Vasiliki Siozopoulou

New insights in the immuno- and molecular biology of soft tissue and bone tumors with therapeutic implications

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“New insights in the immuno- and molecular biology of soft tissue and bone tumors with therapeutic implications”

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LIST OF ABBREVIATIONS

ALK	Anaplastic Lymphoma Kinase
ALT	Atypical Lipomatous Tumor
APC	Antigen Presenting Cells
ARMS	Alveolar Rhabdomyosarcoma
ASPS	Alveolar Soft Part Sarcoma
CB	Core Biopsy
CBR	Clinical Benefit Rate CCS Clear Cell Sarcoma
CD	Cluster of Differentiation
CDK4	Cyclin-Dependent Kinase 4
CPS	Combined Positive Score
CR	Complete Response
CTLA-4	Cytotoxic T lymphocyte antigen-4
DC	Dendritic Cells
DDLPS	Dedifferentiated Liposarcoma
DFSP	Dermatofibrosarcoma Protuberans
DoR	Duration Of Response
DSRCT	Desmoplastic Small Round Cell Tumor
DT	Desmoid Tumors
FAP	Familial Adenomatous Polyposis
FDA	Food and Drug Administration
FFPE	Formalin fixed And Paraffin-Embedded
FISH	Fluorescence in Situ Hybridization
FNA	Fine Needs Aspiration
FNCLCC	Fédèration Nationale des Centres de Lutte Contre le Cancer
GIST	Gastrointestinal Stromal Tumor
HE	Hematoxylin and Eosin
HEV	High Endothelial Venules
HIV	Human Immunodeficiency Virus
HPF	High-Power Fields
HRMA	High-resolution melting analysis
IB	Incision biopsy
IC	Immune Checkpoint
ICB	Immune Checkpoint Blockade
ICI	Immune Checkpoint Inhibitor

IDO	Indoleamine-Pyrrole 2,3-Dioxygenase
IF	Infantile Fibrosarcoma
IHC	Immunohistochemistry
IMT	Inflammatory Myofibroblastic Tumor
irAE	Immune Related Adverse Event
KIT	Tyrosine-Protein Kinase
KS	Kaposi Sarcoma
LA	Lymphoid Aggregates
LMS	Leiomyosarcoma
LN	Lymph node
mAb	Monoclonal Antibodies
MC	Metronomic Cyclophosphamide
MDM2	Murine Double Minute
MHC	Major Histocompatibility Complex
MPNST	Malignant Peripheral Nerve Sheath Tumor
MSI	Microsatellite Instability
MSS	Microsatellite Stable
MTD	Maximum Tolerable Dose
NCI	National Cancer Institute
NGS	Next-Generation Sequencing
NK	Natural Killer
NOS	Not Otherwise Specified
NTRK	Neurotrophic Tyrosine Receptor Kinase Genes
OR	Objective Response
OS	Overall Survival
PD-1	Programmed death-1
PD-L1	Programmed death – ligand 1
PET-CT	Positron Emission Tomography–Computed Tomography
PFS	Progression Free Survival
PR	Partial Response
PTEN	Phosphatase And Tensin Homolog
RR	Response Rate
RT-PCR	Real Time Polymerase Chain Reaction
RTU	Ready-to-use
SD	Stable Disease
SIRPa	Signal-Regulatory Protein Alpha
SLO	Secondary Lymphoid Organ
SMRT	Smarca4-Malignant Rhabdoid Tumor
STS	Soft Tissue Sarcoma

T-VEC	Talimogene Laherparepvec
TAM	Tumor Associated Macrophage
Tef	Effector T Cells
TH1	T Helper Type 1
TIL	Tumor-Infiltrating Lymphocyte
TKI	Tyrosine Kinase Inhibitor
TLO	Tertiary Lymphoid Organ
TMB	Tumor Mutational Burden
TME	Tumor Microenvironment
TNF	Tumor Necrosis Factor
trAE	treatment-related Adverse Event
Tregs	Regulatory T cells
TRK	Tropomyosin Receptor Kinase
UPS	Undifferentiated Pleiomorphic Sarcoma
WDLPS	Well-Differentiated Liposarcoma

CHAPTER 1

INTRODUCTION - PART I: THE BIOLOGY OF SOFT TISSUE TUMORS

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1. Introduction

Soft tissue tumors are rare entities which result in difficulties defining the right criteria for a correct diagnosis. Although they comprise <1% of all adult malignancies and 12 percent of pediatric cancers, there exists more than 100 benign and malignant subtypes, which makes those tumors diagnostically challenging and difficult[1,2]. The role of the pathologist is in first line to differentiate malignant from benign tumors and pseudotumors. There are also entities such as solitary fibrous tumor, whose prognosis is from pathologic point of view unpredictable. If possible, subclassification of the lesion will add to a more specific and correct diagnosis. Moreover, in cases of malignancy, histologic grade is very important as it has been shown to be one of the best predictors of outcome, including both metastatic risk and disease-free survival, in adult soft tissue sarcomas[3-10].

Immunohistochemistry came to support and enhance the role of the histomorphology. Over the years this has led to a better understanding of the tumor´s nature by identifying and specifying the cell lineage of the particular lesions which resulted in further classification of the tumors. Nevertheless, no immunohistochemical staining is specific to a single lesion or cell type, therefore the need for new diagnostic strategies became necessary.

In recent years the advances in cytogenetic and molecular pathology have not only helped us understand the underlying biology of these neoplasms but have also proven to be powerful diagnostic and predictive markers. It has to be emphasized that the final diagnosis is made based not only on pathologic data as clinical and radiological information are just as important as pathology. Especially in cases of biopsy, radiology provides to the pathologist very important information about location, size, consistency, growth pattern, presence or absence of necrosis etc. A typical example is myxoid liposarcoma that almost never occurs primarily in retroperitoneum. Without knowledge of clinical and radiological imaging data, the pathologist could be make a wrong diagnosis. Decision about diagnosis and treatment choices require therefore a multidisciplinary approach.

2. *Tissue sample*

a. Adequate tissue sampling

A pathologist quote: “tissue is the issue” emphases the importance of adequate tissue sampling for a correct diagnosis. Adequate tissue means first of all representative tumor material. Many tumors are inhomogeneous, containing low and high grade areas, which means that material from different areas of the tumor is needed for the correct diagnosis but also for the correct grading of the tumor. Biopsy material containing exclusively low grade areas may create diagnostic confusion as whether the tumor is benign or malignant. These can lead to erroneous treatment choices. Necrosis is also a predominant feature of many malignant soft tissue lesions; the presence of necrotic components in the biopsy specimen could - in addition to tumor morphology - indicate malignancy. On the other hand, if the biopsy sample consists solely of necrosis, the tissue inappropriate for further assessment, as it does not allow evaluation of the cellular components. In cases of tumors treated with chemo- and/or radiotherapy the presence and percentage of necrosis indicates response to the particular therapy. Therefore, the presence of necrosis in the sample could in many cases be of diagnostic and prognostic value. Adequate tissue means also enough amount of tumor material in order to perform special techniques, such as immunohistochemistry and molecular testing, for the (sub)classification of the lesion. This underscores the need for appropriate cooperation between pathologists and radiologists for choosing the tumor areas that are best suited for sampling.

Of the techniques used for this purpose, the gold standard was open (incision) biopsy (IB). Given its high cost and complications, the need for other techniques has emerged. Nowadays, Fine Needs Aspiration (FNA) and Core Biopsy (CB) are both increasingly used for diagnostic purposes mostly for superficial masses. As it is apparent, IB provides to the pathologist the best material in terms of adequacy[11-13]. CB provides also very good material for diagnosis, but in comparison to the IB tissue samples are more often nondiagnostic. FNA has also been used for the diagnosis of soft tissue tumors. Despite the fact that from clinical and radiological point of view, it may be a preferable technique, it is not very useful for pathological diagnosis. First of all, it does not provide insights into the architecture and the growth pattern of the tumor cells, which for

soft tissue tumors is a very helpful and important diagnostic tool. Furthermore, given that the most soft tissue tumors form solid masses, the amount of tumor cells extracted with this procedure can be very limited. If the tumor contains necrotic areas that are less firm due to los of cell adhesion and presence of exudative material, it is likely that the sample will contain solely necrotic and inflammatory cells. For all these abovementioned reasons, decision on the biopsy technique in each individual should be discussed in the multidisciplinary team.

b. Treatment of the fresh material

Fresh tissue samples must be delivered to the pathology laboratory frozen and placed in transport media to keep the cells alive or, in other ways, deliver them as soon as possible to prevent autolysis of the cells. Whenever material arrives in a pathology laboratory, it is treated appropriately according to the needs of each particular case. It can range from a tiny biopsy to a large specimen. Biopsies are usually immediately fixated and proceeded for histologic examination. If the material is sufficient enough, tissue could be frozen for the tumor bank. In case of excisional biopsies, the material has first to be macroscopically examined before proceeding to fixation.

There are seven major components in processing a gross specimen:
1. Reliable and rapid transfer of the specimen from surgery to pathology
2. Accurate identification of all specimens
3. Accurate description of original specimens
4. Accurate description of additional specimens received from the same patient
5. Recording normal and abnormal features of the specimen including markers (e.g., sutures) which orientate the specimens
6. Special studies requested and/or needed
7. The location from which specific sections of tissue are taken for histologic evaluation[14].

When received, the specimen has first to be oriented following the instructions of the surgeon (and the radiologist). The surface of the specimen is often covered with special ink that is preserved after fixation in order to mark the margins of the specimen. Different color inks can be used to identify different areas if needed. When sections are made

and processed, the ink will mark the actual margin on the slide. This provides more accurate estimation of the excision margins. Both the specimen itself and the tumor have to be measured.

Another very important step of macroscopy is recognizing and describing the composition of the lesion. Tumors may show solid, myxoid, cystic, or necrotic areas that have to be macroscopically recognized and sampled for further microscopic evaluation. Those areas contain very important information regarding tumor type and grade. Whether the tumor is well demarcated or has infiltrative borders is an important diagnostic parameter. For instance, schwannomas are always very well demarcated and circumscribed, showing sometimes also a fine fibrous capsule on the surface (**figure 1**). Thus, if a neurogenic tumor shows infiltrative growth pattern, it is unlikely to be a schwannoma. On the other hand, a desmoid tumor shows a characteristic infiltrative growth pattern into the surrounding tissue (**figure 2**), making the complete excision of this otherwise benign tumor very difficult. In most centers fresh frozen material will be stored apart for the Tumor Bank. Subsequently the remaining material will be fixated, embedded in paraffin blocks which will be used for further microscopic, immunohistochemical and molecular evaluation of the tissue.

c. Fixation and its role to diagnosis

Adequate fixation of the material plays a crucial role in pathology in order to allow preservation of the cells. Different fixatives have been used such as aldehydes, mercurials, alcohols, oxidizing agents, and picrates. Formaldehyde does not harm the proteins significantly, so that antigenicity is not lost. Therefore, formaldehyde is a good fixative for immunohistochemical techniques. In pathology the most widely used are 10 % and 4 % formaldehyde solutions. The latest has the ability to penetrate the tissue at a rate of 2–4 mm in 24 h[15]. Biopsies and small specimens will be fixated immediately, while bigger specimens have to be cut into thinner slides of maximum 10 mm thickness in order for the fixative to penetrate the tissue. Fixation has to start soon (<30 min) after surgical removal of the tissue, and overfixation (>24–48 h) has to be avoided[16], since this can influence the immunoreactivity of tissue antigens[17]. Also delayed fixation is proven to influence the number of observable mitotic figures in tissues[18,19].

Once the tissue is fixated, it will be embedded in paraffin and stored in blocks, where the tissue can remain for prolonged periods.

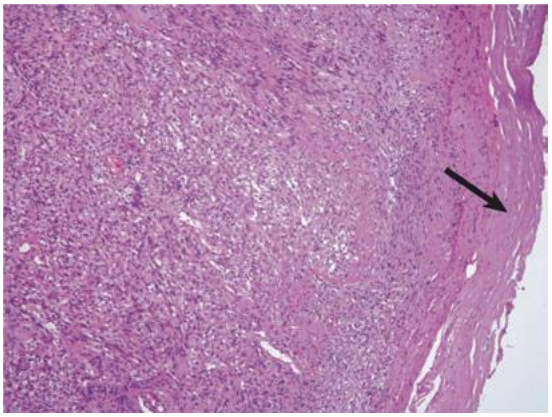

Figure 1. HE, 100x. Schwannomas are well-demarcated lesions surrounded by a fine fibrous capsule (arrow).

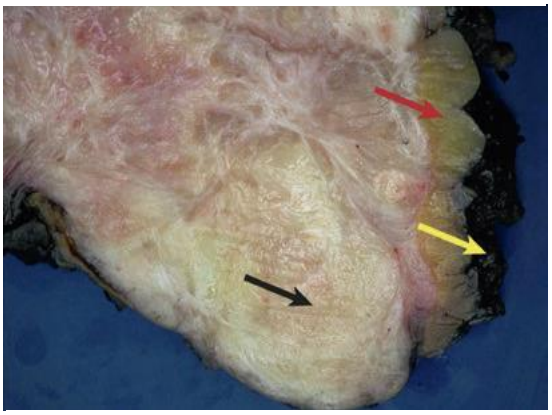

Figure 2. Macroscopic image of desmoid tumor (black arrow) infiltrating the surrounding adipose tissue (red arrow). The margins are demarcated with black ink (yellow arrow).

3. The role of frozen section in soft tissue pathology

Frozen section is one of the most difficult and demanding procedures. It requires well-trained workforce and is costly. The pathologist has to be experienced in the evaluation of soft tissue pathology and has to have a close cooperation with the surgeon and the radiologist. Based on a material which has not undergone fixation and without the ability of any

ancillary investigation, the pathologist should provide a very specific answer in a limited period of time. The surgeon has therefore first to decide if a frozen section will anyway influence the surgical procedure. If not, the procedure is not indicated[20].

The aim of a frozen section is to evaluate whether the biopsy specimen is representative of the lesion and sufficient enough for further permanent examination. An experienced pathologist can also determine the nature of the lesion, particularly to differentiate between benignity and malignancy. However, specific diagnosis and grading of the lesion depends on permanent sections and ancillary investigation may also be required for final diagnosis. Gross specimens submitted for frozen section are examined to evaluate the resection margins in order to intraoperatively determine the level of excision or amputation. Whenever a metastatic spread of the tumor is suspected radiologically, then a frozen section during surgery could determine whether the lesion is metastatic or not. As expected, this requires knowledge of clinical and radiological data, emphasizing once more the need for the collaboration of the different specialties of the multidisciplinary team.

4. Tumorbank

Nowadays, the role of targeted therapy is gaining more and more importance, and individualized therapy is one of the perspectives of cancer research. The aim is to restrict the use of drugs to those patients whose tumor expresses the target, thereby minimizing cost and morbidity. Until today macroscopy, light microscopy, and special techniques in pathology are used to categorize the tumor and determine the stage. Scientists look forward to recognize specific biomarkers for each individual patient that will predict the metastatic risk of his/her disease. The primary objective of Tumor Bank is to collect high-quality biospecimens and associated clinical data to promote scientific cancer research.

The material that is used for this purpose is fresh tissue that is not needed anymore for pathological examination, as pathology must not be compromised. The tissue is frozen in liquid nitrogen and stored in low temperature. It is very important that the tissue will be proceeded for fixation immediately after removal from the human body, because

ischemia can cause degradation of biomarkers. Nevertheless it remains controversial if a prolonged time of cold ischemia after a long warm ischemia is of scientific importance[21]. Studies have indicated that the RNA does not change rapidly after tissues are removed from thebody[22-24] and that degradation of RNA happens more extensively at the time of warm ischemia when the vascular supply to the organs is compromised. When received, the tissue will be frozen in liquid nitrogen and stored in low temperature. Optimal storage temperature is of paramount importance for a high-quality tissue. It is shown that protein activity may persist at low temperatures, even below −80 C[25-31]. Therefore, storage at temperatures at which water particles are still mobile and proteins are still active will result in degradation of the biospecimen[32].

5. The role of light microscopy in the diagnosis of soft tissue tumors

a. Histology

Histology means examination of the tissue on a thin slide stained with hematoxylin and eosin (HE) and remains the gold standard for diagnosis. Histology provides information about the morphology and the architecture of the lesion. Soft tissue tumors in most cases infiltrate diffusely, which is quite consistent for those tumors, in contrast to epithelioid neoplasms, where the cells usually form aggregates. There are of course exceptions to the rules, for instance, a biphasic synovial sarcoma contains also an epithelioid component that is composed of cell groups. On the other hand, an epithelioid neoplasm may also grow diffusely. Some tumors have a very characteristic architecture; examples are solitary fibrous tumors that show a distinctive “patternless pattern” schwannoma with also the distinctive Antoni A and Antoni B tissue, representing compact areas alternating with loosely arranged foci of spindle cells (**figure 3**) and alveolar rhabdomyosarcoma with discrete nests with discohesive cells. As expected, there are atypical forms of these tumors making their diagnosis more difficult and challenging. Of utmost importance is that lymphomas can also infiltrate diffusely, however, lymphomas have other morphological characteristics and a different clinico-radiological presentation.

Cell type can be also be recognized on histology. In general, soft tissue tumors are composed of cells that are elongated or spindled and have eosinophilic cytoplasm and indistinctive cell borders (**figure 4**). The nucleus differs from case to case with the more aggressive and malignant tumors that demonstrate more pleomorphism with enlarged nuclei, abnormal nuclear membrane, and sometimes an evident (macro)nucleolus. Multinucleation may indicate an aggressive cell type, but one has to bear in mind that giant cells are also multinucleated and that some benign tumor cells can also merged into giant forms.

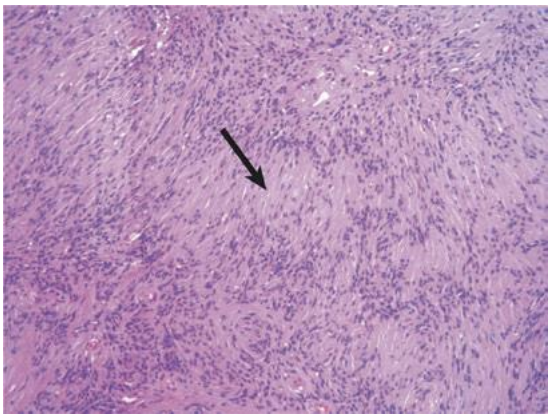

Figure 3. HE, 200x. Nuclear palisading around fibrillary process (Verocay bodies, arrow) in cellular area of a schwannoma.

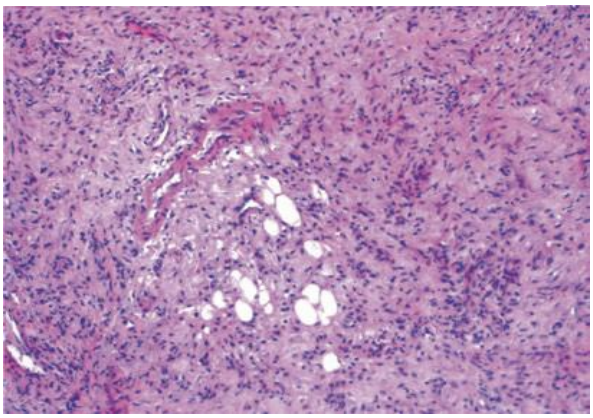

Figure 4. HE, 100x. DDLPS composed mainly of spindle cells without prominent pleomorphism. Centrally scant lipomatous component.

Desmoid tumors exhibit at the edge of the lesion multinucleated cells with strong eosinophilic cytoplasm, which is nothing more than degeneration of entrapped muscle cells when the tumor infiltrates between striated muscle (**figure 5**).

Not all soft tissue tumors are composed of spindle cells. Some neoplasms have an epithelioid morphology, such as epithelioid sarcoma, making the diagnosis more complicated.

It is evident that beyond the morphology of the tumor and the cellular composition, there are also other elements that can lead to the diagnosis. Many neoplasms show a vascular pattern that in many cases is diagnostic for this neoplasm. Thus, a "chicken wire" vascular pattern is described in myxoid lipomas and liposarcomas and a hemangiopericytoma-like pattern in solitary fibrous tumor, while schwannomas show hyalinization of the vascular wall.

Histology serves not only in tissue-specific diagnosis but plays also an important role in grading the tumor by recognizing and counting, for instance, the mitotic activity of the cells as well as identifying necrotic areas (**figure 6**). On histology one can also estimate the resection margins of a specimen.

Special histochemical techniques can be used to reveal material in the cell itself as well as in the tumor background (e.g., mucin). In alveolar soft part sarcoma (**figure 7**), the cells contain rod-shaped intracytoplasmic crystals that can be easily demonstrated with PAS staining (**figure 8**), which is pathognomonic of the lesion.

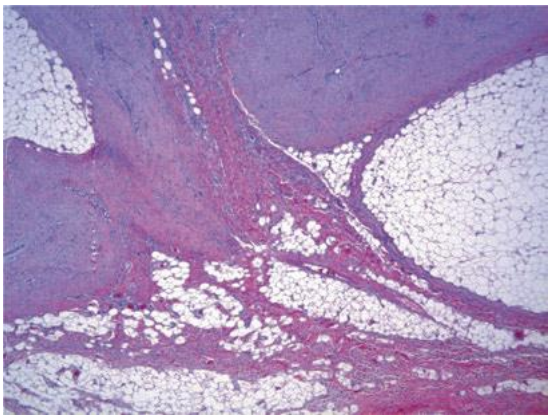

Figure 5. HE, 40x. Desmoid tumor with characteristic infiltrating growth pattern in the surrounding fibro-adipose

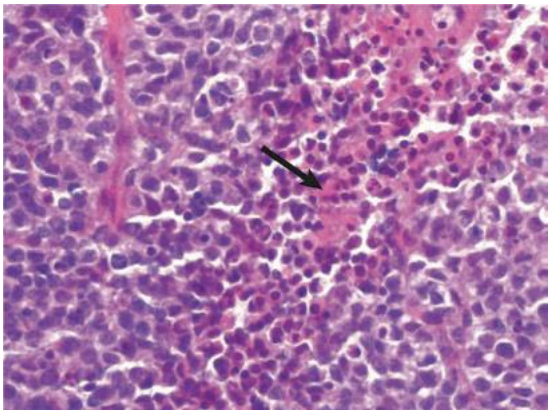

Figure 6. HE, 400x. A case of a small blue round cell tumor (in this case alveolar rhabdomyosarcoma) with a central area of necrosis (arrow).

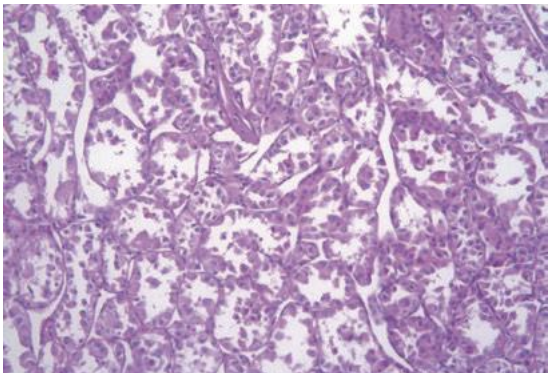

Figure 7. HE, 200x. Alveolar growth pattern in a case of alveolar soft part sarcoma.

