenance chemotherapy followed. In January 2021, there was a further increase in ctDNA, and radiological examinations also showed metastases in the humerus and spine including bone marrow. Subsequently, another cycle of chemotherapy was administered, and the humerus and spine were irradiated accompanied by a slight decrease in tumor DNA. With further metastasis to the bone with meningeal involvement and to the lung and pleura, ctDNA also peaked at 4204 copies/ml, increasing 20-fold in just 24 days.

The isolated blood volume was 2 ml for all samples.

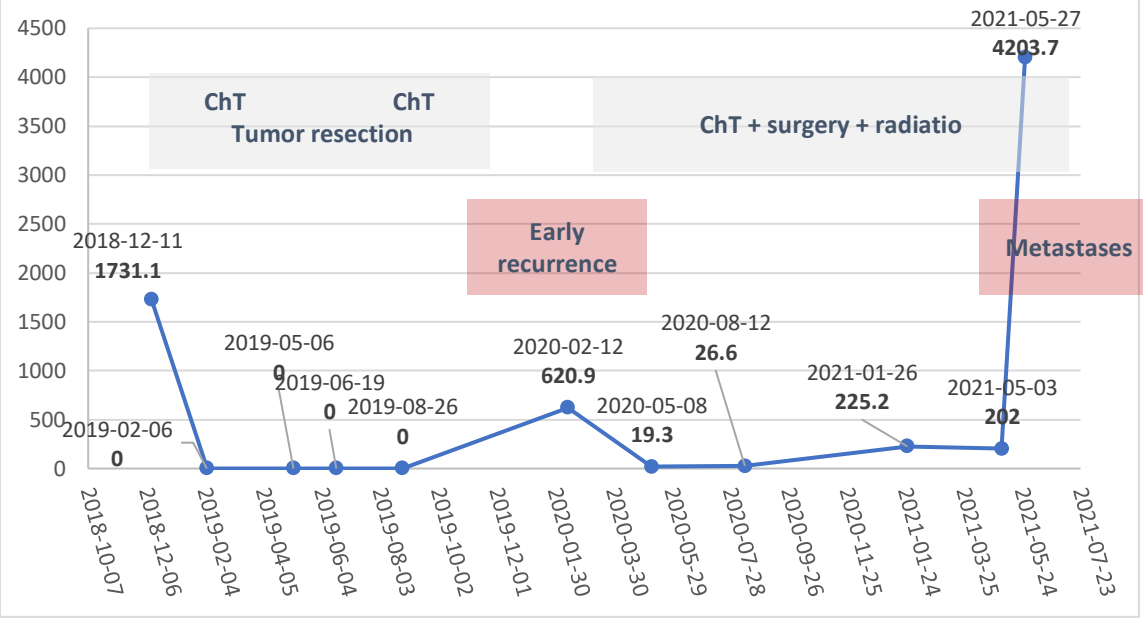


Figure 16: Progress Chart of Patient S121

4 Discussion

Survival of sarcoma patients has been stagnant for several years, unlike other cancers. There is a great need for ways and techniques to detect treatment failure early to improve survival. Liquid biopsy appears to have good potential in this regard, furthermore, it can advance personalized medicine by finding the optimal therapy for each individual patient.

Since children are frequently affected by sarcomas, Liquid Biopsy, as a non-invasive method, is of particular importance to improve the quality of life of these children.

Standardization of assays for detection and quantification of ctDNA is still pending. One question that arises is whether it is more useful to take the ctDNA molecules per ml of examined sample as a value or better the allele frequency. The amount of cfDNA in plasma depends on many factors. Besides infections, correct preanalytical handling is essential. Because if the blood is drawn too slowly, hemolysis occurs, which in turn leads to an increased number of cfDNA. It is also important to consider that radiation leads to inflammation in addition to tumor cell decay, which also leads to an increase in cfDNA. If cfDNA is increased, the proportion of ctDNA will decrease and could lead to a false assumption of a response to therapy.

Therefore, the number of molecules of ctDNA per ml seems to be the better value.