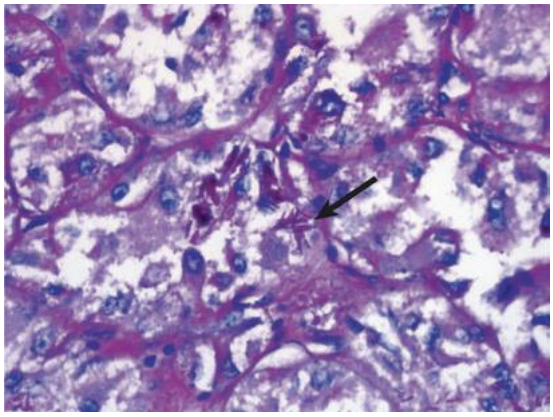

Figure 8. PAS, 400x. Alveolar soft part sarcoma with rodshaped intracytoplasmic crystals (arrow).

b. Immunohistochemistry

As it is already mentioned, morphology is the gold standard of diagnosis. For many years the diagnosis is made solely on the morphology. It is apparent that many of the subtypes that exist nowadays would not be recognized if there was no possibility of further examination. Immunohistochemistry uses antibodies that are specific to epitopes located on the cells of interest. The antibodies are not specific for malignancy with some exceptions that will be discussed further. That means antibodies indicate the type of cell differentiation in nonmalignant cells but also in tumor cells arising from this type, which will also stain for the same antibody. For instance, endothelial cells stain for CD31; thus, angiosarcomas will stain for the same marker. Combining morphology and immunohistochemistry may allow a more precise diagnosis. However, many tumors present nonspecific positive staining for markers of another differentiation lineage, and unfortunately most antibodies are not specific to one differentiation line. A typical example is S100, an antibody that is widely used in everyday practice. In soft tissue pathology, S100 is known for its strong and diffuse positivity in schwannomas (**figure 9**). It stains in neural crest-derived tissue (such as Schwann cells, melanocytes, glial cells), in adipocytes, chondrocytes, dendritic cells, Langerhans cells, macrophages, and myoepithelial and melanocytic cells. This makes things complicated because an undifferentiated tumor with S100 positivity comprises a wide differential diagnosis. Furthermore, tumor cells can lose the antibody expression that reveals the differentiation line, making

sometimes the diagnosis impossible or one of exclusion. Less than half of the malignant peripheral nerve sheath tumors (MPNSTs) stain positive for S100, and the staining is usually focal. Diffuse staining for S100 is rarely compatible with conventional MPNSTs and should raise the possibility of other tumors[2].

It is thus of paramount importance to have immunohistochemical markers that are specific and sensitive for the different lines of cell differentiation or for the different tumor types. Nowadays, new antibodies have been added in the armamentarium of a soft tissue pathologist. Some of the most important ones are antiMDM2 and CDK4. Atypical lipomatous tumor/well-differentiated liposarcoma (ALT/WDLPS) and dedifferentiated liposarcoma (DDLPS) display amplification of MDM2 and CDK4 genes that are located in chromosome 12q13-15. By immunohistochemistry, overexpression of MDM2 and CDK4 is indicated by nuclear staining for the corresponding antibodies (**figure 10**). This positivity is very sensitive for ALT/WDLPS and DDLPS and shows a strong correlation with the gene amplification status[33]. Still, as the majority of antibodies are used in pathology, they are not specific to these entities. It has been shown that intimal sarcomas of pulmonary artery show consistent genetic alteration (gains and amplifications in the 12q13-14 region) and overexpression of the MDM2 gene[34]. Very recently it has been demonstrated that almost half of the MPNSTs exhibit loss of histone H3K27 trimethylation (H3K27me3) which can be highlighted immunohistochemically by loss of nuclear staining for the corresponding antibody. H3K27me3 loss although not very sensitive is a highly specific marker for malignant peripheral nerve sheath tumor, and immunohistochemistry may be useful in differential diagnosis from other high-grade spindle cell sarcomas[35].

There are many novel antibodies with very promising results in defining diagnosis, but one has to be aware of their limitations, as most of them are not entirely specific to one entity or differentiation line. Jason Hornick et al. separated the novel antibodies into three categories: (1) lineage restricted transcription factors, (2) protein correlates of molecular alterations, and (3) diagnostic markers identified by gene expression profiling[36], emphasizing on a close correlation of the immunohistochemical profile to the cytogenetic and molecular events in the tumors (**Table 1**) (**figure**s **9**, **10**, **11** and **12**).

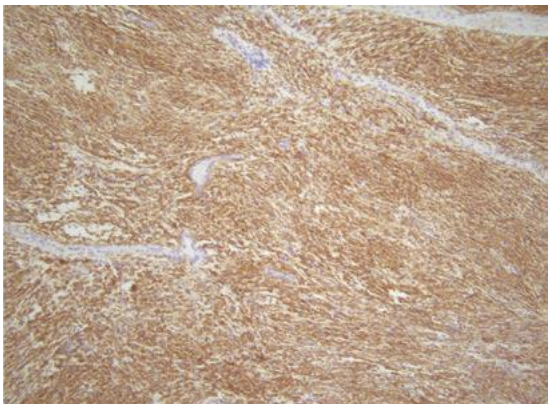

Figure 9. S100 (DAB), 100x. Strong and diffuse, nuclear, and cytoplasmic staining in all tumoral cells, characteristic of a schwannoma.

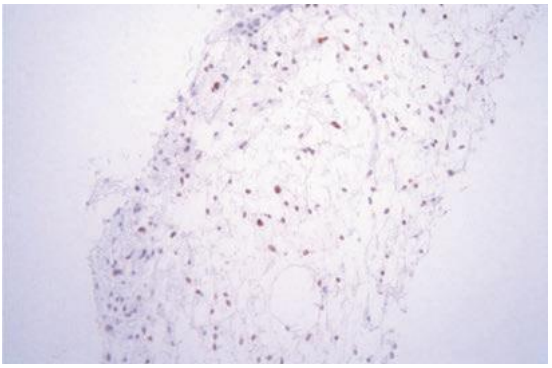

Figure 10. MDM2 (DAB) , 200x. Core biopsy with nuclear immunoreactivity of the cells for MDM2. Case of a WDLPS.

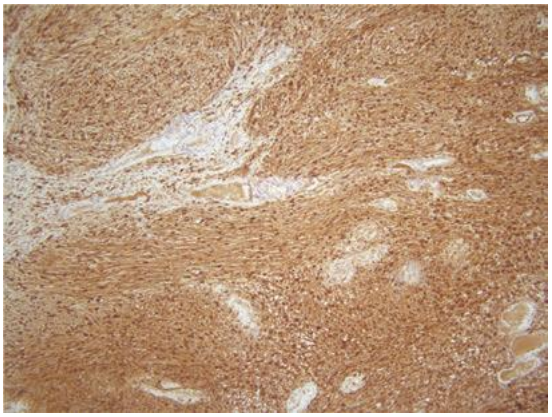

Figure 11. DOG1 (DAB), 100x. This staining is very usefull for the diagnosis of GISTs.

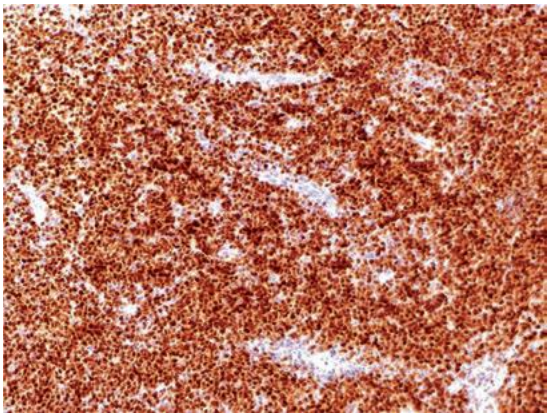

Figure 12. Myogenin (DAB), 200x. Nuclear staining in almost every tumor cell in a case of alveolar rhabdomyosarcoma.

Table 1. Immunohistochemical antibodies and their most common expression in soft tissue

Immunohistochemical marker	Soft tissue tumor - type
Myogenin, MyoD1	Tumor withskeletal muscle differentiation
ERG	Tumors with differentiation towards endothelial cells
Brachyury	Chordoma
β-catenin	Desmoid tumor
INI1(loss of expression)	Epithelioid sarcoma
STAT6	Solitary fibrous tumor
DOG1	GIST
ALK	IMT, Epithelioid FH
MDM2/CDK4	ALT-WDLPS / DDLPS
MUC4	Low grade fibromyxoid sarcoma
GLUT1	Congenital vascular malformation

Abbreviations: GIST; gastrointestinal stromal tumor, ALT ; atypical lipomatous tumor, WDLPS ; well differentiated liposarcoma, DDLPS; dedifferentiated liposarcoma, IMT; inflammatory myofibroblastic tumor, FH; fibrous histiocytoma

6. The role of genetics and molecular studies in the diagnoses of soft tissue tumors

a. Genetic alterations in soft tissue tumors

Over the last few decades, remarkable advances are made in the understanding of molecular biology of the tumors. This paved the way for improving our diagnostic effectiveness, by defining the underlying genes and the corresponding pathways involved in tumorigenesis. Already in the previous WHO edition of 2002, 11% of all benign and malignant tumors presented with a karyotypic abnormality, whereas an additional 19% was also described in the corresponding molecular findings[37].

Many soft tissue tumors harbor a recurrent chromosomal translocation caused by rearrangement of part of genes between nonhomologous chromosomes. This event lead to development of fusion genes which in turn encodes altered proteins that are oncogenic. The most extensively studied translocations relate to Ewing sarcoma. The majority of these sarcomas demonstrate rearrangement between chromosomes 11 and 22,

namely, a t(11;22)(q24;q12)[38] or a t(11;22)(q22;q12)[39] rearrangement that results in EWSR1-FLI1 or EWSR1-ERG fusion gene, respectively. The EWSR1 gene located in the q12 domain of chromosome 22 can rarely present translocations with different partner genes located at different chromosomes resulting in a number of other fusion proteins which are also described in Ewing sarcoma[40]. In addition, EWSR1 gene translocations are involved in a variety of non-Ewing sarcomas. For instance, myxoid liposarcoma can show a t(12;22) (q13;q12) reciprocal translocation resulting in a EWSR1-CHOP fusion protein[41].

Another molecular event that is observed in sarcomas is gene amplification. The most illustrative examples are ALT/WDLPS and DDLPS. These entities are characterized by amplified sequences in the region q14-15 of chromosome 12 where are located the murine double minute (MDM2) and the cyclin-dependent kinase 4 (CDK4) genes. Co-amplification of those genes represents the hallmark for ALT/WDLPS and DDLPS[42], although recently it has also been described in other entities as well, such as intimal sarcoma to name one[43].

A gene mutation is a permanent alteration in the DNA sequence that makes up a gene. It can affect a single base pair or larger segments of the DNA band. Gastrointestinal stromal tumor (GIST) is a mesenchymal neoplasm that is believed to arise from or is differentiated toward interstitial cell of Cajal. It is proven that these neoplasms can harbor a somatic oncogenetically activating mutation. The vast majority demonstrate KIT (which is a proto-oncogene receptor tyrosine kinase) mutations on exon 11. This can be an insertion, a deletion, or a missense mutation. After this initial discovery, three other less frequent hot spots came into light, regarding exons 9, 13, and 17[44]. PDGFRA gene encodes platelet-derived growth factor receptor A. A small percentage of GIST that does not show KIT mutations can demonstrate a PDGFRA mutation. The exons involved in this case are 12, 14, and 18 with the last being the most common one[44]. Both KIT and PDGFRA are driver mutations and are presumed to be the initiating oncogenic event. These mutations are mutually exclusive, namely, when one happens, the other is not present. The overall mutation frequency for KIT and PDGFA is 86% with 14% being wild type. Nowadays it is known that many of those wild-type GISTs contain another mutation, with most extensively described the BRAF V600E mutation[44].

From a genetical point of view, sarcomas are divided in two groups. First is the group with a simple karyotype that presents one main genetic alteration. As previously described, this alteration can be either a somatic mutation, or a gene amplification, or a recurrent translocation, or even an intergene deletion. Second are those presenting with more complex karyotypes[37,45-48]. This last group represents almost two thirds of soft tissue sarcomas and shows aberrant chromosomal events but no recurrent reciprocal translocations. Most demonstrate mutations involving p53 gene and retinoblastoma gene[46-48].

b. Molecular techniques in clinical practice

Three main technical approaches are nowadays used in clinical practice regarding soft tissue tumors. Those are conventional cytogenetic analysis, fluorescence in situ hybridization (FISH) and Reverse Transcription Polymerase Chain Reaction (RT-PCR)[37,47].

Conventional cytogenetic analysis or simply karyotyping aims to detect numerical and/or large structural chromosome abnormalities in metaphase cells. Both primary and secondary changes can be identified. This study is limited to fresh, not fixated, sterile tumor tissue and demands special culture for the tumor cells to grow.

FISH detects the presence, absence, relative positioning, and/or the copy number of specific DNA segments by fluorescence microscopy. It can be performed on either fresh or formalin-fixed and paraffin-embedded (FFPE) tissue. In contrast to the karyotyping, this method requires the knowledge of the specific target examined. FISH testing uses dual-color/fusion or dual color/break-apart probes to detect specific rearrangements, involving a variety of translocation events (**figure 13**).

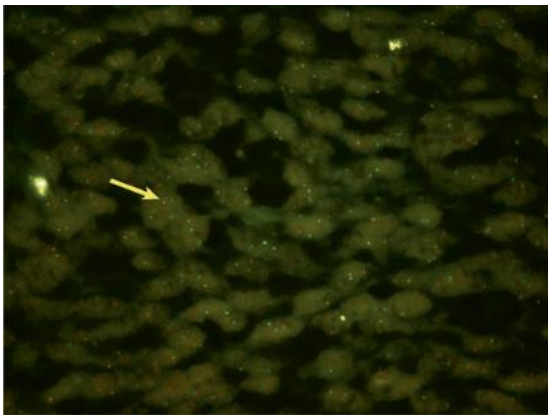

Figure 13. FISH by synovial sarcoma. SYT break-apart probe, showing splitting of red and green signals (By Dr. Karen Zwaenepoel, biomedical scientist, department of pathology, University Hospital of Antwerp)

There are also the locus-specific probes coupled with a control probe usually pointing the centromere of the chromosome and aim to identify gene amplifications or losses. RT-PCR uses specific primers to copy or amplify a small section of a DNA or RNA sequence. It is quick and simple and can be performed on either snap-frozen or FFPE material. Its role in soft tissue pathology is to identify chimeric or fusion genes as well as oncogenic mutations. Next-generation sequencing (NGS) takes nowadays more and more part in molecular biology of human tissue. It is a high-throughput DNA sequencing technique that aims to investigate simultaneously in the same specimen a large number of mutation genes that are proven to cause tumorigenesis. It can be performed on FFPE tissue which makes this technique even more applicable. The advantage of this technique is that different genetic aberrations can be tested simultaneously. This technique requires highquality DNA/RNA.

c. Role of Molecular Biology in Soft Tissue Tumors

i.Diagnosis

The ultimate goal of the pathologist is to give the lesion a name. This happens through three main steps. The first step consists of seeing and recognizing. That means for an experienced pathologist to recognize immediately the morphology that is unique for the lesion and allows him (or her) to classify it as such. Secondly, when the morphology is not typical enough and creates diagnostic doubts to the pathologist, the use

of immunohistochemistry is mandatory. In any case a confident diagnosis is made by correlating pathological findings with the clinical and radiological image. Yet for a relative large amount of entities, the diagnosis remains very difficult, as they present with similar morphological and immunohistochemical characteristics. For instance, most soft tissue neoplasms present with a spindle cell morphology. A monophasic synovial sarcoma is thus a spindle cell neoplasm with no striking pleomorphism and with no specific immunohistochemical profile. It can be positive for CD99 which is also expressed in a large variety of other tumors. The expression of EMA and cytokeratin can be very limited or even absent in cases of monophasic synovial sarcoma. Given the right clinical and radiological information, one can suspect the diagnosis. The identification of the molecular event that happens in these tumors is often necessary for establishing the definitive diagnosis: in this case a t(X;18) rearrangement results in a SS18-SSX fusion gene that is highly specific and sensitive for synovial sarcomas. Moreover, molecular analysis can help to differentiate between benign and not benign entities. For instance, a spindle cell/pleomorphic lipoma can sometimes be confused with an ALT/ WDLPS or rarely with DDLPS when the lipomatous component is very scant. Co-amplification of the MDM2 and CDK4 genes is observed only in ALT/WDLPS and DDLPS, and in this way, a pleomorphic lipoma can be excluded. Low-grade fibromyxoid sarcoma is a morphological indolent entity consisting of slender, spindle cells with alternating hypocellular areas, resembling perineurioma. More interestingly these tumors can express EMA, as do perineuriomas. In the past low-grade fibromyxoid sarcomas were often misdiagnosed as perineuriomas or other benign fibrous or neural proliferations. We now know that those are malignant tumors that eventually can metastasize even after decade(s). RT-PCR or FISH for detection of FUS-CREB3L2 fusion can be useful to distinguish those entities[47].

ii.Prognosis

In addition, the knowledge of the genetic profile of a tumor can be of prognostic value. Alveolar rhabdomyosarcoma (ARMS) is an aggressive malignant neoplasm of the childhood. There are two main fusion proteins described in ARMS, both involving FOXO1 on chromosome 13, either with PAX3 on chromosome 2 or with PAX7 on

chromosome 1. The presence of a PAX3-FOXO1 fusion was associated with a worse prognosis compared to the PAX7-FOXO1 fusion[37,45,47,49,50]. Moreover, ARMS without a PAX3- or PAX7-FOXO1 fusion had a more favorable outcome, similar to embryonal rhabdomyosarcomas when given therapy designed for intermediate-risk rhabdbdomyosarcomas[37,51,52].

In Ewing sarcomas type 1 EWS-FLI1 fusion, which is the most common fusion type, is a positive predictor of overall survival compared to other fusion types[47,48,53].

iii.Treatment

Treatment of soft tissue tumors is a difficult task that requires a multidisciplinary approach with surgeons, radiologists, oncologists, and pathologists. The gold standard of treatment is surgery for the excision of the lesion. Yet, in many cases of high-grade or unrespectable tumors, a combination with chemotherapy and/or radiotherapy must be considered. Advances in the understanding of molecular mechanisms of soft tissue tumors can lead in the implementation of targeted therapy. Imatinib mesylate, was originally developed to target BCR-ABL kinase in chronic myelogenous leukemia. Imatinib can also inhibit a small number of related kinases, such as KIT, PDGFRA, and PDGFB. The identification of KIT and PDGFA mutations in the majority of GISTs makes these tumors a good candidate for this therapy[44].

In addition, dermatofibrosarcoma protuberans (DFSPs) show a t(17;22)(q22;q13) translocation. This generates a COL1A1-PDGFB fusion gene which results in the constitutional upregulation of PDGFB expression. DFSPs have hence been treated with imatinib with promising results[54] (**Table 2**).

iv.Summary

Molecular analysis opens a new way in diagnosing and classifying soft tissue tumors. The impact of molecular characterization of these tumors is still growing and is now an integrated part in the diagnosis of these tumors.

7. Pathology grading and staging

One of the most important objectives of pathology in addition to tissue diagnosis or characterization is to determine the tumors aggressiveness, namely, its metastatic potential, as well as to provide information about the most effective treatment for the patient. It is shown that grade and stage are better predictors of outcome than histologic type.

a. Common Used Grading Classifications

There are two main grading systems, provided by the National Cancer Institute (NCI)[55] and the Fédèration Nationale des Centres de Lutte Contre le Cancer (FNCLCC)[56]. NCI is based on histologic type, location, and necrosis, while FNCLCC is based on histologic type/ differentiation, necrosis, and mitotic activity. Both are three-grade system, where grade 1 represents the most and grade 3 the least favorable edge of the scale. In a comparative study, the FNCLCC system showed slightly increased ability to predict distant metastasis development and tumor mortality[57]. The weakness of this system is the definition of differentiation, since some tumors have no normal tissue counterpart, e.g., epithelioid sarcoma[58]. Mitotic activity is counted in ten consecutive high-power fields (HPF) in a hot spot area. Hypocellular areas as well as areas with necrosis and ulceration should be avoided[3] as the number of mitosis in those last ones can be misleadingly increased. Fixation status as previously mentioned can also affect the number of mitoses. Necrosis should be evaluated macroscopically and microscopically. One has to bear in mind that previous chemotherapy or radiation therapy can influence grading by reducing the amount of mitoses and increasing necrosis. Grading should not be applied to tumors of intermediate malignancy. Furthermore, there are some rare sarcomas, such as epithelioid sarcoma, clear cell sarcoma, and alveolar soft part sarcoma, that are very difficult to grade. It seems that for those types, the histologic classification plays a more important role than grade in prognosis.

Table 2. Current genetics in soft tissue tumors.

Soft tissue tumor	Most common genetic alteration	Gene(s) involved
ALT/WDLP Dedifferentiated liposarcoma	Amplified sequencing in the 12q14-15 region	Amplification of MDM2 and CDK4 genes
Myxoid liposarcoma	t(12;16)(q13;p11)	FUS-DDIT3 fusion
Nodular fasciitis	t(17;22)(p13;q13)	MYH9-USP6 fusion
Desmoid-type fibromatosis	Sporadic lesions: mutations in the gene encoding β-catenin (CTNNB1) Gardner-type FAP: mutations of the APC gene	
DFSP	t(17;22)(q22;q13)	COL1A1-PDGFB fusion
IMT	2p23 rearrangements with different partners	ALK gene
Low-grade fibromyxoid sarcoma	t(7;16)(q33;p11)	FUS-CREB3L2 fusion
Infantile fibrosarcoma	t(12;15)(p13;q25)	ETV6-NTRK3 fusion
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14) t(1;13)(p36;q14)	PAX3-FOXO1A fusion PAX7-FOXO1A fusion
Epithelioid haemangioendothelioma	t(1;3)(p36;q25)	WWTR1-CAMTA1 fusion
Gastrointestinal Stromal Tumor	Mutations in the KIT and PDGFRA genes	
Neurofibroma MPNST	In patients with NF1: 17q11.2 region	Germline alterations of the NF1 gene
Angiomatoid fibrous histiocytoma	t(12;16)(q13;p11)	FUS-ATF1 fusion
Myoepithemioma	t(6;22)(p21;q12) t(1;22)(q23;q12)	EWSR1-POU5F1 fusion EWSR1-PBX1 fusion
Synovial sarcoma	t(X;18)(p11:q11)	SS18-SSX(1,2 or 4)

Alveolar soft part sarcoma	t(X;17)(p11;q25)	TFE3-ASPL fusion
Clear cell sarcoma of soft tissue	t(12;22)(q13;q12)	EWSR1-ATF1 fusion
Extrasceletal myxoid chondrosarcoma	t(9;22)(q22;q12)	EWSR1-NR4A3 fusion
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	EWSR1-WT1 fusion
Intimal sarcoma	amplified sequencing in the 12q12-15 region	amplification of MDM2 and CDK4 genes
Ewing´s sarcoma/ PNET	t(11;22)(q24;q12) t(21;22)(q22;q12)	EWSR1-FLI1 fusion EWSR1-ERG fusion

Abbreviations: ALT; atypical lipomatous tumor, DFSP; Dermatofibrosarcoma protuberans, FAP; familial adenomatous polyposis, IMT ; Inflammatory myofibroblastic tumor, MPNST; Malignant peripheral nerve sheath tumor, PNET; primitive neuroectodermal tumor, WDLPS; well differentiated liposarcoma

The same applies to pediatric sarcomas, namely, rhabdomyosarcomas and Ewing/PNET as the number of mitosis in those last ones can be misleadingly increased. Fixation status as previously mentioned can also affect the number of mitoses. Necrosis should be evaluated macroscopically and microscopically. One has to bear in mind that previous chemotherapy or radiation therapy can influence grading by reducing the amount of mitoses and increasing necrosis. Grading should not be applied to tumors of intermediate malignancy. Furthermore, there are some rare sarcomas, such as epithelioid sarcoma, clear cell sarcoma, and alveolar soft part sarcoma, that are very difficult to grade. It seems that for those types, the histologic classification plays a more important role than grade in prognosis. The same applies to pediatric sarcomas, namely, rhabdomyosarcomas and Ewing/PNET tumorstumors[4,58]. Especially for rhabdomyosarcomas, subclassification provides also prognostic information, as botryoid and spindle cell/sclerosing show better clinical outcome than alveolar rhabdomyosarcoma. The application of grade on core needle biopsies has been an issue of controversy over the years. With the universal use of core needle biopsy, pathologists are more and more asked to apply a grading system on a

restricted amount of material. Although studies have shown that Tru-cut biopsy is sensitive and sensitive in subtyping and grading soft tissue sarcomas[59], this of course depends on how representative the tissue sample is. Given that the same tumor can show different degrees of differentiation ranging from low to high grade, it is obvious that only high-grade tumors can be successfully graded. A seemingly histologically low-grade tumor can radiologically show high-grade features. Moreover, some tumors may show high mitotic rate indicating malignancy but are otherwise benign in nature (e.g., nodular fasciitis) and therefore excluded from grading. Like previously mentioned, the final report depends largely on correlation with the clinical and imaging data, and multidisciplinary approach is therefore recommended in this regard (**Table 3**).