Another limitation that must be overcome is the small amount of ctDNA circulating in the blood. The mutation frequency is often <1%, implying a high wild-type DNA background. This can be overcome to some extent by more sampling and higher sensitivity of the analytical methods used. It is limited by the number of input molecules, the tumor target, and the analytical sensitivity. However, even with a near-perfect sensitivity assay, the ctDNA must be present in the sample in question. Therefore, in a disease with a low disease burden, it is reasonable to look for multiple typical mutations. However, in sarcomas, there is usually a specific translocation, and here one approach is to screen multiple replicates with a small number of molecules to detect the mutation in at least one sample, which we have done using digital droplet PCR. Further increase in sensitivity can be achieved by using different markers. One approach could be to combine liquid biopsy with radiological examination methods. In the case of undetectable ctDNA, this could minimize false negative results.

In addition to technical factors, the characteristics of the tumor also play a major role in the ability to detect ctDNA. It is believed that the amount of DNA released depends on the tumor histiotype and tumor burden. However, little is known to date about the different shedding

capacities. It is also still unclear to what extent the cfDNA concentration varies over the course of the day. If ctDNA is to be used to assess treatment response, it must be clear on what factors the release of ctDNA depends on and whether it is transferable to different tumors of the same subtype or whether certain subclones release more tumor DNA than others and whether it is also biased by blood flow and metabolic activity.

Our approach has yielded good results in all Ewing sarcoma patients and in one patient with synovial sarcoma. No ctDNA was detected in the blood of the other subtypes. However, since it worked in all Ewing sarcoma, it can be assumed that it was not due to the analytical method, but that less or no ctDNA is released by other subtypes. It seems reasonable to look for other circulating biomarkers by liquid biopsy. (23)

Due to the rarity of the various sarcoma subtypes, multi-center collaboration and sample sharing seems necessary for progress in this field. This will also facilitate the demonstration of economic benefit of Liquid Biopsy, which is essential for clinical implementation. This will require randomized trials comparing standard therapy decisions with ctDNA-based decision making.

Due to increasing technical development, further price reductions can also be expected for WGS. Automated processes will also make it increasingly easy to handle the large amounts of data generated in the process.

Pilot studies such as this one that provide proof of concept will be the starting point for demonstrating the clinical utility of ctDNA in monitoring sarcoma patients in further large prospective studies with more candidates and follow-ups.

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52. El Heliebi A. Studienprotokoll: „Pilotstudie“ - Plasma Biomarker bei Patientinnen und Patienten mit mesenchymalen Tumoren2019.

FB-OSL-04(06)
Konzeptformular für wissenschaftliche Arbeiten

(Arbeits-)Titel *Das Thema der Diplomarbeit ist einem der im Curriculum festgelegten Prüfungsfächer zu entnehmen.*	Circulating tumor DNA in sarcoma patients: liquid biopsy as a non-invasive method for treatment monitoring?
Untertitel (optional)	Development of a translocation-specific sarcoma ddPCR assay
Konzept erstellt von: Erstellt am: Revisionsdatum bei Änderungen: Version: *(Erste eingereichte Version ist „01“)* Matrikelnummer: Studienkennzahl:	Korting, Julia 06-12-2020 01 11775053
Betreuer/in: Institut/Klinik: Kontakt: *(Adresse, Telefonnummer, E-Mail)* Zweite/r Betreuer/in: Kontakt: *(Adresse, Telefonnummer, E-Mail)* MitarbeiterInnen:	Kashofer, Karl; Priv.-Doz. Mag. Mag. Dr.phil Diagnostik- und Forschungsinstitut für Pathologie Diagnostik- und Forschungszentrum für molekulare Biomedizin Neue Stiftingtalgasse 6, 8010 Graz karl.kashofer@medunigraz.at
Kernfrage und Zielsetzung *Wie lautet die Fragestellung?* *Warum ist diese Frage von Bedeutung?* *Welche Ergebnisse sind im Wesentlichen zu erwarten?* *Worin besteht der theoretische Kern der Arbeit?* Sind die Forschungsfrage und die mit dem Projekt angestrebte Zielsetzung für Männer und Frauen gleichermaßen bedeutsam? Detaillierte Angaben!	Ziel der Arbeit ist es, eine valide Labormethode zu etablieren, um mittels Liquid Biopsy bei Patienten mit translokationsassoziierten mesenchymalen Tumoren spezifisch veränderte ctDNA im Blut nachzuweisen. Sarkome bilden eine seltene und mit über 100 verschiedenen histologischen Subtypen eine sehr heterogene Gruppe von malignen Tumoren. Zwei Drittel der Tumoren weisen spezifische chromosomale Translokationen und Mutationen auf. Die Diagnose und Therapieentscheidung beruht aktuell auf Gewebebiopsien und radiologischer Bildgebung. Diese Methoden sind jedoch stark limitiert. Methoden zur Therapieüberwachung sowie zur Erkennung von frühen Rezidiven fehlen. Die Liquid Biopsy bietet mit der Analyse des Blutes auf ctDNA eine nicht-invasive Methode. Mittels standardisierter Verfahren kann auch das Ansprechen auf neue Therapieansätze in Studien miteinander verglichen werden. Die Analyse von Translokationen aus der ctDNA ist komplex, da der genaue genomische Bruchpunkt patientenspezifisch und üblicherweise nicht bekannt ist.