Table 3. FNCLCC Grading System: Definition of Parameters.

Tumor differentiation
Score 1: Sarcomas closely resembling normal adult mesenchymal tissue (eg, well-differentiated liposarcoma)
Score 2: Sarcomas for which histologic typing is certain (eg, myxoid liposarcoma)
Score 3: Embryonal and undifferentiated sarcomas, sarcomas of doubtful type, synovial sarcomas, osteosarcomas, PNET
Mitotic count
Score 1: 0–9 mitoses per 10 HPF
Score 2: 10–19 mitoses per 10 HPF
Score 3: ≥20 mitoses per 10 HPF
Tumor necrosis
Score 0: No necrosis
Score 1: <50% tumor necrosis
Score 2: ≥50% tumor necrosis
Histologic grade
Grade 1: Total score 2, 3
Grade 2: Total score 4, 5
Grade 3: Total score 6, 7, 8

Abbreviations: FNCLCC; french federation of cancer centers sarcoma group, HPF; high power field

b. Staging

The staging system that is widely used is the TNM classification. The letter “T” refers to the characteristics of the tumor, “N” stands for the

lymph nodes status, and “M” stands for the presence or absence of metastasis. The “T” category includes the size and the depth of the lesion. The cutoff for size is the 5 cm maximum diameter of the lesion. Tumors that are 5 cm or less are classified as T1, while tumors more than 5 cm are classified as T2. T1 and T2 are subdivided into T1a and T2a for superficial located tumors and into T1b and T2b for deep-seated tumors. The anatomical margin between superficial and deep tumors is the superficial fascia. One can use also the prefixes “m,” “r,” and “y.” “m” is applied for multiple tumors of the same type, “r” for recurrent, and “y” for tumors that have previously been treated.

8. Key points

It is important for the pathologist to describe all the parameters contributing to the correct diagnosis, such as microscopical (e.g. size) and microscopical features (e.g. mitotic activity, necrosis if present) of the tumor and ancillary techniques (immunohistochemistry, molecular testing). Depending on the quality and the amount of the material, the histologic type, subtype and grade (in case of malignancy) should be mentioned in the report. A multidisciplinary cooperation is needed for obtaining correct tissue samples, grading and characterization of soft tissue tumors. In small biopsies in which the material is not representative, the pathologist has to make clear that no diagnosis can be achieved and ask for more tissue. This may avoid diagnostic errors that might be harmful for the treatment of the patient.

The resection margins have to be described in large specimens. If not possible to estimate, this has to be discussed with other members of the multidisciplinary team.

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CHAPTER 1

INTRODUCTION - PART II : THE BIOLOGY OF BONE TUMORS

1. Introduction

Abnormal (neoplastic) growth within a bone results in bone tumors. The World Health Organization (WHO) of soft tissue and bone tumors[1] divides bone tumors into different categories with regard tissue can either be benign or malignant. Benign tumors are more common than malignant ones[2]. Bone tumors can arise anywhere in the human body but are most commonly seen in bones of the pelvis or in the long bones of the extremities[3].

The latest WHO classifies bone tumors according to the biological potential, into: the benign, the intermediate / locally aggressive, the intermediate / rarely metastatic and the malignant category. Benign bone tumors do not have the capacity to spread. They can be treated with surgery and if completely excised, they do not recur. In the locally aggressive category are tumors that can disrupt the bone and infiltrate the surrounding tissue. They usually relapse locally. There is no apparent metastatic potential and wide local excision is recommended for negative margins. The rarely metastasizing intermediate tumors are locally aggressive, while their metastatic potential is estimated to be less than 2%. The metastatic risk is not correlated with lesion morphology. The metastatic potential of the malignant tumors exceeds 20%. Even tumors with a lower metastatic potential can acquire high-grade features. The main location of the most frequent bone tumors is depicted in **figure 1**.

Among the benign category, non-ossifying fibroma is the most common tumor. Cartilaginous lesions, like enchondroma, are also among the most frequent[4]. Although benign neoplasms do not show metastatic capacity, they can cause symptoms such as pain or even give rise to fractures, hence treatment may be required. The standard treatment of choice for this category is surgery.

Malignant bone tumors, also referred to as bone sarcomas, are rare entities. They constitute less than 0,2% of all malignant tumors[5]. They can be divided into primary and secondary tumors[2]. This is especially significant because metastatic bone disease is more common than primary[6]. The tumor types that have a predilection for bone metastasis are kidney, breast and prostate carcinomas.

The most important categories of bone sarcomas are chondrosarcoma, osteosarcoma and Ewing sarcomas. Chondrosarcoma arises from cartilaginous tissue that covers the end of the bones and lines the joints. They can arise de novo or in a pre-existing benign chondrogenic skeletal lesion[1]. Chondrosarcoma occurs mainly in older patients and the risk of developing the tumor increases with age[7]. Osteosarcoma on the other hand is a tumor of younger ages, mostly seen in children, and young adults[8]. It arises from bone-forming cells. Osteosarcoma has been linked to radiation, however a direct cause has not been identified. People with Paget disease have also an increased risk of developing osteosarcoma[2]. Ewing sarcoma is an aggressive tumor belonging to the small blue round cell tumors and arises primarily in the bone but also in the soft tissues[1]. The tumor affects mostly young people and can also be seen in children.

There are many other bone sarcomas, like fibrosarcoma or angiosarcoma of the bone, chordoma etc, but those neoplasms are exceedingly rare. As already mentioned, neoplasm of the intermediate category can occasionally display an aggressive behavior [9]. Giant cell tumor of bone for instance, which typically occurs in young and middle-aged adults, is a locally aggressive neoplasm, causing bone. In rare cases they can give rise to metastatic disease, often to the lungs[2].

The treatment of bone sarcomas depends on the type, the grade, the stage as well as the location. Surgery remains the gold standard for localized and low-grade tumors[2]. Osteosarcoma and Ewing sarcoma are sensitive in treatment with chemotherapeutic agents, hence chemotherapy can be used in these cases in a neo-adjuvant setting[3,10]. In cases of uncontrolled disease, radiation can play a role in neo-adjuvant or adjuvant application, alone or in combination with chemotherapy[3,10]. On the other hand, nor chemotherapy either radiation are traditionally used for local or distant control of chondrosarcomas[3,11].

The prognosis of bone tumors is also depended on the tumor type, as well as the stage of the disease. In general, the overall survival rate ranges from 60% to 80%, while in metastatic disease, the rate drops to nearly 15%[12].

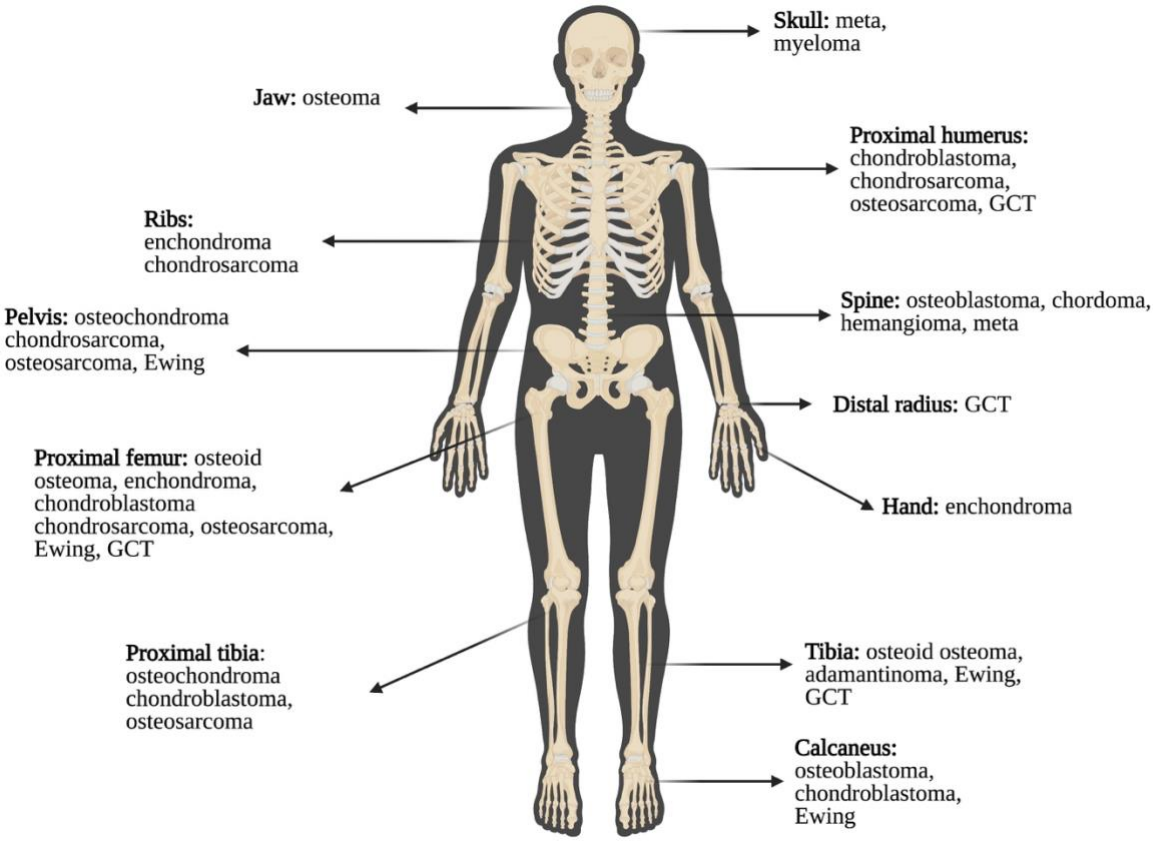

Figure 1. Distribution of different bone tumor types in the skeleton.
Abbreviations: meta; metastasis, GCT; giant cell tumor.
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2. The role of the pathologist in the diagnosis of bone tumors

The diagnosis of bone tumors can be very challenging for the pathologist. This is mainly due to their rarity in combination with the many different subtypes, that makes diagnostic criteria difficult to access. Still classification of the bone tumors is very important, not only for giving the patient the opportunity for an effective treatment, but furthermore for assessing reproducible diagnostic criteria, for better determinable biological potential, for understanding the intrinsic biology, and also because it reflects the conceptual evolution. Patients with a bone tumor should be discussed in a multidisciplinary team meeting, wherein all disciplines involved in the diagnosis and the treatment take part[10]. It is recommended that staging investigation takes place prior to biopsy for two important reasons. Firstly, biopsy can lead to reactive tissue changes which will make radiological diagnosis difficult. Second, radiography is more specific in the initial diagnosis of

bone tumors[13] and in some instances pathological diagnosis is impossible without radiological correlation[14].

Morphology is the cornerstone for the histological diagnosis and to date the gold standard. In few instances, immunohistochemistry can reveal the differentiation line of the lesion. As for instance S100 protein that indicate cartilaginous differentiation[15]. In general, and in contrast to soft tissue tumors, immunohistochemistry has limited value in differentiating bone tumors. However, unraveling the molecular pathways of many bone tumors has led to investigation of immunohistochemical surrogates for these molecular alterations[16] (**table 1**). Of note, most of these immunohistochemical markers are not pathognomonic for each separate sarcoma but can also be found in other sarcomatous or non-sarcomatous tumors.

In most tumor types, the diagnosis is followed by the grading. As already mentioned in previous section, grade is the most important prognostic factor for soft tissue sarcomas. However, this is not the case for bone sarcomas. Although guidelines for grading and reporting of bone tumors exist, they are not generally used[1,17-19]. This is because these tumors are very heterogenous and histological subtype determines the clinical behavior thus also the grade[1].

3. Molecular classification of bone tumors

Recent advances in molecular techniques can contribute to the diagnosis and accurate classification of bone tumors. Like soft tissue tumors, bone tumors are divided in two major categories: (1) those with a simple karyotype, where a single genetic alteration contributes the driver oncogenic mechanisms and (2) those with a complex karyotype, with no specific multiple genetical alterations[20]. Examples of the latest category are chondrosarcomas and osteosarcomas.

In **table 1** we summarize the most important molecular alterations that are described in bone tumors and the immunohistochemical surrogates that correlate in each case to the molecular alteration. These molecular alterations have clarified the genetic landscape for many entities. The same morphological picture has now been attributed to different entities with different clinical behavior. For instance, round cell tumors that

morphologically resemble Ewing sarcomas, have proven to harbor other rearrangements than those described in classical Ewing sarcomas, such us CIC or BCOR rearrengements[21-23]. Tumors with these genetic alterations respond different to the classical Ewing treatment protocols and have usually a more aggressive course. Therefore, systematic recognition of these tumors will help to standardize the diagnostic criteria and establish effective treatment options.

4. Conclusions

Bone tumors are rare entities that are difficult to diagnose. The diagnosis relays mainly on the histomorphology in correlation with the clinical and radiological image. Molecular techniques have helped to understand the biology of many tumor types and to recognize novel entities. Still, the treatment options for the malignant bone tumors remains limited, while the survival rates for metastatic disease do not exceed 15%-20%. It is apparent that insights for new therapeutic strategies have to be investigated.

Table1: Molecular mechanisms and immunohistochemical surrogates in the most important histological sarcoma types.

Mechanism	**Molecular Alteration**	**IHC marker**	**Tumor type**
Gene rearrangements	EWSR-FLI1 EWSR-ERG EWRS-ETV1 FUS-FEV FUS-ERG	NKX2.2	Ewing Sarcoma
	CIC-NUTM1 CIC-FOXO4 CIC-LEUTX CIC-DUX4	WT1 and ETV4	CIC-rearranged round cell sarcomas
	FOS-various fusion partners	FOS	Osteoblastoma
			Osteoid osteoma
	BCOR-CCNB3 BCOR-MAML3 ZC3H-BCOR	BCOR	BCOR-rearranged round cell sarcomas
	EWSR1-SP3 EWSR1-SMARCA5 EWSR1-NFATc2 EWSR1-PATZ1		Round cell sarcoma with EWS-non-ETS fusion

Mutation	H3F3B	G34W	Giant cell tumor
		K36M	Chodroblastoma
	IDH1/IDH2 loss of function	IDH1	Enchondroma
	GNAS		Fibrous dysplasia
Amplification	12q13-15	MDM2 / CDK4	Low grade juctacortical osteosarcoma

Abbreviations: IHC, immunohistochemical

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CHAPTER 2

RATIONALE AND AIMS OF THE STUDY

1. Rationale

Soft tissue and bone tumors are rare entities. This results in difficulties to identify criteria for their diagnosis. As we already discussed in previous sections (chapter 1, part I and II), advantages in molecular profiling techniques led to a better clarifying and understanding of the nature of these tumors, which could help in identifying more effective targeted therapies. However, very specific types of mesenchymal tumors can be targeted with biology-driven therapies, while chemotherapy is still the golden standard for the majority of mesenchymal malignancies with an aggressive behavior.

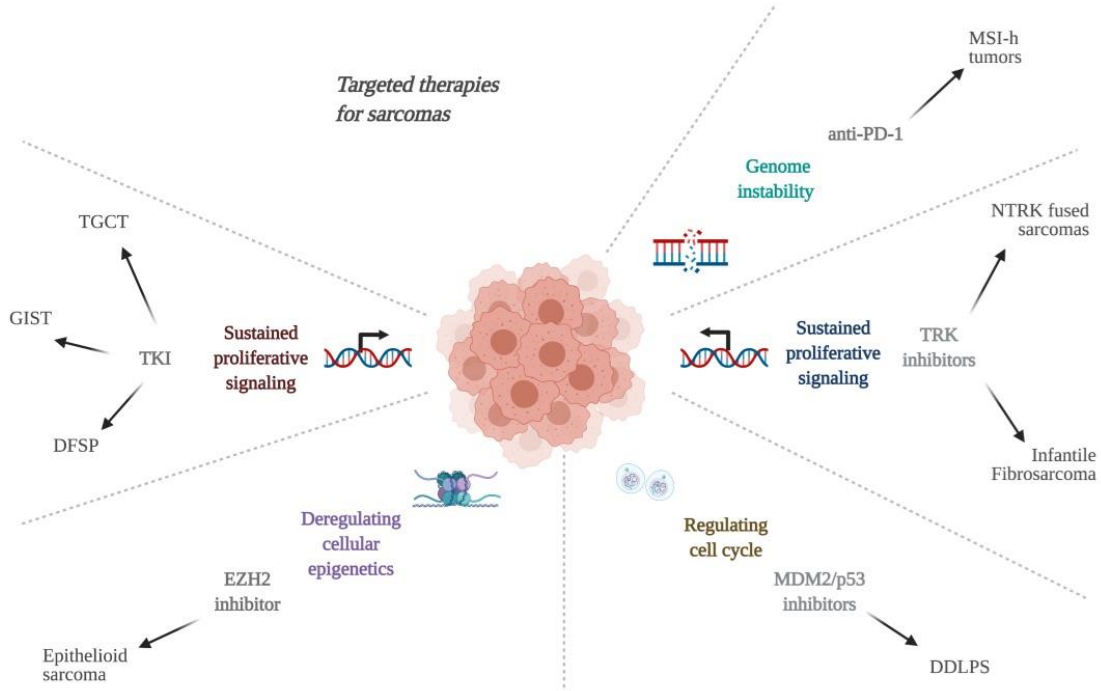

Figure 1. Schematic representation of targeted therapies used for specific sarcoma types.
Abbreviations: PD; programmed death, MSI; microsatellite instability, TRK; tyrosine receptor kinase, NTRK; neurotrophic TRK, MDM2; mouse double minute 2, p53; protein 53, DDLPS; dedifferentiated liposarcoma, EZH2; enhancer of zeste homolog 2; TKI; tyrosine kinase inhibitor, TGCT; tenosynovial giant cell tumor, GIST; gastrointestinal stromal tumor, DFSP; dermatofibrosarcoma protuberans
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Immunotherapy is one of the main breakthroughs of the last decade. It uses the patient's immune system to selectively target tumor cells. However, tumors can develop defense mechanisms, for instance by producing specific proteins, such as immune checkpoint, which suppress the body's immune response. This way cancer cells can avoid elimination by activated immune cells. Immune checkpoint inhibitors (ICIs) are developed to block this tumor defense mechanism. Different categories of those inhibitors are nowadays used in daily routine practice for the treatment of diverse tumor types, among them melanoma and lung carcinoma, with good response rates and limited side effects. It is noteworthy that despite this progress, there are no promising data on the treatment of mesenchymal tumors with ICIs.

2. Aims

The general aim of this thesis was to unravel the possible reasons why ICIs is at present not a treatment of choice for sarcomas. Therefore, I have investigated the expression of different immune checkpoints, namely PD1, PD-L1, CD70 and IDO in different mesenchymal tumors. The composition of the tumor's immune microenvironment, which appears to be also a key component of the tumor, has also been part of my research. Tumors with microsatellite instability (MSI) can be treated with immunotherapy regardless the tissue of origin. Hence, I likewise examined different mesenchymal malignancies for their microsatellite status. Finally, given the recent advantages on targeting tumors with specific molecular alterations as *NTRK* fusions, my aim was to investigate the expression of TRK on sarcomas and the correlation with the presence of a *NTRK* fusion.

3. Outline of the study

In order to start the story, **chapter 3** provides an overview of immune checkpoint inhibitory therapy in diverse sarcoma types. For this review I did an extensive research on "clinicaltrials.gov" and on PubMed and I have gathered all the clinical trials with published results. My main focus was the outcome of each clinical trial in relation to the primary endpoint. Special attention was given to the description of predictive biomarkers, such as the expression of PD1, PD-L1 or IDO on tissue

samples of responders versus non-responders. In the second part of this review I have summarized all the possible predictive parameters that could be used in selecting the sarcoma patients that will most benefit from immune checkpoint inhibitory therapy.

In **chapter 4** I investigate the role of the tumor microenvironment in anti-tumor activity. For this research I used desmoid tumors as the paradigm. Desmoid tumors have a special immune microenvironment with formation of lymphoid aggregates at the periphery of the tumor. Those lymphoid aggregates represent tertiary lymphoid organs and help as vehicles for the transport of immune cells in and around the tumor tissue. I have found interesting data on the composition of the tumor microenvironment of those tumors and as well as the expression of PD-1 and PD-L1 on immune cells and tumor cells.

In following chapter (**chapter 5**) I provide data on the expression of immune checkpoint inhibitors, namely PD-1, PD-L1, IDO and CD70 in different soft tissue and bone tumors. Herewith I could provide evidence that specific tumor types disserve further investigations as they could be promising candidates for immune checkpoint inhibition. Moreover, I analyze the microsatellite status of all tumors in our study and found an interesting tumor that showed MSI. This patient can benefit from immune checkpoint inhibitory therapy in case of relapse or dissemination of the tumor.

Moving forward to the molecular aspects of sarcomas, in **chapter 6,** I present an overview on *NTRK* fusions in sarcomas. Patients with *NTRK* fusions can be treated with TRK inhibitors regardless tissue of origin, as in cases of MSI. *NTRK* fusions in sarcomas are very rare and identifying the patients that harbor these genetical alterations is of outmost importance. Hence, this review focuses on the clinical, pathological and diagnostical aspects concerning *NTRK* fusions in soft tissue and bone tumors. Finally, according to the data presented in this review, I propose an algorithm on how to select cases that need further molecular investigation for the detection of fusions of the *NTRK* gene.

Taking into consideration the outcome of the review, in **chapter 7**, I describe the correlation of TRK immunohistochemical expression on tissue samples with the presence of *NTRK* fusion in different sarcoma

types. Despite their rarity, I describe here two cases among 70 samples with particular interest in terms of their morphological image and molecular profile.

Finally, in **chapter 8**, the research results will be discussed and future perspectives will be mentioned, while **chapter 9** summarizes what has been discussed in all the previous chapters.

CHAPTER 3

IMMUNE CHECKPOINT INHIBITORY THERAPY IN SARCOMAS: IS THERE LIGHT AT THE END OF THE TUNNEL?

Vasiliki Siozopoulou, Andreas Domen, Karen Zwaenepoel, Annelies Van Beeck, Evelien Smits, Patrick Pauwels
Immune Checkpoint Inhibitory Therapy In Sarcomas: Is There Light At The End Of The Tunnel?
Cancers (Basel) 2021 Jan 19;13(2):360. Doi: 10.3390/Cancers13020360.

1. Abstract

Soft tissue and bone sarcomas are a very heterogeneous group of tumors with many subtypes for which diagnosis and treatment remains a very challenging task. On top of that, the treatment choices are limited, and the prognosis of aggressive sarcomas remains poor. Immune checkpoint inhibitors (ICIs) have drawn a lot of attention last years because of their promising response rates and their durable effects. ICIs are currently widely used in the daily routine practice for the treatment of a different malignancies, such as melanoma, Hodgkin lymphoma, and non-small cell lung carcinoma. Still, ICIs are not included in the standard treatment protocols of the different sarcoma types. However, a plethora of clinical trials investigates the clinical benefit of ICIs in sarcomas. There is clear need to develop predictive biomarkers to determine which sarcoma patients are most likely to benefit from immune checkpoint blockade. This review will focus on (i) the clinical trial results on the use of ICIs in different sarcoma types; and on (ii) possible biomarkers predictive for the effectiveness of these drugs in sarcomas.

2. Introduction

a. Sarcomas

Soft tissue and bone sarcomas are very rare neoplasms and account for less than 1%[1-3] of all malignancies. Although we refer to mesenchymal tumors as an entity, there are more than 200 distinct categories recognized and described in the latest World Health Organization classification of tumors book[4]. The rarity of these tumors leads into difficulties defining the right criteria for diagnosis and precise treatment. Moreover, the heterogeneity makes prognosis difficult to assess. Many different parameters have been investigated in order to establish prognostic criteria for sarcomas. Among them, the tumor grade has been proven to be one of the best predictors of metastatic risk and progression free survival[5].

The etiology of most sarcomas remains unknown; however, genetic events have been attributed as being the main cause of mesenchymal tumorigenesis. According to their genetic alterations, sarcomas can be subdivided in two main categories. In the first category, sarcomas display a simple karyotype which can be a somatic mutation, a gene

translocation or amplification that represents the driver oncogenic mechanism of the tumor. The second category includes sarcomas with a complex karyotype showing multiple aberrant chromosomal alterations[6-8] and represents almost two thirds of the sarcomas.

Some of the molecular events found in sarcomas are druggable, such as tyrosine-protein kinase (KIT) mutations in gastrointestinal stromal tumors (GISTs) and in a minority of other mesenchymal tumors. Unfortunately, today most of the oncogenic driver alterations are undruggable.

Surgery remains the golden standard for the treatment of localized disease. In case of larger tumors that cannot be resected completely, adjuvant radiation therapy can be applied to control the local aggressiveness. Unresectable sarcomas can also be treated with radiotherapy and/or chemotherapy in neoadjuvant setting. Chemotherapy is used in specific subtypes, such as rhabdomyosarcoma, osteosarcoma, and Ewing sarcoma. However, effective treatment of advanced sarcoma remains a challenge. Moreover, the five-year survival rates for the metastatic setting does not exceed 16%, thereby highlighting the need for new therapeutic strategies in sarcoma.

b. Immunotherapy

Immune checkpoint blockade (ICB) with immune checkpoint inhibitors (ICIs) is a well-known immunotherapeutic approach widely used due to the promising results in several cancer types. Inhibitory immune checkpoints (ICs) are responsible for controlling and inactivating the immune system in order to avoid autoimmunity. ICs are expressed under normal physiological conditions by different immune cell types[9]. Unfortunately, tumor cells can hijack this system. This results in T-cell exhaustion, immune tolerance and eventually suppression of the anti-tumor immune response. By blocking ICs, silenced anti-tumor responses will be reactivated[10,11].

A broad range of different ICs has been identified to date. One of the most commonly known is programmed death-1 (PD-1) expressed a.o. on T-cells. It can bind to programmed death ligand-1 (PD-L1) expressed on tumor cells and other cells. Today several drugs have been developed

that can block the interaction between PD-L1 and PD-1, thereby reactivating silenced immune responses. The PD-1 and PD-L1 blockers that are widely used in the clinical practice are nivolumab, pembrolizumab, atezolizumab, durvalumab, avelumab, and cemiplimab, while many new ones are tested in clinicals trials. Another example of an IC is cytotoxic T-lymphocyte-associated antigen4 (CTLA-4) which binds with B7 on the antigen presenting cells (APC)[12]. In this context, ipilimumab is a widely known CTLA-4 blocking antibody that has been extensively used for the treatment of metastatic melanoma[13]. Ipilimumab in combination with nivolumab has also been approved as first-line treatment for advanced renal cell carcinoma and non-small cell lung cancer[14,15].

Although we will focus on ICB in this review, it is important to mention that the term immunotherapy refers to a broad range of different therapies. According to the National Cancer Institute (NCI), there are five main categories within immunotherapy: (1) T-cell transfer therapy, (2) monoclonal antibodies, (3) cancer treatment vaccines, (4) immune system modulators, and (5) ICB[16]. In addition to these categories, there are also new generation immune checkpoints with stimulatory effect, or checkpoints concerning innate immunity or natural killer cells, which gain more and more research interest in the field of immunotherapy[17].

Despite the rapid developments in the field of immunotherapy the last decade, ICB is not included in the standard treatment protocols of sarcomas. However, immunotherapy is currently under investigation in several clinical trials that include sarcomas. So far, no effective immunotherapeutic strategy for sarcoma has been identified. Given that ICB is a widely used immune therapeutic strategy in daily clinical practice together with the imperative need for new therapeutic alternatives for sarcomas, this review will (i) outline the results of clinical trials on the application of ICB in sarcomas, and (ii) discuss the possible mechanisms why this treatment has not been proven effective for sarcomas at present.

3. *Online Searching Method*

a. ClinicalTrials.gov

For this review we started with an extensive search on ClinicalTrials.gov for clinical trials that describe results in the application of ICB in different types of soft tissue and bone tumors, until 11 November 2020. For our primary search we used the following three search terms: the first term was "sarcoma" AND the second term was variable, being "immune check point inhibitory therapy" (4 results) OR "immune checkpoint inhibitory therapy" (4 results) OR "immune checkpoint blockade" (0 results) OR "CTLA-4" (33 results) OR "anti-CTLA4" (14 results) OR "PD-L1" (62 results) OR "anti-PD-L1" (14 results) OR "PD-1" (70 results) OR "anti-PD1" (31 results). We also checked other checkpoint inhibitors such as "VISTA" (0 results), "LAG-3" (5 results) and "TIM-3" (4 results) and saw that these 9 studies were already covered by our broad primary search. Moreover, as second term we also used the approved IC blocking antibodies that are used in the daily routine practice at the moment, being "ipilimumab" (28 results) OR "nivolumab" (43 results) OR "pembrolizumab" (34 results) OR "atezolizumab" (13 results) OR "avelumab" (9 results) OR "durvalumab" (15 results) OR "cemiplimab" (2 results). When comparing all outcomes from the second searching term, there was considerable overlap of the clinical trials and we finally ended up with 149 different clinicals trials in sarcomas treated with ICB. As a third search term we used "study with results" in the pool of 149 clinical trials and only 5 clinical trials were left.

b. Pubmed

The number of clinical trials with results on ClinicalTrials.gov seemed very low. Therefore, we investigated each of the 149 clinical trials in PubMed by NCT number for any published results and we found publications for 26 of our NCT numbers. Three out of those 26 numbers overlap the 5 clinical trials reported with results on ClinicalTrials.gov, resulting in 28 clinical trials with results.

c. Summary

After thoroughly searching, only 14 out of these 28 clinical trials with results met the searching terms *sarcoma* AND any of the *IC blocking antibodies* listed above AND *study with results* on ClinicalTrials.gov and/or PubMed. Table 1 summarizes these 14 clinical trials and the results in terms of the primary endpoint, while Table 2 gives an overview of all the predictive biomarkers that are investigated in each one of those clinical trials.

Table 1. This table summarizes all the clinical trials and their results in terms of the primary endpoint.

Study	Phase	Medication	Targeted Group	Number of Eligible Patients	Tumor Type	Primary Endpoint	Results According to Primary Endpoint
NCT02301039	II	Pembrolizumab	12 years or older	80	Metastatic or surgically unresectable locally advanced soft tissue and bone sarcoma	OR	17.5% soft tissue 5% bone sarcomas
NCT02304458	I-II	Nivolumab	Children and young adults	85	Relapsed or refractory Rhabdomyosarcoma, Ewing sarcoma, osteosarcoma	Tolerability, systemic exposure, MTD and antitumor activity	3 mg/kg every 2 weeks well tolerated No OR
NCT02428192	II	Nivolumab	Adults	12	Advanced UMLS	ORR	0%
NCT01445379	I	Ipilimumab	Children and adolescence	37	Refractory or recurrent sarcomas (and other solid non-sarcoma tumors)	Tolerance and toxicity	Higher grade irAE with increasing dose Better response in patients with high irAE
NCT03623581	II	Geptanolimab	Adults	31	Unresectable, recurrent, or metastatic ASPS	ORR	37.8%
NCT02595866	I	Pembrolizumab	HIV patients	6	Kaposi Sarcoma	Safety of drug	AE similar to non-HIV patients

NCT02500797	II	Nivolumab ± ipilimumab	Adults	76	Metastatic sarcoma	ORR	5% for monotherapy 16% for combination therapy
NCT02406781 (osteosarcoma study)	II	Pembrolizumab + MC	Adults	15	Osteosarcoma	Non-progression and OR at 6 months	Non-progression: 13.3%
NCT02406781 (STS study)	II	Pembrolizumab + MC	Adults	50	LMS UPS Other sarcoma types GIST	Non-progression and OR at 6 months	Non-progression: 0% for LMS/UPS 14.3% for other sarcoma types 11.1% for GIST OR: one patient (2%)
NCT02888665	I-II	Pembrolizumab + Doxorubicin	Adults	37	Advanced Anthracycline-I Sarcoma	ORR	19% for phase I 13% for phase II
NCT02636725	II	Pembrolizumab + Axitinib	16 years and older	33	Advanced or metastatic sarcoma	3-months PFS	65.6%
NCT03359018	II	Carmelizumab + apatinib	11 years and older	41	Advanced osteosarcoma	6-months PFS and CBR	PFS: 50.9% CBR: 30.2%

NCT01643278	Ib	Ipilimumab + dasatinib	Adults	28	Refractory GIST and advanced sarcomas	Safety profile and MTD	MDT: dasatinib 140 mg/day + ipilimumab 3 mg/kg
NCT03069378	II	Pembrolizumab + T-VEC	Adults	20	Locally advanced or metastatic sarcoma	Best ORR at 24 weeks	30%

Abbreviations: AE; adverse event, ASPS; alveolar soft part sarcoma, CBR; clinical benefit rate, irAE; immunotherapy associated adverse event, MC; metronomic cyclophosphamide, MTD; maximum tolerated dose, LMS; leiomyosarcoma, OR; objective response, ORR; objective response rate, STS; soft tissue sarcoma, UPS; undifferentiated pleiomorphic sarcoma

Table 2. Overview of the predictive biomarkers and their clinical importance investigated in the clinical trials described in this review.

Study	Phase	Drug(s)	Predictive Biomarker	Interesting Findings	Clinical Relevance
NCT02301039	II	Pembrolizumab	PD-L1 on TCs Cut-off ≥ 1%	4% PD-L1$^+$, all positive samples were UPS	From positive patients: 1 CR and 1 PR
NCT02304458	I–II	Nivolumab	PD-L1 on TCs Cut-off ≥ 1% TME	Low PD-L1 on TCs PD-L1 expression mostly in macrophages	-
NCT02428192	II	Nivolumab	PD-L1 on TCs and ICs PD-1 on Ics	No results available	-
NCT01445379	I	Ipilimumab	Circulating and activated T-cells after ipilimumab administration	Increase of CD4$^+$HLA-DR$^+$ T cells	No correlation with irAE
NCT03623581	II	Geptanolimab	PD-L1 on TCs Cut-off CPS ≥ 1 MSI TMB Baseline lymphocyte composition	No difference in response between PD-L1 positive and negative TCs Higher percentage CD4$^+$ T cells in non-responders	Baseline % CD4$^+$ T-cells was negatively associated with patient response
NCT02595866	I	Pembrolizumab	CD4$^+$ T-cell count before and after drug administration	CD4$^+$ T-cell counts tended to increase	The increases were not statistically significant

NCT02500797	II	Nivolumab ± ipilimumab	PD-L1 TILs TMB T-cell receptor clonality	No results available (ongoing)	-
NCT02406781 (osteosarcoma study)	II	Pembrolizumab + MC	PD-L1 expression on TCs and ICs. Cut-off ≥ 1%	TC positivity in 14.3% IC positivity in 7.1%	No correlation of PD-L1 status and clinical response
NCT02406781 (STS study)	II	Pembrolizumab + MC	PD-L1 expression on TCs and ICs (cut-off ≥ 1%) Correlation of M2 macrophage, $CD8^+$ and IDO densities	One patient with PR had PD-L1≥10, mild IDO^+ ICs, $CD68^+$ cell density below the median and very high $CD8^+$ cell density. The majority of tumors had M2 macrophage that expresses IDO	M2/IDO pathway possibly important mechanism for primary resistance to PD-1 inhibition
NCT02888665	I–II	Pembrolizumab + Doxorubicin	PD-L1 expression (H-Score/MPS) TILs based on morphology Gene expression profile	Expression of PD-L1 was not associated with PFS or OS TILSs present in 29% No gene was significantly associated with PFS	Presence of TILs associated with inferior PFS
NCT02636725	II	Pembrolizumab + Axitinib	PD-L1 expression Presence of TILs	Investigated ASPS tissue samples showed PD-L1 expression and a high TIL score	No PD-L1 and TIL score correlation with PFS (>6 months) or PR

NCT03359018	II	Carmelizumab + apatinib	PD-L1 expression Cut-off ≥ 5% in TCs	No ORR benefit in PD-L1 positive tumors	Prolonged PFS in patients with PD-L1-expressing tumors
NCT01643278	Ib	Ipilimumab + dasatinib	Levels of IDO before and after therapy	IDO suppression in 1 patient with GIST	IDO suppression may potentially correlate with antitumor efficacy in GIST
NCT03069378	II	Pembrolizumab + T-VEC	PD-L1 in TCs Cut-off ≥ 1% TIL score	55% had a turn from PD-L1^{-} at baseline to PD-L1^{+} after treatment Among the responders, one patient with PD-L1^{+} at baseline and 4/9 with PD-L1^{+} posttreatment had PR	All responded patients had higher TIL score mostly in the form of CD3^{+}/CD8^{+} aggregates, at the periphery of the tumor

Abbreviations: CD; cluster of differentiation, CPS; combined positive score, CR; complete response, Ics, immune cells, H-score; "histo" score, semiquantitative immunohistochemical scoring, HLA; human leukocyte antigen, IDO; indoleamine-pyrrole 2,3-dioxygenase, MPS; modified proportion score, MSI; microsatellite instability, ORR; objective response rate, PFS; progression free survival, PD1; programmed death 1, PD-L1; programmed death-ligand 1, PR; partial response, STS; soft tissue sarcoma, TCs; tumor cells, TIL; tumor-infiltrating lymphocyte, TMB; tumor mutational burden, TME; tumor microenvironment, UPS; undifferentiated pleiomorphic sarcoma

4. Results

a. Immune Checkpoint Inhibitors as Monotherapy

One of the largest studies is the SARC028 multi-institutional phase II study that assessed the safety and activity of pembrolizumab in patients with advanced sarcoma (NCT02301039)[18]. The trial included 80 patients divided equally in two treatment groups, one including patients with soft tissue and one including patients with bone sarcoma. In the soft tissue category patients with leiomyosarcoma (LMS), poorly differentiated/dedifferentiated liposarcoma (DDLPS), undifferentiated pleiomorphic sarcoma (UPS)/malignant fibrous histiocytoma, and synovial sarcoma were enrolled. The bone tumor category consisted of osteosarcoma, chondrosarcoma, and Ewing sarcoma. Pembrolizumab was used as monotherapy and was administered at 200 mg intravenously every three weeks for both groups, until disease progression or unacceptable toxicity.

Primary outcome of the study was investigator-assessed objective response (OR) according to response evaluation criteria in solid tumors (RECIST) version 1.1. This was defined as the proportion of patients in each cohort with a best overall response of complete (CR) or partial (PR) response. The trial did not reach the prespecified OR of 25%. Seven of 40 patients (17.5%) with soft tissue sarcoma achieved an OR. The median progression free survival (PFS) was 18 weeks. The 12-week PFS was 55%, suggesting clinical activity for soft tissue sarcomas with the majority being UPSs and DDLPSs. For UPS, the median duration of response was 49 weeks, suggesting that ICIs can have durable effects, especially for this group. Only one patient with synovial sarcoma showed a short-lived PR, while no patient with leiomyosarcoma displayed OR.

Confirmed PR was observed in 2 of 40 bone sarcomas showing a substantial shrinkage of tumor volume and a durable effect of more than six months. Median PFS was eight weeks. Anemia and decreased lymphocyte count were the most persistent toxic events often resulting in grade 3 or worse toxicity. The investigators tried to find out if there is a correlation between immunohistochemical (IHC) expression of PD-L1 and response to therapy. Three samples, all from UPS patients, had

a PD-L1 expression in more than 1% of the tumor cells, still in general no statistical correlation could be shown. The investigators concluded that ICIs induced durable responses and showed meaningful clinical activity in patients with soft tissue sarcoma, in particular UPS and DDLPS.

The ADVL1412 (NCT02304458)[19] study is another multicentric, single arm, phase I–II trial investigating the safety, pharmacokinetics, and anti-tumor activity of nivolumab as monotherapy in children and young adults with recurrent or therapy refractory tumors, including soft tissue and bone sarcomas (particularly rhabdomyosarcoma, Ewing and osteosarcomas). The study had several primary objectives: (1) to determine the tolerability and describe the toxicity of nivolumab at the adult recommended dose; (2) to determine the systemic exposure of nivolumab in children compared to that in adults; (3) to determine the maximum tolerated dose in children; and (4) to explore the anti-tumor activity of nivolumab in selected childhood solid tumors or lymphoma. Secondary objectives included investigating the presence of infiltrating lymphocytes and PD-L1 expression in tumor specimens from patients.

Nivolumab 3 mg/kg was well tolerated and confirmed as the pediatric recommended phase II dose. To investigate dose-expansion the primary outcomes were tolerability, systemic exposure, maximum tolerated dose, and anti-tumor activity of nivolumab at the adult recommended dose in children and young adults.

A higher frequency of hematological toxicity was found in children but in general, administration of 3 mg/kg every 14 days was well tolerated. The most common immune related adverse events (irAEs) were increased lipase levels and cardiac and pleural effusion. No OR was observed and nivolumab as monotherapy did not show significant anti-tumor activity in the pediatric tumors. The study showed a low PD-L1 expression on the different sarcoma types and a paucity of infiltrating T-cells, emphasizing the possibility that other factors/mechanisms, such as tumor mutational burden (TMB), might play a role in the response.

Nivolumab as single agent was also investigated in a single-center phase II trial in patients with uterine LMS (NCT02428192)[20]. Twelve patients with metastatic or unresectable disease that were previously treated with

chemotherapy were included. The primary endpoint was objective response rate (ORR). Secondary, they investigated the correlation between response to nivolumab and PD-1, PD-L1 and PD-L2 expression on available tissue samples. The patients received 3 mg/kg intravenous nivolumab every two weeks. None of the 12 patients experienced OR. The overall median PFS was 1.8 months while the median overall survival (OS) was not met. Clear correlation with IHC expression of PD-1, PD-L1 and PD-L2 could not be documented. Nine of 12 patients had grade 3 AEs or higher, with an increase of serum amylase and lipase being correlated to the drug administration.

Another humanized anti-PD1 antibody, geptanolimab, was investigated in Gxplore-005 phase II study (NCT03623581)[21] in adult patients with unresectable, recurrent or metastatic alveolar soft part sarcoma (ASPS) and provides evidence that suggest that immunotherapy could play an important role in the treatment of ASPS. Primary end point was ORR. Thirty-seven patients were included, which received 3 mg/kg intravenous geptanolimab every 2 weeks until disease progression or significant toxicity occurred. OR was 37.8%, which is significantly higher than the 10% expected from chemotherapy[22]. Limited patients developed grade 3 AEs, such as anemia, fever, and hypophisitis. In this study the expression of different biomarkers, such as PD-L1 status, microsatellite instability (MSI), TMB and immune infiltration with $CD4^+$ cells, was compared with response to therapy. About 30% of the patients had PD-L1 combined positive score (CPS) of ≥ 1 in tissue samples; however, there was no difference in response to ICB among positive and negative patients. The percentage of baseline $CD4^+$ T-cells was significantly higher in non-responders than in responders, indicating that non-responding tumors may be rich in regulatory T-cells (T-regs) that suppress immune response. No MSI was present and all samples showed very low TMB.

ASPS was also a field of interest of the phase II clinical trial from Japan. The OSCAR study investigated the possible role of nivolumab in the treatment of advanced clear cell sarcoma (CCS) and ASPS[23]. The trial presented its preliminary results in CTOS 2020. Eleven CCS and 14 ASPS patients received nivolumab 240 mg every two weeks until disease progression or intolerable drug toxicity. The primary endpoint, which was response rate (RR), was not met. Nevertheless, encouraging

was that the disease control rate reached 64% for unresectable CCS and ASPS. Median PFS was 4.9 months and median OS was 15.8 months.

The NCI 08-C-0007 (NCT01445379)[24] phase I study investigated the safety and pharmacokinetics of ipilimumab. Thirty-one patients between 2 and 21 years old with refractory or recurrent solid tumors were included. Seventeen of them had sarcoma of various histological types. The primary endpoint was to determine the tolerance and toxicity of ipilimumab in the young population. Secondarily, the trial aimed to quantify the anti-tumor effects of ipilimumab in the target group. Patients received ipilimumab with a dose escalation from 1 mg/kg up to 10 mg/kg. Grade 3 and 4 irAEs were seen with 5 mg/kg and 10 mg/kg dose, mostly related to gastrointestinal and liver toxicity. Because an increase in irAEs was seen in children under 12 years old treated with 10 mg/kg, the cohort of this age group was expanded. Interestingly, OS was better in patients with irAEs than in patients without irAEs, suggesting that irAE is an undesirable side effect of a desired result, i.e., the activation of the immune response. The investigators concluded that given the toxicities and inability to predict toxicity or response, ipilimumab as single agent in the pediatric tumors has no leading role.

The investigators of the nonrandomized phase I study (NCT02595866)[25] had a unique scope. They focused on safety of administrating pembrolizumab in human immunodeficiency virus (HIV)-positive patients who developed diverse non-HIV and HIV related malignancies, such as Kaposi sarcoma (KS). The secondary endpoint was to evaluate the tumor response. The study included 30 patients, 6 of which had KS.

In general, irAEs were similar to those described in non-HIV patients that received ICI for any other FDA-approved indications. Five out of six KS patients demonstrated tumor regression, yet did not meet the criteria for PR. One patient with pretreatment KS-herpesvirus viremia died through a polyclonal KS-herpesvirus-associated B-cell lymphoproliferative disease. The investigators concluded that ICIs can be safely administrated in patients with HIV, but caution for patients with active viremia.