	Maligne mesenchymale Tumore treten mit der gleichen Inzidenz bei Männern und Frauen auf. Damit ist die Forschungsfrage für beide Geschlechter gleich relevant. In der Kohorte sind die Geschlechter annähernd gleichverteilt, somit lässt sich das Ergebnis auf Männer und Frauen gleichermaßen anwenden.
Kurzbeschreibung (max. 20 Zeilen) *Worin besteht der Neuigkeitswert?*	Das Potential der Liquid Biopsy zur frühen Erkennung von Therapieresistenzen, Rückfallen und Rezidiven wurde bereits in vielen Studien gezeigt. Über die Bedeutung der Liquid Biopsy bei Patienten mit Sarkomen ist bisher allerdings nur wenig bekannt. Ein Grund dafür ist, dass Translokationen diagnostisch über die Analyse der RNA aus dem Tumor durchgeführt werden. Diese Analyse gibt Aufschluss über die beteiligten Exons, nicht aber über den genauen genomischen Bruchpunkt. In der Liquid Biopsy wird üblicherweise DNA analysiert da sie in der Zirkulation stabiler als RNA ist. Um die Analyse der ctDNA zu ermöglichen, muss also der patientenspezifische genomische Bruchpunkt vorab bestimmt werden. In diesem Projekt werden die Bruchpunkte einer Kohorte von Sarkomen mittels Sequenzierung des gesamten Genoms (whole genome sequencing) detektiert. Diese Bruchpunkte dienen als Grundlage für die Entwicklung von hochsensitiven digital droplet PCR Assays. Damit soll die Möglichkeit für eine bessere Therapieüberwachung bei Patienten mit translokationsassoziierten Sarkomen geschaffen werden.
Methodenwahl *Welche Methoden stehen zur Beantwortung der Frage zur Verfügung? Wieso wählen Sie genau diese Methode?*	Methoden der Molekulardiagnostik von Nucleinsäuren: Next Generation Sequencing, Whole Genome Sequencing, digital PCR
Ethikkommissionsvotum *Ist ein Votum der Ethikkommission erforderlich?* *Siehe Informationsblatt „Genehmigung Ethikkommission“*	☐ Erforderlich ☐ Nicht erforderlich ☒ Bereits vorhanden
Datenerhebung (falls zutreffend) *Werden aufgrund der oben genannten Methodenwahl medizinische Daten benötigt? Wenn ja, welche?* *Mit welcher Fallzahl ist zu rechnen? Wie wurde die Fallzahl ermittelt?* *Wie ist das PatientInnenkollektiv zu beschreiben (Mindest-/Höchstalter, Geschlechtsverteilung, Begleiterkrankungen, etc.)?* *Bitte beachten Sie, dass eine Weitergabe der Daten an projektfremde Personen gemäß Datenschutzgesetz nicht zulässig ist. Das Bekanntwerden von PatientInnendaten ist durch Pseudonymisierung (Codierung mit fortlaufender Nummer) und ggf. Zugriffsbeschränkungen zu verhindern.*	Die Kohorte besteht aus 136 Patienten mit unterschiedlichen histologischen Subtypen mesenchymaler Tumore von denen ca. 35 Fälle translokationsassoziiert sind. Die benötigten Daten umfassen den histologischen Subtyp des Sarkoms und die Blutproben. Die Blutprobenentnahmen finden vor und nach der operativen Entfernung der Tumore durch die Abteilung der Orthopädie und Traumatologie der Medizinischen Universität Graz statt.
Datenauswertung *Welche Hauptzielgröße wird analysiert (z.B. Alter bei Diagnosestellung/Alter bei Operation/Diagnose, etc.)? Wie wird die Hauptzielgröße analysiert?* *Welche Nebenzielparameter sollen betrachtet werden? Mit welchen Methoden erfolgt die Auswertung?*	Die Hauptzielgröße ist der Nachweis von sarkom-spezifischen Translokationen in der ctDNA im peripheren Blut von Patienten mit Sarkomen. Es werden Blutproben vor und nach operativer Therapie analysiert. Die Analyse erfolgt mittels digital droplet PCR.
Zeitplan (grob strukturiert) *Wann wird mit der Arbeit begonnen? Wann wird ein Antrag bei der Ethikkommission gestellt, sofern ein Votum erforderlich ist?* *Welche Meilensteine wurden zwischen dem/der Studierenden und den BetreuerInnen vereinbart?* *Wann ist voraussichtlich mit der Beendigung der Arbeit zu rechnen?*	Der Beginn der Diplomarbeit findet im Dezember 2020 statt. Ein Ethikvotum ist bereits vorhanden. Als Meilensteine wurden die Literaturrecherche bis Januar 2021 vereinbart sowie die Durchführung der Laborarbeit im Februar 2021. Die Fertigstellung der Arbeit ist mit Ende des Sommersemesters 2021 geplant.