Response to pembrolizumab was investigated also in patients with endemic and classic type KS in a prospective phase II clinical trial

(NCT03469804)[26], published its results at the ESMO 2020. The study included 17 patients, which were given pembrolizumab 200 mg intravenously every 3 weeks for up to 6 months. The primary endpoint was best ORR. Almost 71% of the patients experienced an OR, while another 24% had SD. The irAE were tolerable with only one grade 3. A key finding of the study is that patients with lack of PD-L1 expression on tumor and immune cells on baseline tissue samples, had a limited effect with pembrolizumab treatment. This raises the question whether PD-L1 could become a predictive factor for response of endemic/classic type KS to ICB.

The AcSé study is a non-randomized, phase II clinical trial (NCT03012620) that investigated the response of pembrolizumab on different sarcoma histologic subtypes and demonstrated its results at the ESMO 2020[27]. Twenty-four of the included patients had chordoma, 13 had ASPS, 6 had desmoplastic small round cell tumor (DSRCT), another 6 smarca4-malignant rhabdoid tumor (SMRT), and 31 had other histologic subtypes. The patients received pembrolizumab 200 mg intravenously every three weeks for up to two years. Best response was PR in 16% and SD in 36%. The investigators of this study highlighted the importance of histological type in response to treatment, as 50% of responses were observed in SMRT and 39% in ASPS patients.

b. Immune Checkpoint Inhibitors as Combination Therapy

The Alliance A091401 (NCT02500797)[28] is a randomized phase II trial investigating nivolumab with or without ipilimumab in patients with metastatic or unresectable sarcoma who received at least one previous line of systemic therapy. Nivolumab 3 mg/kg was given every two weeks or nivolumab 3 mg/kg and ipilimumab 1 mg/kg every three weeks until disease progression or up to two years after registration.

The primary endpoint was confirmed OR defined as CR and PR by RECIST version 1.1. Secondary endpoints were duration of response, the proportion of patients achieving a clinical benefit, PFS and OS.

All patients receiving treatment experienced irAEs. The most common grade 3 or worse irAEs in both cohorts were anemia and increased serum lipase levels. Monotherapy was generally tolerated better compared to

the combination therapy. Two patients of the monotherapy group had confirmed PR, one with ASPS and one with non-uterine LMS, resulting in RR of 5%. The RR in the combination group was higher, reaching 16%. The median PFS was 1.7 months with monotherapy versus 4.1 months with the combination therapy. The clinical benefit of nivolumab monotherapy was not equal when compared to the currently available treatment options[29]. Moreover, nivolumab monotherapy did not meet the predefined primary endpoint. The combination therapy met its predefined primary endpoint, with median OS of 14.3 months while OS described for similar patient populations treated with selective tyrosine kinase inhibitor (TKI) is approximately 11–15 months[30]. These findings suggest that nivolumab as a single agent may not be active, and that only the combination therapy shows efficacy that may justify further studies as a treatment option for metastatic sarcoma patients.

A prospective, phase II clinical trial (SWONG S1609, cohort 51), that presented its results at the ASCO 2020 (NCT02834013)[31], investigated the combination of ipilimumab 1 mg/kg every six weeks and nivolumab 240 mg every two weeks, both administrated intravenously, in patients with metastatic or unresectable angiosarcoma. The primary endpoint was ORR, while the secondary endpoints were multiple, including PFS and OS. There were nine cutaneous and seven non-cutaneous angiosarcomas. ORR was 25% and six-month PFS was 38% regardless the primary localization, while 60% of the cutaneous angiosarcomas had a confirmed OR. The investigators made here also a comment about UV light exposure DNA mutational signature in cutaneous angiosarcomas, implying that this may interfere with the drug efficacy.

c. Immune Checkpoint Inhibitors Combined with Chemotherapy

Chemotherapy, particularly metronomic cyclophosphamide (MC), has been described to show immunological properties by depleting regulatory cells and restoring T- and natural killer- (NK) effector factors in cancer patients[32]. The PEMBROSARC was a single-arm, phase II, multicenter clinical trial, which aimed to target osteosarcomas with pembrolizumab in combination with MC (NCT02406781)[33].

The study included 17 patients, 15 of which were assessable for the primary efficacy endpoint. In order to be included, patients should

present with metastatic or unresectable tumor the last six months before entering the study. The primary endpoint was dual-pointed at non-progression and OR at six months. PR was seen only in one patient. Two patients had stable disease (SD). The non-progression rate was not met, reaching only 13.3%. The median PFS was 1.4 months and the median OS was 5.6 months. The most frequent AEs were fatigue and anemia. Fourteen patients had available tissue samples for PD-L1 expression analysis. Two samples showed PD-L1 ≥ 1% in the tumor cells and 1 sample in the immune cells. Four patients showed tumor shrinkage but no one of them expressed any positivity for PD-L1. The authors concluded that this combination of anti-PD1 with MC had insignificant activity in advanced osteosarcoma.

The same investigational team examined the effect of pembrolizumab combined with CM in patients with advanced soft tissue sarcomas (NCT02406781)[34]. The primary endpoints were the same as in the PEMBROSARC study. The study had four arms according to the histological type of the tumor: LMS, UPS, other histological types, and gastrointestinal stromal tumors (GIST). Fifty patients were assessable for the primary efficacy endpoint. The AEs were similar as reported for the PEMBROSARC study. Three patients were progression free at 6 months, while 31 showed disease progression and 16 SD. One patient showed OR. The six-months non-progression was 0%, 0%, 14.3%, and 11.1% for each category, respectively. The median PFS was 1.4 months for each cohort. The median OS was 9.2, 5.6, and 7.1 for the first three categories, respectively, but was not reached for the GIST patients. PD-L1 ≥ 1% was observed on the tumor cells in 12% and on the immune cells in 40% of cases. Interestingly, immune cells were positive in 64% of UPS cases. Only one patient with PR demonstrated a PD-L1 ≥ 10% on immune cells.

The investigators also examined the composition of the tumor microenvironment (TME) and found that the tumors had a high proportion of $CD163^+$ macrophages, associated with M2 phenotype known to play a role in immune suppression. This composition ranged from 31% in the LMS arm and reached up to 73% in the UPS arm. In addition, the tumor-associated $CD163^+$ macrophages expressed indoleamine-pyrrole 2,3-dioxygenase (IDO1), reaching again 73% in UPSs. Overall, PD-L1 expression in immune cells was significantly

positively associated with $CD8^+$ cell density and IDO expression. The authors concluded that the M2/IDO suppressor pathway present in most of the investigated sarcomas might play an important role in the resistance to the therapy.

Doxorubicin, a chemotherapeutic agent, alone or in combination with other chemotherapeutic agents, constitutes standard first line systemic treatment for advanced sarcomas[35]. A nonrandomized study addressed the combination of doxorubicin with pembrolizumab in advanced, anthracycline naïve sarcoma patients (NCT02888665)[36]. The study consisted of two phases; in phase I dose-escalation of doxorubicin was examined, starting at 45 mg/m^2 and increasing up to 75 mg/m^2 which is the standard treatment dose for sarcomas. A 75 mg/m^2 dose was well tolerated with no AEs higher than grade 3. In the following phase II patients received 200 mg pembrolizumab and 75 mg/m^2 doxorubicin every three weeks for up to seven cycles. Thereafter, patients could continue with pembrolizumab as single agent for up to two years. Thirty-seven patients were included in the study, with LMS being the most frequent tumor (11 cases). The primary endpoint was ORR in 15% of the patients, which was not reached, ending in 13% for phase II. On the other hand, encouraging results were found for OS and PFS, being 27.6 and 8.1 months, respectively. PFS at 12 months was 27%. In particular, three out of four patients with UPS and two out of four patients with DDLPS had durable PR, and three out of four patients with chondrosarcoma had tumor regression. PD-L1 expression was very low in almost 70% of the evaluated samples. Of note, a strong association between tumor-infiltrating lymphocytes (TILs) and inferior PFS was seen. Nevertheless, the study did not extensively analyze the composition of the TME.

d. Immune Checkpoint Inhibitors Combined with Molecular Targeted Therapy

The synergistic effect of targeted therapy and immunotherapy assumes that targeted therapy can have an immunomodulatory effect that increases clinical responses[37].

The single arm phase II trial aimed to combine axitinib, a selective TKI, and pembrolizumab in patients with advanced or metastatic sarcomas

(NCT02636725)[38]. Thirty-three patients were enrolled; 36% had ASPS, while the remaining 64% were divided into several histological sarcoma subtypes. Patients received 5 mg axitinib two times a day continuously and 200 mg pembrolizumab every three weeks for cycles of six weeks for up to two years. The investigators applied an intrapatient dose-escalation and de-escalation of axitinib ranging from 2 mg to 10 mg twice daily per cycle. The primary endpoint was PFS at three months. Other endpoints were the rate of participants achieving OR, clinical benefit, OS and the safety and toxicity profile of the drugs. The three-months PFS rate for all patients that received therapy was 65.6%, but most patients ultimately progressed. Most patients with PR had ASPS. The investigators analyzed ASPS and non-ASPS patients separately for the PFS, OS, and OR. Notably, in non-ASPS the median PFS was similar and the OR was only slightly higher compared to that of patients receiving other types of monotherapy, including axitinib, posing the dilemma whether the addition of an ICI has any value. Still, the six-month PFS is favorable for patients treated with the combined therapy and the investigators concluded that this may be due to the delayed anti-tumor effect of ICIs. The median PFS of the ASPS population is not favorable compared to monotherapy with other broad spectrum TKIs, as reported in literature[39-41]. On the other hand, the proportion of patients achieving an OR exceeded the highest previously reported OR of any given monotherapy[39].All patients with ASPS showed tumor positivity for PD-L1 and a high TIL score, nevertheless, this could not be correlated with a PFS longer than six months nor with a PR. The most frequent AEs were fatigue and thyroid disorders. Grade 3 and 4 AEs were autoimmune toxic effects, diarrhea, and liver disfunction but the authors concluded that the toxic effects are acceptable and those can be brought under control by axitinib dose escalation.

An investigational team of the University of Miami Miller School of Medicine identified seven patients with angiosarcoma within their institute which were treated with ICIs as monotherapy or combination therapy with other ICIs or TKIs in the context of a clinical trial or off label; after gathering all the available information of each patient, they performed a retrospective study[42]. One of those patients was from the previous described clinical trial that used axitinib and pembrolizumab (NCT02636725)[38]. Five patients showed PR at 12 weeks and 1 showed a CR as best overall response. This patient was treated with anti-CTLA-

4 and got different kinds of chemotherapeutic agents in the past. Tissue material gathered from this patient 12 days after the first dose of anti-CTLA-4 revealed that the TME consisted mainly of central memory $CD4^+$ and $CD8^+$ T-cells, underlining the importance of these cells in the patient's durable response. The tumor also expressed many novel gene fusions and cancer-testis antigens, which can serve as neoantigens and induce immune response but had a low TMB. The investigators hypothesized that in particular cutaneous angiosarcomas display a comparable mutational signature to ultraviolet-induced skin cancer, such as melanoma, which generally responds well to ICB.

The single-arm, phase II trial (NCT03359018)[43] investigated the synergistic effect of TKIs in combination with ICIs in chemotherapy refractory osteosarcomas. Studies have shown that expression of PD-L1 associates significantly with the presence of T-cells and dendritic cells in the tumor, but also with a poorer five-year event free survival in patients with osteosarcoma[44]. The research combined 500 mg daily of apatinib, a TKI against vascular endothelial growth factor receptor-2[45], with 250 mg intravenously carmelizumab given once every two weeks, until disease progression or unacceptable toxicity. The primary endpoint of the study was PFS and the clinical benefit rate (CBR) at six months. Forty-one patients were included. The median PFS was 6.2 months, with a CBR of 30.2%. For the patients with available pretreatment tissue sample for PD-L1 expression, investigators reported a statistically significant PFS in case of PD-L1 $\geq$ 5%. However, the study did not reach its primary endpoint with a 6-months PFS of 50.9%, which was much lower than the prespecified target of 60%. Moreover, compared to apatinib monotherapy in advanced osteosarcoma[46], the combination treatment did not show superiority. The AEs were in general consistent with the safety profile of the TKIs.

Gastrointestinal stromal tumors (GISTs) nearly always carry activating mutations of c-KIT, a proto-oncogene or platelet-derived growth factor receptor-α gene, giving ground to treatment with TKIs. A phase Ib study of the TKI dasatinib combined with ipilimumab was administrated in 20 GIST and 8 non-GIST sarcoma patients with advanced disease (NCT01643278)[47]. The primary objective was to evaluate the safety profile and to identify the maximum tolerable dose (MTD) of the combination therapy. A dose of 70 mg/day dasatinib and 10 mg/kg

ipilimumab was well tolerated with gastric hemorrhage and anemia as the worst grade 3 AEs. After dose escalation of both therapeutic agents, MTD was 140 mg/day dasatinib and 3 mg/kg ipilimumab. The median PFS was 2.8 months and the median OS 13.5 months. No PR or CR was noted. Interestingly, comparing a pre- and two consecutive post-treatment biopsies collected from four patients, IDO was suppressed at the second post-treatment biopsy in a GIST-patient with stable disease for 19 weeks. As such, the investigators suggested IDO suppression might play a role in anti-tumor activity in GISTs. However, this study could not provide convincing evidence for a synergistic effect of dasatinib with ipilimumab for the treatment of GISTs.

e. Immune Checkpoint Inhibitors Combined with Oncolytic Virus

The effectiveness of talimogene laherparepvec (T-VEC) in combination with pembrolizumab was investigated in patients with advanced or metastatic sarcoma, who had received at least one prior standard therapy line (NCT03069378)[48]. T-VEC is a genetically engineered herpes virus and generates a systemic anti-tumor immune response[49]. Both drugs were administrated every 3 weeks for up to 12 months. This open-label, phase II trial aimed to investigate the ORR at 24 weeks determined by the RECIST version 1.1. Twenty patients with various histological subtypes of sarcoma were included. The primary endpoint was met with an ORR of 30% at 24 weeks. The median time to response was 14.4 weeks while the median duration of response was 56.1 weeks. Epithelioid sarcomas, cutaneous angiosarcomas and unclassified sarcomas were among the histologic types that showed response. Overall, the AEs were not severe and combination of T-VEC with pembrolizumab was well tolerated. Tumor tissue samples from 11 patients were investigated to identify prognostic markers. TIL-score was higher in the responders compared to the refractory group. All responders had CD3+/CD8+ aggregates at the periphery of the tumor while non-responders did not display this phenotype. In six patients with available pre- and post-treatment tissue, negative PD-L1 expression in pretreatment samples turned positive in post-treatment samples. Compared to the ORR of neoadjuvant chemotherapy in sarcomas that ranges from 16% to 28%[50-52], the investigators concluded that T-VEC in combination with pembrolizumab may have a role in treatment of specific histological sarcoma subtypes.

5. Discussion

Soft tissue and bone sarcomas are a rare and very heterogenous group of tumors with many subtypes for which diagnosis and treatment remains a very challenging task. On top of that, the treatment choices are limited, and the prognosis of metastatic sarcomas remains poor. Checkpoint inhibitors have drawn a lot of attention in recent years because of their promising response rates and their durable effects[53]. Nevertheless, ICIs are not a standard treatment choice for sarcomas. Very little data also emerges from the published clinical trials. UPS, DDLPS, and ASPS seem to be good candidates, but it is generally unclear whether ICB, as mono- or combination therapy, is an appropriate treatment for all types of sarcomas.

The possible predictive factors that may play a role in sarcomas' response to ICB still remain undetermined and require further investigation, as we will discuss. Some sarcomas express PD-L1. The reported expression varies among different studies. The SARC028 clinical trial (NCT02301039)[18] showed PD-L1 expression in UPS cells, corresponding with an OR after treatment with ICI monotherapy. Moreover, apatinib combined with pembrolizumab in osteosarcomas (NCT03359018)[43] showed that tumors with PD-L1 ≥ 5% correlated significantly with PFS. On the other hand, axitinib in combination with pembrolizumab (NCT02636725)[38] showed PD-L1 expression in all patients with ASPS, but no correlation with PFS for more than 6 months. Furthermore, the study with geptanolimab in patients with advanced ASPS (NCT03623581)[21] demonstrated that almost 1/3 of the tissue samples were positive for PD-L1, but there was no correlation with response. The study with T-VEC and pembrolizumab (NCT03069378)[48] showed expression changes of PD-L1 from negative in pre-treatment samples to positive in post-treatment samples from the same patients. A recent study has shown that the proportion of PD-L1 positive osteosarcomas was higher in metastatic than in primary samples[54], emphasizing the ability of the tumor to adapt in order to escape immune response. The remaining trials discussed in this review showed either low PD-L1 IHC expression or no correlation with response. IHC may not represent the actual status of the PD-L1 expression due to the tumor heterogeneity[55]. Moreover, at present, the prognostic and predictive

significance of PD-L1 expression in sarcomas is largely unknown. This, together with the variability of expression between the different histological subtypes, poses a challenge in the use of PD-L1 expression as a single predictive biomarker. All these data make it clear that PD-L1 expression in sarcomas disserves to be studied as a separate predictive factor within separate homogenous subtypes of sarcoma.

In addition to PD-L1 expression, the presence of tumor microenvironmental factors also appears to be largely responsible for the response to ICB. Tumors may display a "hot" or a "cold" inflammation signature[56]. Hot tumors are T-cell infiltrated and show a strong immune response to eradicate the tumor. In the SARC028 study (NCT02301039)[18] an inflamed phenotype has been observed in undifferentiated sarcoma which could explain the clinical activity of pembrolizumab. The presence of a strong immune cell infiltrate also suggests activation of immune-suppression pathways that can be targeted[56]. Sarcomas with a complex karyotype such us DDLPS, LMS, and UPS can display an inflamed phenotype and this on its own has been highly associated with clinical response[57]. Moreover, PD-L1 expression has been correlated with T-cell infiltration in UPS[58-60]. On the other hand, "cold" tumors have an exhausted or a desert T-cell phenotype. In this regard, activation of the immune response may be the primary scope for tumor elimination. Specific biological mechanisms, such as activation of β-catenin seem to be responsible for T-cell exhaustion and resistance to anti-PD1 therapy[56,61]. Desmoid tumors (DT) are known for showing mutations of the CTNNB1 gene, resulting in activation of the β-catenin pathway. A recent study on DT demonstrated that the tumors have a strong immune infiltrate at the periphery but not within the tumor and do not show a PD-L1 driven immune suppression[62]. Patients with activation of β-catenin are hence very unlikely to benefit from ICB, and β-catenin could be a potential negative predictor biomarker candidate. In melanoma patients it is demonstrated that T cell-mediated cell death can be inhibited by loss of phosphatase and tensin homolog (PTEN) protein[63]. Loss of PTEN is also documented in LMSs and osteosarcomas[64] and this could be a potential mechanism of resistance to ICB. Moreover, limited response of LMS to ICB is confirmed by the studies SARC028 (NCT02301039)[18] and NCT02428192[20]. Given that PI3K-AKT-mTOR pathway has been proven to be dysregulated via several genetic mechanisms, among them mutations of the PTEN[65],

combination therapy of ICB with PI3K-AKT pathway inhibitors may have a role in the treatment of certain sarcomas[63,66,67]. In general, the inflammation signature of sarcomas is not yet clearly described.

In addition to the inflammation signature, also the composition of the TME is of great importance. The first line of defense against the tumor are the cytotoxic $CD8^+$ T-cells which presence has been positively correlated with prognosis in different tumor types[68]. However, continuous activation of this defense mechanism can lead to an exhausted T-cell phenotype. On the other hand, the upregulation of T-regs may induce immunologic tolerance[69]. The study of geptanolimab (NCT03623581)[21] demonstrated increased $CD4^+$ T-cells in non-responders, whereas $CD4^+$ T-cells decreased after treatment in patients with a response. This suggests that $CD4^+$ cells might be T-regs and that geptanolimab plays an immunomodulatory role in patients with advanced ASPS. Another important cell type in the TME are the tumor associated macrophages (TAMs). There are two major types of TAMs of which the type 2 (M2) has been described to act in the suppression of the TME and progression of disease[70]. High numbers of TAMs correlate with tumor progression and metastases and have been associated with poor prognosis in gynecological LMS[71,72]. The presence of M2/IDO suppressor pathway in sarcomas might lead to resistance to ICB, according to the PEMBROSARC study (NCT02406781)[34]. Taken together, it is clear that composition of the TME is dynamic, emphasizing that the response to immunotherapy can be altered. The important question that arises here is how we can monitor such a dynamic change of the TME.

Even if the tumor expresses PD-L1 and the TME seems potent to eliminate the tumor, there are many other factors that can sabotage this process. One of them concerns tumor recognition by the immune system. In order for the immune cells to attack the tumor, the major histocompatibility complex (MHC) has to present neoantigens on the tumor cell surface. As such, loss of MHC could be a possible reason on why tumor cells are not recognized by cytotoxic T-cells. Osteosarcomas can demonstrate variable expression of PD-L1, which could be a potential target, however they also show loss of MHC class I protein indicating immune escape[73].

Secretory factors seem also to interfere with anti-tumor host immunity. Among them, interferon-gamma (IFN-γ) is recently described as an anti-tumor cytokine with an essential role in the polarization of T-helper 1 cells and activation of $CD8^+$ cytotoxic T-cells. However, this activation has a negative effect, as the interferon released by those cells can induce expression of PD-L1 by the tumor cells, ultimately stimulating escape from the immune response[74]. A study of Hyuang Kyu Park et al.[75] on sarcomas presented that treatment with IFN-γ increased PD-L1 mRNA levels in different types of sarcoma cell lines. Hence, combination of IFN-γ with anti-PD-L1 agents is a therapeutic possibility for sarcomas that needs further investigation.

The molecular status of tumors gained great interest in the field of immunology the past years. TMB refers to the total number of somatic mutations on coding areas of the tumor genome per megabase. The number of mutations varies among different tumor types. Tumor specific mutations may give rise to neoantigens which can be targeted by T-cells[76]. Statistically, the higher the number of neoantigens, the greater the response to treatment. Although TMB is a promising prognostic biomarker for response to immunotherapy, it does not represent direct evidence of immunogenicity and does not accurately predict the dynamic immune response[77]. Most of the clinical trials discussed in this review did not investigate the role of TMB in sarcomas. The survival benefit of axitinib when combined with pembrolizumab in metastatic sarcomas showed in the NCT02636725 study[38] could not be explained by the high percentage of $PD\text{-}L1^+$ tumor cells nor by the high TIL scores. Although those tumors lack a high TMB, neoantigens arising from the ASPL-TFE3 fusion with which they are known, could have an immunogenic function[78]. Moreover, the study from the University of Miami[42], found that angiosarcoma patients that showed CR after ICI monotherapy treatment had a very low TMB, but showed novel protein fusions and cancer-testis antigens. On the other hand, all ASPS investigated tissue samples in the study with geptanolimab (NCT03623581)[21], had a very low TMB. Although not all fusions are immunogenic, it has recently been shown that specific fusion-derived neoantigens elicit a cytotoxic T-response including tumors with low TMB. Hence, the possible immunogenicity depends on the expressed fusion protein[79].