Welche formalen Schritte sind für die Umsetzung der Diplomarbeit notwendig?	
Referenzen Welche Literatur ist relevant? Gibt es Vergleichsstudien?	1. Wei J, Liu X, Li T, Xing P, Zhang C, Yang J. The new horizon of liquid biopsy in sarcoma: the potential utility of circulating tumor nucleic acids. J Cancer. 2020 Jul 9;11(18):5293-5308. doi: 10.7150/jca.42816. PMID: 32742476; PMCID: PMC7391194. 2. Mihály D, Nagy N, Papp G, Pápai Z, Sápi Z. Release of circulating tumor cells and cell-free nucleic acids is an infrequent event in synovial sarcoma: liquid biopsy analysis of 15 patients diagnosed with synovial sarcoma. Diagn Pathol. 2018 Oct 17;13(1):81. doi: 10.1186/s13000-018-0756-2. PMID: 30326929; PMCID: PMC6191904. 3. Fletcher CD, Hogendoorn P, Mertens F, Bridge J. WHO Classification of Tumours of Soft Tissue and Bone. 4th ed. Lyon, France: IARC Press; 2013. 4. Heitzer E, Haque IS, Roberts CES, Speicher MR. Current and future perspectives of liquid biopsies in genomics-driven oncology. Nat Rev Genet. 2019 Feb;20(2):71-88. doi: 10.1038/s41576-018-0071-5. PMID: 30410101. 5. Heitzer E. Circulating Tumor DNA for Modern Cancer Management. Clin Chem. 2019 Oct 31:clinchem.2019.304774. doi: 10.1373/clinchem.2019.304774. Epub ahead of print. PMID: 31672857.
Benötigte Ressourcen *Werden Geld- oder Sachmittel von Einrichtungen der Med Uni Graz benötigt?* Die Vergabe ist nur zulässig, wenn die Leiterin/der Leiter dieser Einrichtung über die beabsichtigte Vergabe informiert wurde und diese nicht binnen eines Monats untersagt hat.	Die notwendigen Mittel sind im Rahmen eines Research Grants des Zentrums für molekulare Biomedizin vorhanden.