A specific type of high TMB tumors demonstrate MSI that induce a hypermutated phenotype. Those tumors generate numerous neoantigens and are highly sensitive to ICB regardless of the tissue of origin[80]. The FDA has granted accelerated approval to pembrolizumab for pediatric and adult patients with MSI-high or mismatch repair-deficient solid tumors[81]. This is the first time the agency has approved a cancer treatment based on a common biomarker rather than an organ-based approach. In this review, only the study with geptanolimab in advanced ASPSs (NCT03623581)[21] investigated tissue samples for MSI, but all tumors were microsatellite stable. Nevertheless, this biomarker seems to play a pivotal role in selecting patients for immunotherapy and further investigation in the context of sarcoma is therefore needed.

As already mentioned, sarcomas are a very heterogenous group of tumors with different subtypes, many of which represent unique diseases with distinct biology. Generally speaking, to date there are no clearly defined biomarkers that predict clinical response of specific histologic subtypes to ICIs. Moreover, given the rarity of sarcomas, it is very difficult to investigate the different histological subtypes separately and come to definite conclusions. What one could summarize from the clinical trials described in this review is that for some histologic sarcoma types that responded in treatment with ICB, specific characteristics might be predictive to the response. Hence, PD-L1 status seems to correlate with better response of endemic/classic type KS. UPS, DDLPS, and LMS can display a "hot" immune signature, implying that the TME might be a predictive factor for these tumor subtypes. An UV-light gene signature in cutaneous angiosarcomas is mentioned as possible indicator for response to ICB. The ASPL-TFE3 gene fusion displayed in ASPSs might be partly responsible for the general good response in most clinical trials. We think that this field disserves further investigation.

6. Conclusions

Immune checkpoint blockade gains substantial ground in cancer treatment. So far, it has shown controversial results in sarcomas. The clinical trials described in this review do not reach a common conclusion or provide strong evidence for the use of any kind of immunotherapy. There are many different etiological leads that could explain those

controversies. IHC expression of PD-L1 in sarcomas does not sufficiently correlate with response to ICB in order to be used as biomarker. The potential role of IFN-γ on PD-L1 expression by sarcoma cells deserves further attention. The TME composition and TIL counts are very interesting research items for their predictive power, yet not used in daily practice. Specific mutations seem to have a predictive role, such as mutations of the CTNNBN1 or PTEN genes. TMB started as a promising factor, but nowadays we know that it cannot be used as a unique but rather as a complementary marker. MSI strongly correlates to response to ICB and patients with mismatch repair—deficient tumors can benefit from treatment with pembrolizumab regardless of tumor type. Given this complexity and the interaction of various factors in tumors in general, but especially in sarcomas, we believe that in the future a multicomponent predictive biomarker, that will determine which patients are more likely to benefit from treatment with ICIs, will be introduced.

7. References

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CHAPTER 4

RESULTS - PART I: THE ROLE OF THE TUMOR MICROENVIRONMENT IN ANTI-TUMOR ACTIVITY; THE PARADIGM OF DESMOID TUMORS

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Desmoid tumors display a strong immune infiltration at the tumor margins and no PD-L1-driven immune suppression
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1. Abstract

Desmoid tumors (DTs) are local aggressive neoplasms, whose therapeutic approach has remained unsolved so far and is controversial in many instances. Nowadays, immunotherapy appears to play a leading role in the treatment of various tumor types. Characterization of the tumor immune microenvironment (TME) and immune checkpoints can possibly help identify new immunotherapeutic targets for DTs. We performed immunohistochemistry (IHC) on 33 formalin-fixed paraffin-embedded (FFPE) tissue sections from DT samples to characterize the TME and the immune checkpoint expression profile. We stained for CD3, CD4, CD8, CD20, FoxP3, CD45RO, CD56, CD68, NKp46, granzyme B, CD27, CD70, PD-1 and PD-L1. We investigated the expression of the markers in the tumoral stroma, as well as at the periphery of the tumor. We found that most of the tumors showed organization of lymphocytes into lymphoid aggregates at the periphery of the tumor, strongly resembling tertiary lymphoid organs (TLOs). The tumor expressed a significant number of memory T cells, both at the periphery and in the tumoral stroma. In the lymphoid aggregates, we also recognized a significant proportion of regulatory T cells. The immune checkpoint ligand PD-L1 was negative on the tumor cells in almost all samples. On the other hand, PD-1 was partially expressed in lymphocytes at the periphery of the tumor. To conclude, we are the first to show that DTs display a strong immune infiltration at the tumor margins, with formation of lymphoid aggregates. Moreover, we demonstrated that there is no PD-L1-driven immune suppression present in the tumor cells.

2. Introduction

Desmoid tumors (DTs), also known as aggressive fibromatosis, represent a monoclonal proliferation of myofibroblasts arising from the musculoaponeurotic stromal element, which can develop at virtually any anatomic site. According to the newest WHO classification of soft tissue and bone tumors, DTs are neoplasms of intermediate malignant potential (locally aggressive) characterized by an infiltrative growth pattern into the surrounding normal structures, sometimes in a disruptive manner, but with no metastatic potential[1]. Nevertheless, they can be lethal for the patient due to destruction of vital tissues. Morphologically, the tumor is

composed of bland small, slender spindle cells without distinctive cytoplasmic borders. This tumor type is morphologically rather bland, since there is no nuclear pleomorphism, mitotic activity or necrosis. The aggressiveness of DTs has been proven by the infiltrative character of the neoplastic cells which invade and entrap the surrounding normal structures, with imminent risk when growing next to large vessels or other vital organs. Many things about the pathogenesis of these tumors still remain unknown. The majority of DTs are sporadic, sometimes described after local trauma, pregnancy or injury[2-4]. In a recent study, 85% of DTs have shown somatic mutations of the beta catenin gene, CTNNB1[3]. They can also develop as part of hereditary diseases, like familial adenomatous polyposis (FAP) syndrome. In FAP, a germline mutation involving the APC gene is the causative agent of the disease[2,4,5]. DTs associated with FAP syndrome, also referred to as the Gardner syndrome[6], have a predilection for abdominal localization and patients with FAP show an 80% risk for developing DTs. Both the CTNNB1 and APC genes are part of the Wnt signaling pathway[2]. Mutation of those genes can result in upregulation of β-catenin and accumulation into the nucleus where it activates Wnt pathway transcription factors[5]. Accordingly, nuclear positivity for β-catenin is an important diagnostic tool in the armamentarium of the soft tissue pathologist. Nuclear staining with immunohistochemistry (IHC) is reported in nearly 67–80% of cases[2,7]. Given the rarity of this tumor and its ability to appear anywhere in the body, the therapeutic approaches are broad. Depending on the localization of the lesion and the overall condition of the patient, different treatment options are described, ranging from surgery to chemotherapy and radiotherapy[6]. However, in many instances the tumor recurs more aggressively after treatment. Therefore, the most preferable option for stable and asymptomatic disease is close monitoring of the patient, also called “watchful waiting”[2,8]. Since treatment of DTs still remains challenging and due to its potential aggressive behavior, novel therapeutic strategies are required.

Over the past years, immunotherapy gained more and more interest among the therapeutic options for cancer treatment. Immunomodulation refers to the recruitment and activation of innate and adaptive immune cells to control the tumor growth and its metastatic potential. More recently, blockade of the immune checkpoints, cytotoxic T lymphocyte

antigen-4 (CTLA-4) and programmed death-1 (PD-1), has proven to be effective in the treatment of different tumor types, such as metastatic melanoma and renal cell carcinoma[9,10]. Given the advantages of immunotherapy in other solid tumors, sarcomas might also be a good candidate. To date, there are few randomized trials and small studies about immunotherapy in sarcomas, including osteosarcoma, synovial sarcoma and rhabdomyosarcoma (reviewed ref [11]). However, surgery for localized tumors and cytotoxic therapy for more advanced diseases remain first-line treatment for soft tissue and bone tumors. To identify new therapeutic targets for soft tissue tumors, we characterized the immune cell composition of DT tissue samples and looked at the expression of immune checkpoints in the tumor microenvironment (TME) using IHC (**figure 1**).

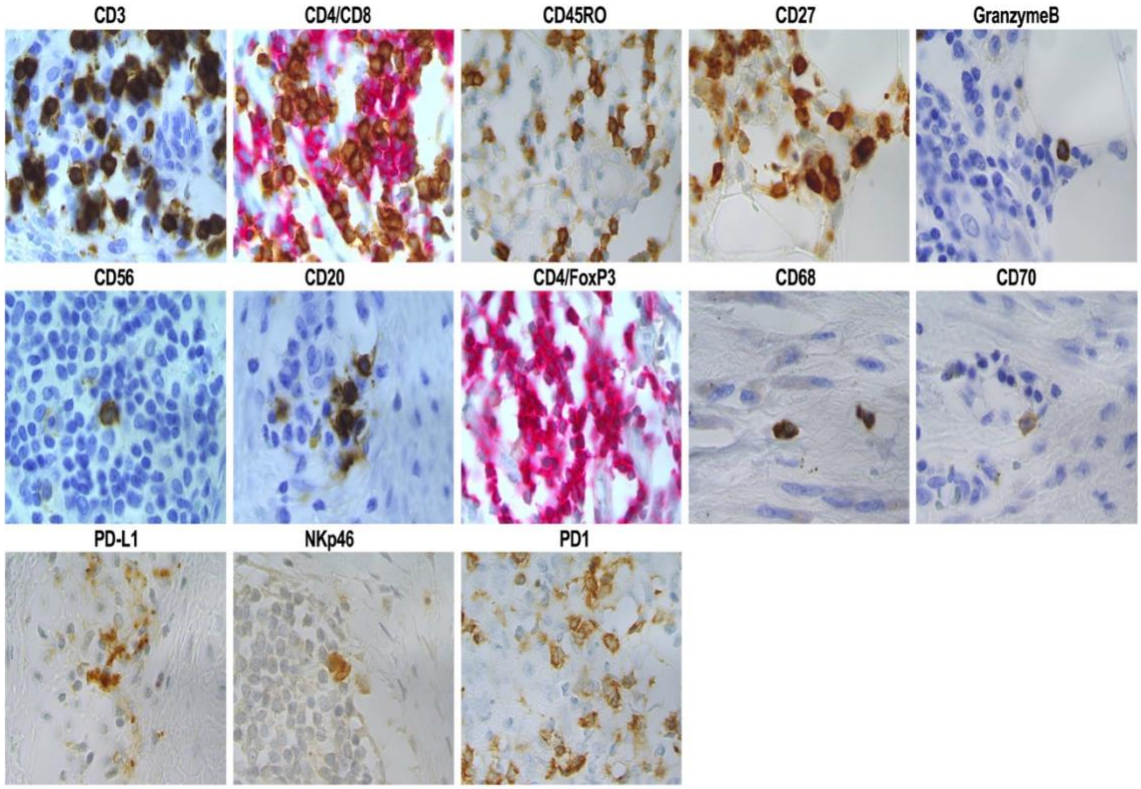

Figure 1. Representative immunohistochemical staining patterns of different immunomarkers in DT tissue samples. Dual staining has been used for CD4 (red, cytoplasmic) / CD8 (brown, cytoplasmic) as well as for CD4 (red, cytoplasmic) / FoxP3 (brown, nuclear). All tissue sections were counterstained with hematoxylin (blue color). Original magnification 1000x.

3. Materials and methods

a. Patient selection and samples

Thirty-three (33) formalin-fixed paraffin-embedded (FFPE) archival tissue samples from 27 different patients with DTs were collected. Thirty (30) of those samples were retrieved from the Department of Pathology at the Antwerp University Hospital and they were collected between 2009 and 2016. Three (3) other samples were kindly provided by the Laboratory for Pathology and Medical Microbiology (PAMM) at the Catharina Hospital in Eindhoven, collected in 2015. Twenty-four (24) of the 33 samples were excision specimens, while the other 9 samples were biopsy material (**figure 2**). The biopsy samples were fixed in 4% formaldehyde for up to 12 h, while the excision samples were fixed for up to 32 h and paraffin embedded on a routine basis.

b. Immunohistochemistry

Five-µm-thick sections were prepared from FFPE tissue blocks and baked in an oven for 2 h at 60 °C prior to staining. Following IHC, stainings were performed on a Benchmark Ultra XT autostainer (Ventana Medical Systems Inc, Roche) according to the manufacturer's datasheets: anti-CD3 (clone 2GV6, ready-to-use (RTU), Ventana, anti-CD4 (clone SP35, RTU, Ventana), anti-CD8 (clone SP57, RTU, Ventana), anti-PD-1 (clone NAT105, RTU, Ventana) and antiCD45RO (clone UCHL-1, RTU, Ventana).The FOXP3, PD-L1, CD27 and NKp46 IHC were also performed on the Benchmark Ultra XT with a protocol that was slightly adapted from the datasheet: anti-FoxP3 (clone 236A/E7, 1/50 for 40 min, Abcam) in combination with a mild pretreatment with CC1 and the UltraView detection kit, anti-PD-L1 (clone E1L3 N 1/150 for 44 min, Cell Signaling Technologies) in combination with a mild pretreatment with CC1 and the OptiView detection and Amplifcation kit, anti-NKp46 (clone 195314, 1/50 for 40 min, R&D systems) in combination with mild CC1 pretreatment and UltraView detection, anti-CD27 (clone 137B4, 1/25 for 40 min, Thermo Fisher Scientifc) in combination with a mild CC1 pretreatment and the OptiView detection and OptiView Amplifcation kit. The CD68, B catenin 1, CD20, CD56 and granzyme B IHC were performed on a Dako Omnis instrument (Agilent) in combination with the Envision Flex detection system (Agilent) with minor alterations to the manufacturer's

datasheet: anti-CD68 (clone KP-1, RTU for 25 min, Dako), anti-granzyme B (clone GrB-7 1./50 for 32 min, Dako), anti-CD20 (clone L26, RTU for 12.5 min, DAKO), anti-CD56 (clone 123C3, RTU for 30 min, Dako) and anti-beta catenin (clone beta-catenin-1, RTU for 22.5 min, Dako). CD70 IHC was performed on the Dako PT Link and Autostainer Link using the anti-CD70 (CD27 Ligand, Clone 301731, 1/40 for 20 minutes, R&D systems) as described by Jacobs et al.[12]. Upon staining, the sections were counterstained with hematoxylin as part of the automated staining protocol. After staining, slides were washed in reaction bufer (Ventana), dehydrated in graded alcohol, cleared in xylene, mounted with Quick-D Mounting Medium (Klinipath) and cover slipped. Positive controls were included in each staining and consisted of tonsil tissue or placenta tissue (specifically for PDL-1). All stained slides were assessed and scored independently by two pathologists and one scientist, as described by Marcq et al.[13]. We looked at the presence of tumor infiltrating lymphocytes and lymphoid aggregates (score 0 = 0 aggregates; 1 = 1–5 aggregates; 2=5–10 aggregates; 3=>10 aggregates). Expression of each marker in the tissue was divided into five categories (0= 1% positive cells, with specific staining of any intensity (0 = no expression, 1 = weak, 2= moderate, 3=strong) and any distribution (membrane and/or cytoplasm). These criteria were used for IHC scoring of all the different markers[13]. An Olympus BX41 microscope was used for scoring of the tissue sections. Pictures were made using the Leica acquisition software v4.

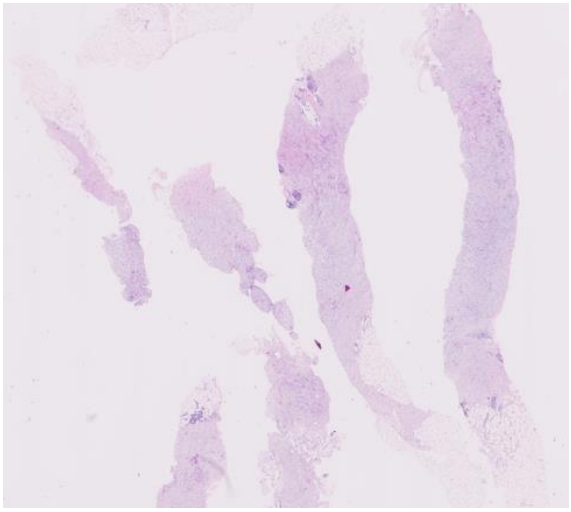

Figure 2. Overview Hematoxylin/Eosin staining of a biopsy specimen (5x).

c. Molecular test

For CTNNB1 mutation analysis, DNA was extracted from FFPE tissue blocks using the QIAmp DNA FFPE tissue kit (Qiagen), according to the manufacturer's instructions. CTNNB1 mutations in exon 3 were investigated using highresolution melting analysis (HRMA) on a lightCycler 480 instrument (Roche Diagnostics Gmbh), using the following primers: 5′-GTAAAACGACGGCCAGAGTCACTGGCA GCAACAGT C-3′ and 5′-AGCGGATAACAATTTCAC ACAGGTCTTCCTCAGGATTGCCTT-3′. High-resolution melt analysis (HRMA was assessed using GeneScanning software (Roche). HRMA products with a deviating CTNNB melting pattern were directly purifed using ExoSAP-IT (Afymetrix) and the purifed PCR product was used as template for direct Sanger sequencing (Big Dye Terminator v1.1 kit (Applied Biosystems) using M13 tag primers (Eurogentec, Seraing, Belgium) on a 3130 XL Genetic Analyzer (Applied Biosystems)). Sequencing data were analyzed using SeqScanner software (Applied Biosystems). The material harboring a mutation was further analyzed with next-generation sequencing (NGS) to retrieve the mutation status of the tumors. Next-generation sequencing was performed using a HaloPlex custom panel (Agilent) on a MiSeq instrument (Illumina). Variant analysis was performed using SeqNext software (JSI).

d. Statistics

Associations of immunological markers, such as CD3, CD4 and CD20, with clinicopathological parameters of DT patients were investigated by $\chi 2$ analysis or Fisher's exact test (when appropriate). Spearman correlation coefficients (R) were calculated to investigate the correlation between the expression of immunological markers within DT specimens. All analyses were performed using SPSS version 23 and significance was reached if $P < 0.05$ (two-tailed).

4. Results

a. Patient characteristics

The clinicopathological characteristics of our patient cohort are summarized in **table 1**. Thirty-three samples from 27 different patients were included in this study. Within our patient cohort, there was a slight

female predominance with a female/male ratio of almost 1.5/1. The age at the time of the initial diagnosis ranged from 18 to 70 years with a median of 44.8 years. Almost half of the patients (13/27) had a tumor that was located intra-abdominally (abdomen and mediastinum). Some of the tumors seem to have occurred after intra-abdominal surgery, including gastric bypass, whipple surgery and liver transplantation. For the extra-abdominal DTs, five were located on the proximal arm and shoulder, three on the proximal leg and two on the breast. Both breast tumors occurred after operation in this region, one after a breast prosthesis and one after breast amputation. The rest of the DTs were in different locations. All but one of the extra-abdominal tumors were located in the subcutis or within the muscle and only one was cutaneous. Two patients were known to have FAP syndrome.

The diagnosis of all the tumors was confirmed histologically by morphology and by IHC for the beta-catenin antibody. Also, molecular analysis was performed. The materials were all examined for mutations on exon 3 of the CTNNB1 gene. One sample was not informative. Of the informative samples, almost 60% of the patients had the mutation, while 40% were wild type. IHC showed at least focal nuclear positivity of beta-catenin in the tumor cells, confirming the diagnosis in all cases, and also those with negative molecular results.

Table 1: clinicopathological parameters.

CHARACTERISTICS	**N**
Age (years)	
Median	44,8
Range	18-70
Sex	
Male	11
Female	16
Localisation tumor	
Extra-abdominal	14
Intra-abdominal	13
Tumor size	
<5 cm	17
≥5 cm	8
NA	2

Treatment	
Excision and FU	23
Systemic	4
History	
Not relevant	19
Relevant	6
FAP syndrome	2
Mutation	
Wt	10
Mutated	16
NI	1
Total number	27

Abbreviations: NA; not applicable, FU; follow up, FAP; familial adenomatous polyposis, NI; not informative

b. Immune composition of the tumor samples

Of all 33 samples, 24 came from excision specimens and 9 from biopsies. The tumor itself displayed no remarkable inflammatory infiltrate. According to our observation, a less described although constant finding in DTs is the presence of a lymphoid infiltrate in the form of lymphoid aggregates, usually at the periphery of the tumor adjacent to the surrounding tissue. Those lymphoid aggregates, defined as a group of 50 or more inflammatory cells[13], are mostly seen in the immediate proximity with small- to medium-sized blood vessels and it usually spreads in the tissue between adjacent vessels (**figure 3**). In our cases, we found such an inflammatory response at the periphery of the tumor in 88% of tumor specimens in the form of small lymphoid aggregates. The presence of germinal centers within the lymphoid aggregates was noted in two of our samples. B cell marker CD20 was present in almost 86% of the specimens. There was a remarkable contrast between CD20 expression in lymphoid aggregates and stromal lymphocytes (**figure 3**). Those 86% of the cases showed at least moderate CD20 positivity in the lymphoid aggregates. On the other hand, in only 12% of the cases, stromal lymphocytes demonstrated low CD20 (score 1) immune reactivity.

All other samples showed no expression for CD20. T cell marker CD3 was found in all samples, on lymphocytes in the aggregates as well as in

the stroma. Although CD4 and CD8 were almost equally strongly expressed on lymphocytes in the lymphoid aggregates (**figure 3**), this was not the case for stromal lymphocytes (**Figure 4, Table 2**). The majority of samples (73%) had no CD4+ stromal lymphocytes, while another 97% of samples did display CD8+ stromal lymphocytes. Regulatory T cells, identified by CD4+FoxP3+ cells, were seen in 83% of the lymphoid aggregates, while no CD4+FoxP3+ cells were found in the stromal lymphocytes. CD45RO, a marker for effector and memory T cells, was expressed on the lymphocytes of the lymphoid aggregates in all our samples (**figure 3**) and on the stromal lymphocytes in nearly all of the samples (**Figure 4, Table 2**). Regarding the lymphoid aggregates, CD45RO was strongly present (score 4) in almost 93% and moderately present (score 3) in the rest of the cases. In the stroma, the CD45RO scores were distributed between low to strong expression. A significant positive correlation between CD8 and CD45RO expression ($p=0.009$, $R2=0.448$) could be found in the stroma, while there was a negative correlation between CD8 and CD4 expression ($p=0.000015$, $R2=-0.678$) in the stroma. This may indicate that the TILs in the stroma are CD8+ memory T cells. We have also found a strong correlation between CD4 and CD45RO expression in the lymphoid aggregates ($p=0.056$, $R2=0.358$) which suggests the presence of CD4+ memory T cells.

Although no CD68+ cells were found in the stroma, almost all samples (97%) showed CD68 expression in lymphoid aggregates (**figure 3**). The morphology of those CD68+ cells was consistent with those of histiocytes. A significant correlation between CD68 and CD4+/FoxP3+ cells were found ($p=0.038$, $R2=0.386$) in the lymphoid aggregates.

Natural killer (NK) cells were not remarkably present in our samples. This was shown both with CD56, a homophilic binding glycoprotein whose expression is strongly associated with NK cells, and with NKp46. NKp46, a killer activation receptor expressed on the plasmatic membrane of NK cells, was not expressed on stromal lymphocytes and only low (score 1) expression was demonstrated in 14% of the aggregates. CD56 showed similar results with only one case showing mild expression of CD56 in the aggregates. Comparable results could be demonstrated for granzyme B, a protease expressed in the granules of different type of cells as cytotoxic lymphocytes and NK cells. There was

no positivity for granzyme B in the stroma and only scattered positive cells could be observed in the aggregates.

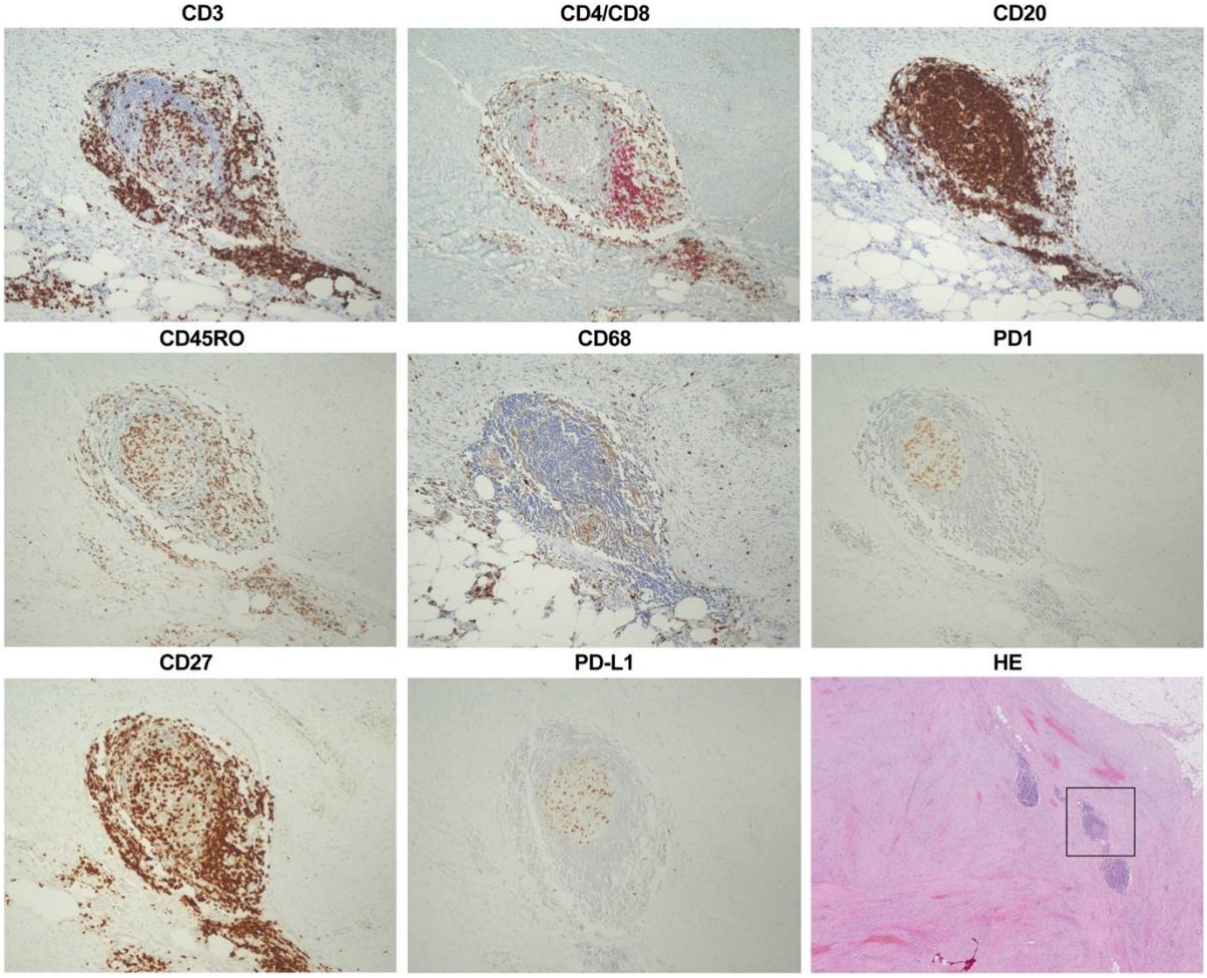

Figure 3. Immune composition of a representative lymphoid aggregate at the periphery of the tumor. Dual staining was used for CD4 (red, cytoplasmic)/CD8 (brown, cytoplasmic). All tissue sections for immunohistochemistry were counterstained with hematoxylin (blue color). Original magnification Å~ 200. An overview picture of the hematoxylin/eosin (HE) staining is depicted on the right, below. Original magnification 50x.

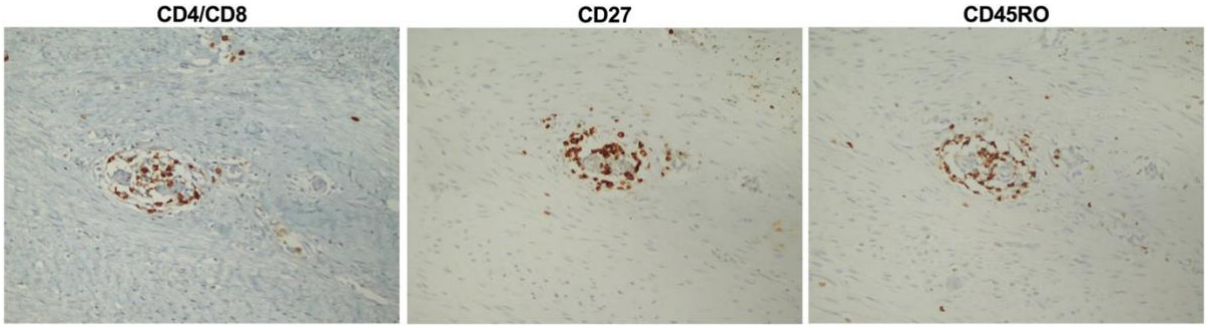

Figure 4. Immune composition of the lymphocytic infiltrate in the tumoral stroma. Dual staining was used for CD4 (red, cytoplasmic)/CD8 (brown, cytoplasmic). No CD4 positivity could be detected in the tumoral stroma. All tissue sections were counterstained with hematoxylin (blue color). Original magnification 200x.

c. Immune checkpoint expression

CD27, a TNF receptor superfamily member, was also a very important component of the lymphocytes, mainly those present in the lymphoid aggregates (**figure 2**): 93% of the samples had CD27-positive aggregates (**Table 2**). Interestingly, 39% of the cases had no CD27+ stromal lymphocytes (**figure 4**). CD70 is a ligand for CD27 and is transiently expressed on activated lymphocytes. In our samples, CD70 was mild to moderately positive (score 2 and 3) in almost 52% of the aggregates, while it was absent in the stromal lymphocytes in all but one case. Moreover, no expression of CD70 on the tumor cells was seen. CD70 positivity in the lymphoid aggregates was strongly correlated with CD68 ($p=0.032$, $R2=0.400$), CD20 ($p=0.009$, $R2=0.475$) and granzyme ($p=0.021$, $R2=0.467$). On the other hand, CD27 in the tumoral stroma was correlated with CD45RO ($p=0.008$, $R2=0.457$) and CD20 ($p=0.082$, $R2=0.308$). The expression of the immune checkpoint PD-1 and its ligand PD-L1 was evaluated both in the lymphocytes and in tumor cells. PD-1 showed expression in the lymphoid aggregates (**figure 2**), but rather within the lower scores; no positivity within score 4 was seen. In the tumoral stroma, only 1 out of the 33 cases exhibited mild positivity (score 2), while the rest were negative or very lowly expressed (score 1).

Interestingly enough, 40% of the tumor cells were also positive (33%, mainly score 2). All of the samples with lymphocytes in the stroma as

well as half of the samples with lymphoid aggregates were negative for PD-L1. Tumor cells did also not express PD-L1 in 94% of the cases, while one case displayed score 1 and another one score 3. We found a strong correlation between PD-1 and CD70 expression in het lymphoid aggregates (p=0.015, R2=0.448). Finally, a correlation could be found between PDL1 positivity in the tumor cells and CD27 expression in the tumoral stroma (p=0.030, R2=0.379).

d. Mutation analysis

PCR analysis was performed on all samples. Nineteen samples from 16 patients were mutated. Next-generation sequencing (NGS) analysis showed beta-catenin missense mutations in codons 41 and 45 of exon 3 (identified as c.121 A4G or pThr-41-Ala (T41A), c.133T4C or pSer45Pro (S45P), and c.134C4T or pSer45Phe (S45F)). The T41A mutation was the most common, occurring in 52% of the mutated samples, whereas the S45P and S45F mutations occurred in 16% of the same samples. The other samples were not informative for the NGS. We have identified a significant association between the extent of the lymphocytic infiltrate in the stroma and the mutation status. It was seen that patients with the S45F mutation were lacking an immune infiltrate in the stroma (p=0.052). Moreover, all mutated samples (n=16), independently of the codon that was involved, showed a very low PD-L1 expression in lymphoid aggregates (p=0.022).

e. Clinicopathological parameters

Within the clinical parameters, the one which is mostly correlated with the immune profile of the lesions is the tumor size. Bigger lesions contain a higher percentage cytotoxic T cell in the lymphoid aggregates, matching with score 4 ($p<0.001$). On the other hand, smaller tumors contain a higher percentage of lymphocytes (p=0.022). Additionally, smaller tumors demonstrate more PD-1+ lymphocytes in the aggregates (p=0.015), as well as more B cells (indicated by IHC for CD20) (p=0.036).

5. *Discussion*

We report a comprehensive study of the TME in DTs. To our knowledge, we are the first to characterize the immune microenvironment in DTs and to investigate the immune checkpoint expression in these tumors. Thereby, we are the first to demonstrate the presence of CD4+ regulatory T cells in the lymphoid aggregates, while in the stroma a predominance of CD8+ memory T cells could be noted. Moreover, we showed that although almost no PD-L1 was seen in the lymphocytes and in the tumor cells, PD-1 was clearly present in both these cell types.

Several categories of immune cells can be found in a tumor and they can be located in the center, in the invasive margin or in the adjacent tertiary lymphoid organs (TLOs). The majority of our samples showed an inflammatory response relying mainly on lymphocytes. In more than 85% of the samples, lymphoid aggregates were seen mainly localized around small- to medium-sized blood vessels at the tumor invasive margin in association with the surrounding normal tissue. Almost 86% of the samples with lymphoid aggregates showed high expression of CD20 (located on the surface of B cells) in the aggregates. T cells, marked by the expression of CD3, were also abundantly present in the lymphoid structures. It is well known that normal lymphoid structures, such as lymph nodes (LN), and other lymphoid organs, such as Payer's patches, are also composed of B and T cells that are required for defense against pathogens. Those structures are known as secondary lymphoid organs (SLO). The organization of lymphoid tissue with B and T lymphocytes around specialized blood vessels, as we also describe in our samples, mimics the SLOs and represents the TLOs or ectopic lymphoid tissue. Those blood vessels in the TLOs are specialized high endothelial venules (HEV)[14]. They seem to play a significant prognostic role in studies for breast carcinoma and melanoma, where the density of HEVs alone predicted patient's outcome[15,16]. TLOs are formed to mimic the SLOs at the lesion border, initiating the recruitment of hematopoietic cells through a complex procedure involving a chemokine-directed positive feedback loop[17,18] and drive adaptive, antigen-specific immune response[17]. Those TLOs attract naive B and T cells which eventually will evolve into memory B and T cells after antigen proceeding. The lymphoid aggregates in our samples have a composition that highly

relate to TLOs. Moreover, all those lymphoid structures in our material contained a high percentage of CD45RO+ cells. The strong associations that we found between CD4 and CD45RO suggest the presence of CD4 memory T cells in the lymphoid aggregates. However, to confirm the presence of TLOs in our DT samples, further characterization is needed because apart from B and T cells, TLOs are further composed of mature dendritic cells (DCs). Especially, the presence of mature DCs in the intratumoral lymphoid structures was proven to be a better predictor of clinical outcome in patients with non-small cell lung cancer[19,20].

Table 2. Expression of immune checkpoints and immune cell markers in desmoid tumors.

n, % samples	CD3	CD20	CD4	CD4/FOXP3	CD8	CD45Ro
total (n=33)	33 (100%)	25 (86,2%)	29 (87,9%)	24 (72,7%)	32 (97%)	33 (100%)
Tumor cells (n=33)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
immune cells in stroma (n=33)	33 (100%)	4 (12,1%)	9 (27,3%)	0 (0%)	32 (97%)	33 (100%)
lymphocytes in lymphoid aggregates (n=29)	29 (100%)	25 (86,2%)	29 (100%)	24 (82,6%)	29 (100%)	29 (100%)

n, % samples	NKp46	CD68	Granzyme	CD27	CD70	PD-1	PD-L1
total (n=33)	4 (12,1%)	28 (84,8%)	13 (39,4%)	28 (84,8%)	15 (45,5%)	27 (81,8%)	15 (45,5%)
tumor cells (n=33)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	13 (39,4%)	2 (6,1%)
immune cells in stroma (n=33)	0 (0%)	0 (0%)	0 (0%)	20 (60,6%)	1 (3%)	10 (30,3%)	0 (0%)
lymphocytes in lymphoid aggregates (n=29)	4 (13,8%)	28 (96,6%)	13 (44,8%)	27 (93,1%)	15 (51,7%)	24 (82,6%)	14 (48,3%)

The development and function of regulatory T cells (Tregs) depend on the transcription factor FoxP3. Naïve CD4+ T cells convert into Tregs after transcription of FoxP3[21]. In our series, a subset of CD4+ T cells was also positive for FoxP3 in the lymphoid aggregates. Those double CD4- and FoxP3-positive lymphocytes in the aggregates suggest the presence of Tregs that are known for their pivotal role in maintaining immunologic tolerance[22]. Moreover, we found a strong association between the presence of Tregs and macrophages, marked by CD68, in the lymphoid structures. The presence of macrophages in the tumor microenvironment and their association with Tregs has been investigated the past years. Macrophages are known for their plasticity. In the tumor environment, they have the ability to transform to tumor-associated macrophages (TAMs) that enhance tumor progression[23,24]. By secreting chemokine CCL22, Tregs are attracted in the tumor environment[23,25,26]. This interaction could very well explain the significant association between CD4+FoxP3+ cells and CD68+ cells that we found in our samples.

Signaling of CD27, a TNF receptor superfamily member, increases T cell expansion and function and is of importance in the maintenance of T cell memory[27-29]. Ruprecht and coworkers showed that in synovial fluid from patients with juvenile idiopathic arthritis, CD27 can also be used to as a marker for Tregs and can differentiate Tregs from activated effector T cells (Tef)[30,31]. Furthermore, Duggleby et al.[32] described that for freshly isolated Tregs, only CD27 expression correlates with regulatory activity and could be used to isolate cells with regulatory activity from CD4+ CD25+ cells. Also, cells expressing high levels of FoxP3 were confined to the CD27+ population. Within our research, we also could demonstrate that the CD4+FoxP3+ cells in the lymphoid aggregates were strongly correlated with CD27 expression in those aggregates, emphasizing the high probability that the observed CD4+FoxP3+ cells are indeed the Tregs. Different observations regarding the immune cell composition were made for the tumoral stroma compared to the lymphoid aggregates. No B cells were found in the stroma and most of the CD3+ cells here were CD8+ T cells. Nevertheless, there was a significant association between the presence of CD3+, CD8+ and CD45RO+ cells in the stroma, which implies the presence of CD8+ memory T cells in the stroma.

The role of cytotoxic memory T cells in tumors has been a great subject of investigation over the recent years. The main research was done on colorectal cancer where Bernard Mlecnik et al.[33] showed in 2011 that the density of CD8+ memory cells was associated with low rates of tumor recurrence and that the assessment of CD8+ memory T cells in combined tumor regions provides an indicator of tumor recurrence beyond that predicted by AJCC/UICCTNM staging. It seems that CD8+ memory T cells have a more direct role in killing the cancer cells, while CD4+ T helper cells function in a more complicated way[34]. Naïve CD4 T cells differentiate into T helper type 1 (TH1) cells and produce interferon gamma, which promotes CD8 T cell-mediated adaptive immunity[35]. Many other studies on different tumor types underscored the participation of CD45RO memory T cells as crucially important for favorable patient outcome[20,33-40]. Further research is needed to establish the prognostic value of stromal CD8+ memory T cells in the DTs.

In our samples, there was only limited CD56 positivity in both the lymphoid aggregates and the stroma, while NKp46 staining was absent. NKp46 receptor is considered to be the major lysis receptor for NK cells, capable of mediating direct killing of virus-infected cells and tumor cells[41]. The absence of NKp46 might be explained by the fact that NK cells are not attracted to being infiltrated in the tumor through inactivation of their receptor ligands or through secretion of immunosuppressive molecules[42]. Tumor size was significantly correlated with the expression of both CD8 and PD-1 in the lymphoid aggregates. Interestingly, those two parameters were negatively correlated with each other and with the tumor size. As such, this suggests that lymphoid aggregates of bigger tumors contain more CD8+ T cells and less PD-1 positivity. Although the presence of CD8 cytotoxic cells in the tumoral stroma has been negatively correlated with the tumor size in patients with breast carcinoma[43], we found that larger tumors contain more CD8 cytotoxic lymphocytes. However, in our cases it concerns CD8 cytotoxic lymphocytes in the lymphoid aggregates at the periphery of the tumors and not in the tumoral stroma. In the cancer–immunity cycle[44], it is described that for the T cells to kill the cancer cells, T cells should recognize the cancer cells. This could in our cases explain why although there are CD8 cells in the lymphoid aggregates, the tumor continues to grow. It is possible because those immune cells do not

recognize the tumor antigens. PD-1 is known to be a marker of exhausted CD8+ cells[45]. In our cases, CD8+ cells do not show PD-1 expression, suggesting that they do not have an exhausted phenotype. Another feature we observed in the tumors we investigated is the correlation of CD45RO with CD27 in the tumoral stroma and PD-L1 in the tumor cells. This may imply that the PD-L1 expression in the tumor cells is associated with the presence of memory T cells.

Lazar et al.[3] described that the S45F mutation is a prognostic factor strongly associated with recurrence in patients suffering from DTs. We found that all patients with mutations of the codon 45, mainly the S45F mutation, show almost no or very limited lymphocytic infiltrate in the tumor. Although our sample size is rather limited, this finding correlates with the results of Lazar et al. A low number of lymphocytes in the tumor microenvironment indicate limited antitumor defense, which may indirectly explain the greater incidence of recurrence in tumors with this mutation.

6. Conclusion

To conclude, we are the first to describe the immune composition in DTs. We showed the presence of lymphoid structures at the periphery of the tumor, strongly resembling TLOs that serve as ectopic lymphoid tissue, helping in recruitment of lymphoid cells at the tumor. Moreover, we demonstrated the presence of memory T cells both in the lymphoid aggregates and in the tumoral stroma. The lymphoid aggregates contain also a significant number of Tregs. However, while immune cells are clearly present in the lymphoid aggregates, a strong stromal antitumor immune response is lacking. There was no PD-L1 expression on tumor cells, and PD-1 was partially expressed in the lymphoid aggregates but very limited in the tumoral stroma, which means that DTs are possibly not the best candidates for immune checkpoint blockade. Thus, we conclude that further research into other immunotherapeutic targets for DTs is needed to trigger the immune system.

7. References

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CHAPTER 5

RESULTS - PART II: PD-1, PD-L1, IDO, CD70 AND MSI AS POTENTIAL TARGETS TO PREVENT IMMUNE EVASION IN SARCOMAS

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PD-1, PD-L1, IDO, CD70 and MSI as potential targets to prevent immune evasion in sarcomas
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1. Abstract

Soft tissue and bone sarcomas are rare entities, hence standardized therapeutic strategies are difficult to assess. Lack of effective therapy results in a low 5-year survival rate for metastatic disease. Immunotherapy shows good response rates in certain tumor types and tumors with Microsatellite Instability (MSI). Finding potential targets to prevent immune evasion in sarcomas could help introduce immunotherapy for these tumors.

We performed immunohistochemistry on 68 sarcoma tissue samples to identify pathways that are involved in immune evasion. We evaluated the expression of PD-1, PD-L1, IDO and CD70 on lymphocytes in the tumor microenvironment and the tumoral stroma, as well as on the tumor cells. We also investigated the MSI status with molecular technique. Finally, we correlated the results with clinicopathological parameters.

We found that sarcoma cells attract immune cells, while lymphocytes often display an exhausted phenotype. All three pathways, PD-1/PD-L1, IDO and CD70 are at play in evasion of sarcomas in general. Soft tissue sarcomas show more often an inflamed phenotype in comparison to bone sarcomas. Specific histologic sarcoma types express the different markers more than other types. Finally, we are the first to present an MSI-high Kaposi sarcoma. Our data show that immune evasion occurs in sarcomas but specific histologic types might be benefit from immunotherapy, for which further investigation is needed.

2. Introduction

a. Current treatments for sarcoma

Soft tissue and bone sarcomas are rare neoplasms accounting for less than 1% of all malignancies[1]. Despite their rarity, both benign and malignant soft tissue and bone tumors consist of more than 200 different entities[2]. Diagnostic criteria, prognostic factors and standardized therapeutic strategies are thus difficult to assess for each separate entity. Localized and low-grade tumors are preferable treated with surgery. Neo-adjuvant radiation or chemotherapy is applied in large tumors where surgery can be mutilating. Radiation and/or chemotherapy can also be used in an adjuvant setting, in cases of unresectable tumors[3,4].

Tumors that are chemo-sensitive are usually treated with chemotherapy in a neo-adjuvant setting, as for instance rhabdomyosarcoma, myxoid liposarcoma, osteosarcoma and Ewing sarcoma[4,5]. A minority of tumors with a known driver oncogenic mechanism can be treated with oncoprotein specific targeted therapy. For example, cKIT/PDGFβ mutant driven Gastrointestinal Stromal Tumors (GISTs) are excellent responders to imatinib[6]. Nevertheless, lack of effective therapy results in a low 5-year survival rate of less than 16% for metastatic sarcomas in general[7].

b. Immunotherapy: immune checkpoints and Microsatellite Instability (MSI)

According to the National Cancer institute there are different categories of immunotherapy, including T-cell transfer therapy, monoclonal antibodies, cancer treatment vaccines, immune system modulators, and immune checkpoint blockade (ICB)[8]. ICB is nowadays used in tumor treatment protocols, increasing the therapeutic options for many patients with diverse solid tumor types such as melanomas[9], lung carcinomas[10] and clear cell renal carcinomas[11] among others.

Programmed death-1 (PD-1) is a receptor expressed on the surface of immune cells, mainly T cells. Binding to its ligand, programmed death – ligand 1 (PD-L1), results in a signal transduction cascade that functionally shuts down an adequate immune response. Under normal circumstances, normal cells use this interaction with the immune cells in order to escape an excessive immune response[12]. However, tumor cells can hijack this mechanism by overexpressing PD-L1 on their surface. Inhibitors that block the interaction between PD-1 and PD-L1 showed substantial and durable response in the treatment of metastatic tumors and were therefore declared as the major breakthrough of the last decade.

Next generation immune checkpoint modulators have also been described[13]. Among them is Cluster of Differentiation 70 (CD70), a type II transmembrane glycoprotein belonging to the tumor necrosis factor (TNF) superfamily. It is expressed on antigen presenting cells and rapidly induced on both T and B cells upon activation of these cells[14]. CD70 has a stimulatory effect, leading to naïve and cytotoxic T cell

proliferation. Upon constitutive overexpression, CD70 can facilitate tumor cell evasion from the immune system by three important mechanisms: induction of T cell apoptosis, T cell exhaustion and increasing the amount of suppressive Tregs[15-17]. The constitutive overexpression of CD70 on tumor cells and its absence on normal tissue has led to the development of two different anti-CD70 monoclonal antibodies (mAb), SGN-CD70A and ARGX-110. Both antibodies are currently being tested in clinical trials[18-20].

Another player in the field is Indoleamine 2,3-dioxygenase (IDO). IDO can suppress the immune cell function by reducing the levels of tryptophan. There are two major IDO pathways. Tumor cells are shown to upregulate IDO expression which initiates the breakdown of tryptophan in the tumor microenvironment (TME) into kynurenine. Kynurenine suppresses effector T cells and promotes the activity of Tregs[21-24] which results in an ineffective anti-tumor immune response. Secondary, it promotes the activity of T regulatory cells (Tregs) that suppresses the immune response[25]. IDO expression is upregulated in several types of cancer[26]. It has been shown that inhibition of subtype IDO1 can synergize with ICB. The main effect of ICB is removing the negative effect of tumor cells on immune cells, but it also stimulates the production of IDO, which switches-off the immune response in a negative feedback loop[27]. Moreover, IDO has been reported to induce resistance to anti-CTLA-4 therapy in mice[28]. Pre-clinical and clinical trials suggest that IDO-targeting drugs should enhance ICB efficacy[27]. Moreover, IDO expression in tumor cells confers resistance to chemotherapy drugs, independent of its immune regulatory function[29,30].

These three different pathways of immune evasion are schematically represented in **figure 1**.

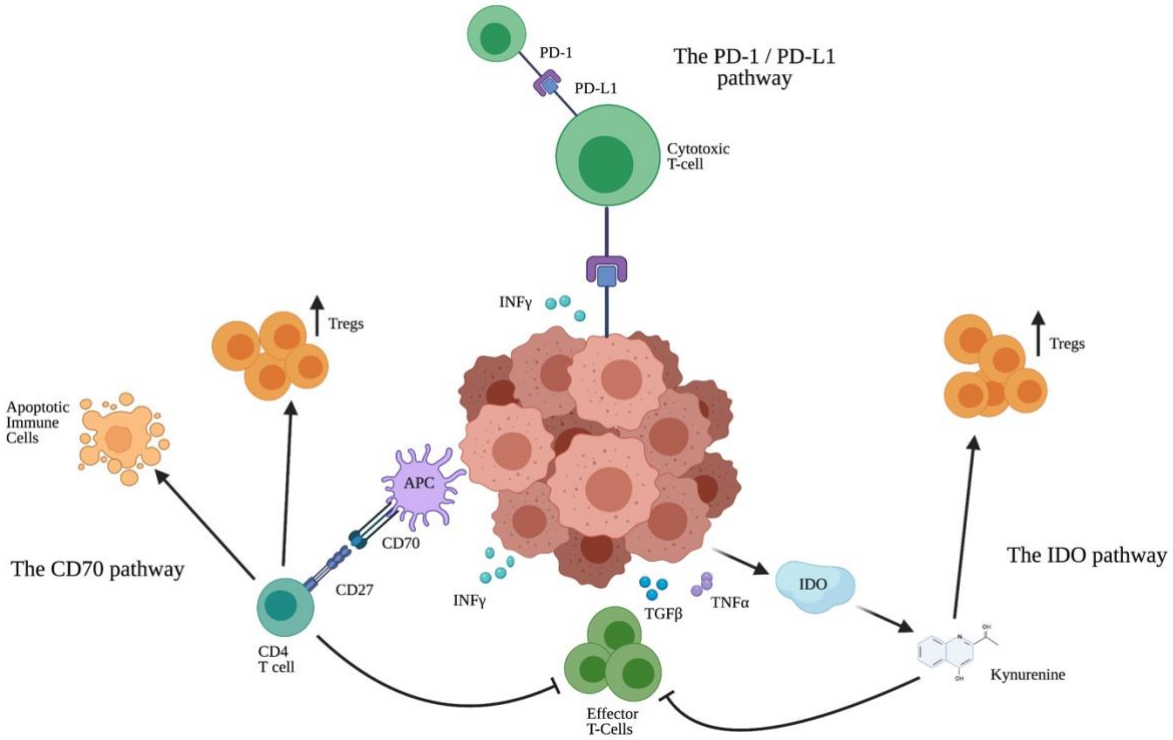

Figure 1. Schematic representation of three pathways of tumor evasion: the CD70, the PD1/PD-L1 and the IDO pathway. *Abbreviations*: Tregs; T regulatory cells, APC; antigen presenting cell
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The molecular status of tumors also plays a role in response to immunotherapy[31]. A specific type of hypermutated tumors are those with Microsatellite Instability (MSI). Given the hypermutated status, those tumors present many neo-antigens that can trigger immune response. Those tumors have shown very good response rates when treated with ICB[32]. Thus, the Food and Drug Administration (FDA) has given approval to pembrolizumab, an anti PD-1 agent, for paediatric and adult patients with MSI-high or mismatch repair-deficient solid tumors[33]. **Figure 2** is a schematic presentation of the role of MSI in immune response and immunotherapy.

Immunotherapy is not a standard therapeutic option in sarcomas. Results from clinical trials were not encouraging[31]. The relationship between response and the presence of known immune modulatory biomarkers is

not well documented. Moreover, the MSI status has not been extensively investigated in sarcomas but given its pivotal role in selecting patients for immunotherapy, investigation of this biomarker in sarcomas is needed. In summary, the immune checkpoints CD70, IDO, PD-1 and PD-L1 can induce immune evasion, while inhibitors of those proteins are used in clinical trials or in daily routine practice for the treatment of many tumor types. Moreover, patients with MSI-high or mismatch repair–deficient solid tumors can benefit from ICB with pembrolizumab (anti PD-1)[32]. Hence, in this paper we aim to investigate the expression of the immune checkpoints CD70, IDO, PD-1 and PD-L1 on sarcoma tumor cells and in the TME by means of immunohistochemistry (IHC). Moreover, we also examined the microsatellite status of the tumors with molecular technique.

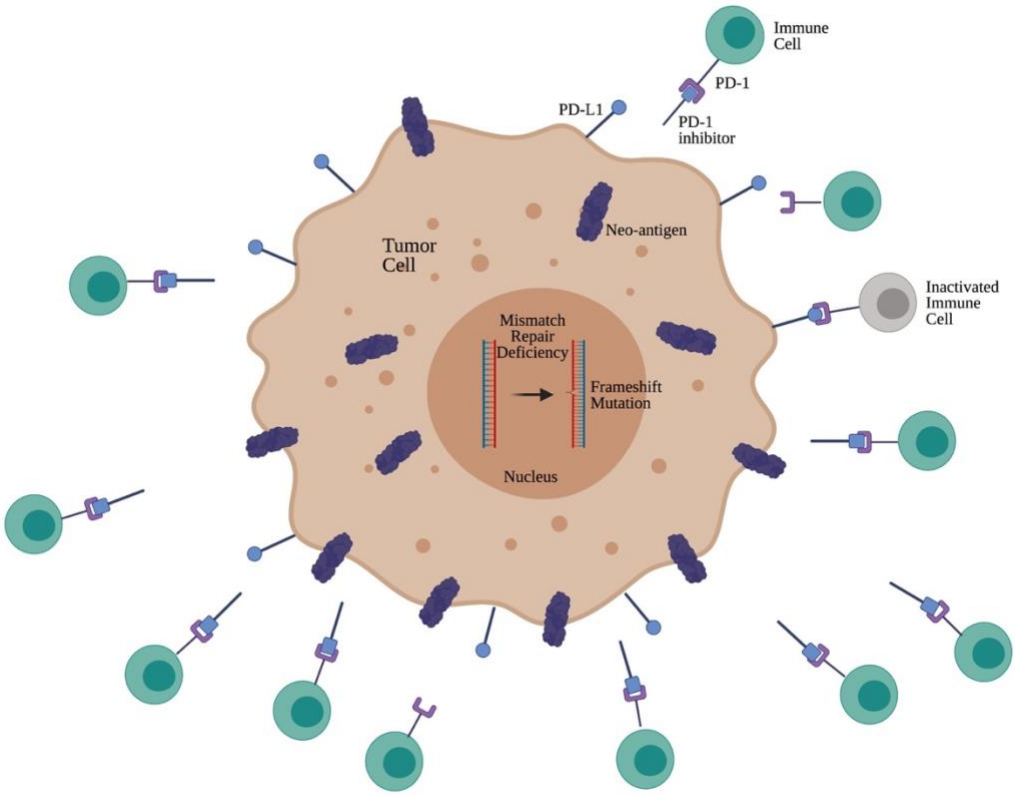

Figure 2. Microsatellite Instability (MSI) and PD-1 blockade. MSI results in production of numerous neo-antigens. Those will elicit immune cells in the tumor microenvironment. Immunotherapy with PD-1 inhibitors aims to block the PD-1 / PD-L1 interaction, keeping immune cells active against tumor cells. Created with BioRender.com

3. Materials and Methods

a. Patient selection and samples

Archival formalin-fixed paraffin embedded (FFPE) tissue samples from 68 patients with soft tissue and bone tumors were retrieved from the Department of Pathology at the Antwerp University Hospital. They were collected between 2016 and 2018. The tissue samples were collected from biopsy material as well as excision specimens. The biopsies were fixed in 4% formaldehyde for up to 12 hours while the excision samples were fixed for up to 32 hours and were afterwards embedded in paraffin on a routine basis.

We received approval by the Ethics Committee of the Antwerp University Hospital/University of Antwerp (EC 18/45/517) to use historical samples. As it was a retrospective study on archival material, no informed consent of the patients could be obtained.

b. Immunohistochemistry

Four µm-thick sections were prepared from FFPE tissue blocks and baked for 15min at 60°C. IDO1 IHC was done with IDO1 AB(SP260 Mab, M5600, SpringBio, 40min AB1/100) with 4 min pretreatment using protease 1 (Ventana). Staining was performed using the Ultraview detection system on a BenchMark Ultra instrument (Ventana Medical Systems Inc, Roche). PD-L1 and CD45 IHC were performed on an Omnis instrument (Agilent) with the PD-L1 AB (28-8 clone, Abcam, 20min AB1/50) and a CD45 AB (clone 2B11+ PD7/26, RTU, Omnis) respectively, in combination with the Envision Flex+ detection system. For PD-L1 an additional 10 min rabbit linker incubation was performed. Pretreatment consisted of Low pH reagent (Agilent, 30min 95°C). For CD45 no rabbit incubation was performed. Pretreatment in this case consisted of High pH reagent (Agilent, 30min 95°C). PD-1 IHC was performed using Anti-PD-1 (NAT105 clone, Cell Marque, 1/200 20 min) on an Omnis Apparatus in combination with the Envision Flex detection kit. Pretreatment consisted of 30min incubation with Low pH at 95°C. CD70 IHC was performed using the CD27 ligand antibody (clone 301731 R&D systems MAB2738, 1/40 for 20min) on an Autostainer48 in combination with the Envisio Flex detection kit. Pretreatment was performed on a PT Link instrument using High pH

reagents (Dako, 30min at 95°C). Upon staining the sections were counterstained with hematoxylin, washed in reaction buffer, air dried for 15 min at 60°C and further processed using the Coverstainer instrument (Agilent).

Tonsil and appendix were used as a positive control for all stainings. All stained slides were assessed and scored independently by two pathologist and one scientist.

First of all, we evaluated the immune response of the tumor. CD45 staining was done to better understand and evaluate the immune infiltrate in the tumor. CD45 is a leukocyte common antigen that is expressed on almost all hematopoietic cells except for mature erythrocytes and megakaryocytes [34]. We looked for immune response in the different compartments of the tumor microenvironment (TME). First of all, we evaluated the lymphocytic infiltrate in the peritumoral stroma (PS), namely the stroma that surrounds the tumor. PS was defined as the area around the tumor, when the tumor reaches to the middle of a 20x microscopic field; the lymphocytic infiltrate was evaluated as follows: score 0 = no lymphocytes; score 1 = low lymphocytic density; score 2 = intermediate lymphocytic density; score 3 = high lymphocytic density. Moreover, we looked at the presence of lymphoid aggregates (LA) within the PS (no or score 0 and yes or score 1). Furthermore, we investigated the presence of tumor infiltrating lymphocytes (TILs), namely the lymphocytic immune infiltrate in the intratumoral stroma (IS), as follows: score 0 = no lymphocytes; score 1 = low lymphocytic density; score 2 = intermediate lymphocytic density; score 3 = high lymphocytic density and presence of lymphoid aggregates.

Afterwards, we evaluated the expression of the different immune checkpoints on the lymphocytes of all previously described compartments of the TME, i.e. the PS, the LA and the IS, but also those expression on the tumor cells. Expression of each marker in the tissue was divided into five categories (0 = <1%; 1 = 1–<5%; 2 = 5–<10%; 3 = 10–<50%: 4 = ≥50%), as described by Marcq E. et al[35]. A cut off value of ≥1% was used to determine positivity of all samples. Samples were considered to be positive in case of ≥1% positive cells, with specific staining of any intensity (0 = no expression, 1 = weak, 2 = moderate, 3 = strong) and any distribution (membrane and/or cytoplasm). These

criteria were used for IHC scoring of all the different markers. All slides were scanned and evaluated using the digital Philips Platform. Pictures were also made using the same platform.

c. Microsatellite instability testing

MSI analysis was performed using the Idylla MSI cartridge on an Idylla platform (Biocartis). As input material 1 FFPE section was used. This test analyses 7 monomorphic biomarkers (ACVR2A, BTBD7, DIDO1, MRE11, RYR3, SEC31A and SULF2). When at least 2 markers were divergent, the sample was considered MSI-high (MSI-H). When none or only 1 marker was divergent, the sample was considered microsatellite stable (MSS).

d. Statistics

The statistical analysis was performed via the SAS 9.4 for Windows (Cary, North Carolina, USA). For the descriptive statistics the complete range of mean value, standard deviation, minimum and maximum values and median are reported when arithmetic variables are presented. For the categorical variables frequencies and relevant percentages are used. For inferential statistics, for the comparison between two groups when arithmetic variables are in question, the t-test or the Mann Whitney U test was applied according to variables normality (examination by the Kolmogorov Smirnov test). For more than two categories we applied the ANOVA or the Kruskal-Wallis test depending on a positive or negative test for normality, respectively. When categorical variables were the subject of comparison, we applied the chi-square test (and if required the Fisher exact test, in cases of expected frequency <5 in more than 80 of the cells), in the case of 2x2 contingency tables Odds Ratio (OR) and the relevant 95% Confidence Interval (CI) is reported. For the identification of relations between arithmetic variables (such as birth weight and gestational age) we applied the Pearson correlation and in cases that normality was not possible to be ensured we calculated the Spearman correlation coefficient. All tests were two sided and the level of significance was set to $p<0.05$ for all study tests.

4. Results

a. Patient characteristics

The clinicopathological characteristics of our sarcoma patients are summarized in **table 1**. All 68 patients were diagnosed between 2016 and 2018. The age of the 68 patients was computed from birth date to the initial diagnosis date. On average the age was 50.4±20.5 years (median: 52.6). The majority of the patients were males, n=44 (64.7%).
Nineteen (19) patients were diagnosed with a bone tumor, while the remaining 49 presented with a soft tissue tumor. Among the soft tissue tumors, 25 were located in the deep soft tissue, from which 16 in the extremities, 5 on the trunk or back and 4 on the head and neck. In 11 patients the tumor was located on the skin or subcutaneous adipose tissue. Deep locations in the abdomen, mediastinum and retroperitoneum were also involved with 6, 5 and 2 cases respectively.

Table 1 : Clinicopathological characteristics of all sarcoma patients in our series.

CHARACTERISTIC	FREQUENCY (n)	PERCENT (%)
Gender		
Male	44	64.7%
Female	24	35.3%
Age		
Average	50.4±20.5 years	NA
Median	52.6	
Tumor location		
Bone	19	27.94%
Deep soft tissue extremities	16	23.53%
Deep soft tissue trunk and back	5	7.35%
Deep soft tissue head and neck	4	5.88%
Skin and subcutaneous fat tissue	11	16.18%
Abdomen	6	8.82%
Mediastinum	5	7.35%
Restoperitoneum	2	2.94%
Histological type		
Chondrosarcoma	10	14.71%
Ewing sarcoma	3	4.41%
Osteosarcoma	5	7.35%
Angiosarcoma	7	10.29%
Kaposi sarcoma	8	11.76%
Leiomyosarcoma	5	7.35%

Liposarcoma	9	13.24%
Myxofibrosarcoma	5	7.35%
Rhabdomyosarcoma	3	4.41%
Synovial sarcoma	3	4.41%
Sarcoma NOS	10	14.7%
Grade		
High grade	47	69.12%
Low grade	17	25%
Not known	4	5.88%
Local or metastatic disease		
Local aggressive	15	22.06%
Monometastatic disease	11	16.87%
Multimetastatic disease	12	17.64%
None of both	30	44.12
Oncogenic mechanism		
Oncogenic mechanism known	14	20.59%
Not known oncogenic mechanism	46	67.65%
Oncogenic virus (HIV)	8	11.76%
Survival		
Alive	40	59.70%
Death from disease	22	32.84%
Death from other cause	6	7.46%
Medical history		
No medical history	54	79.41%
HIV	5	7.35%
Lymphoma + HIV	1	1.47%
Lymphoma and other tumors	1	1.47%
Other epithelial tumors	4	5.88%
Mesothelioma	1	1.47%
Melanoma	1	1.47%
Syndrome	1	1.47%
Therapy		
Excision only	26	38.24%
Excision + adjuvant CHMT	7	10.29%
Excision + adjuvant RT	10	10.41%
Excision + adjuvant CHMT and RT	1	1.47%
Excision + adjuvant ICB	1	1.47%
Excision + adjuvant targeted therapy	1	1.47%
Neoadjuvant CHMT	12	17.64%
Follow-up	2	2.94%

Abbreviations: NA; not applicable, NOS; not otherwise specified, CHMT; chemotherapy, RT; radiotherapy, ICB; immune checkpoint blockade

b. Sarcoma cells elicit immune response and preferentially express CD70

The immunohistochemical expression of the different parameters in the different compartments of the tumor, namely in the peritumoral stroma (PS), in the lymphoid aggregates at the periphery of the tumor (LA), in the intratumoral stroma (IS) and on tumor cells are shown in **table 2**. All samples were examined for the expression of the immune checkpoints IDO, PD-L1, PD-1 and CD70. The immunohistochemical expression of the different antibodies is depicted in **figure 3**.

We evaluated the positivity of each marker in the different compartments as described in our materials and methods. A small 3% of the samples did not have peritumoral tissue available for evaluation. In those specific cases, all parameters referring to this location were excluded from evaluation.

Table 2: Overview of IHC scoring in the various tumor environment sites.

MARKER	COMPARTMENT	SCORE				
		0	**1**	**2**	**3**	**4**
CD45	PS	6.4%	46%	27%	20.6%	-
	LA	NO : 54%			YES : 46%	
	IS	16.7%	28.8%	24.2%	30.3%	-
	TUMOR CELLS	N/A				
IDO	PS	33.9%	46.4%	14.3%	5.4%	0%
	LA	93.1%	6.9%	0%	0%	0%
	IS	32.3%	27.4%	19.4%	16.1%	4.8%
	TUMOR CELLS	90.5%	4.8%	0%	1.6%	3.2%
CD70	PS	33.3%	31.6%	17.5%	12.3%	5.3%
	LA	34.5%	17.2%	17.2%	24.1%	6.9%
	IS	32.3%	27.4%	19.4%	16.1%	4.8%
	TUMOR CELLS	27.7%	15.4%	9.2%	24.6%	23.1%
PD-L1	PS	71.9%	10.5%	14%	1.8%	1.8%
	LA	48.3%	13.8%	24.1%	13.8%	0%
	IS	67.7%	12.9%	11.3%	8.1%	0%
	TUMOR CELLS	76.9%	6.2%	6.2%	9.2%	1.5%
PD-1	PS	47.4%	28.1%	15.8%	8.8%	0%
	LA	34.5%	6.9%	34.5%	20.7%	3.5%
	IS	54.8%	14.5%	16.1%	12.9%	1.6%
	TUMOR CELLS	98.5%	0%	1.5%	0%	0%

Abbreviations: PS: Peritumoral Stroma; LA: lymphoid Aggregates; IS: Intratumoral Stroma

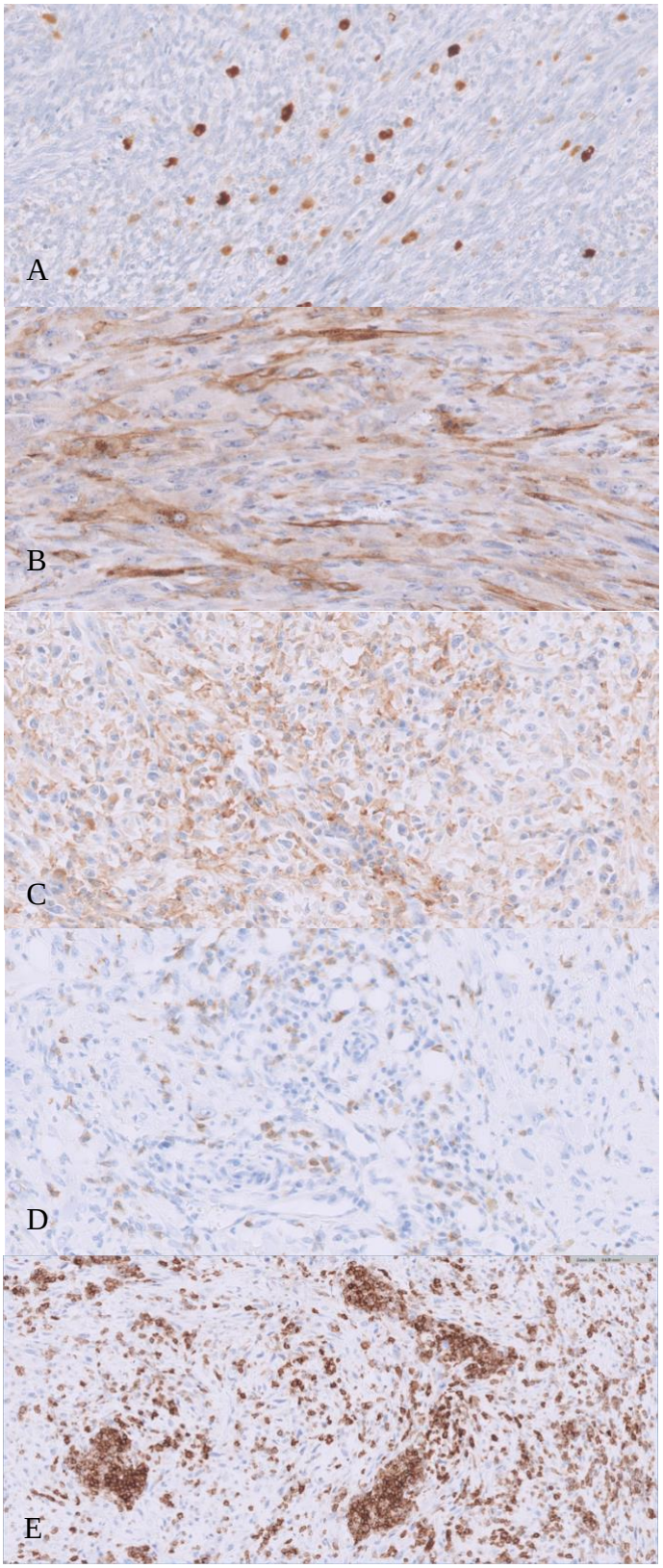

Figure 3. Immunohistochemical staining of the different antibodies. IDO expression was seen in limited cases in tumor cells (A), on the opposite,

tumor cells preferentially expressed CD70 (B). In some cases sarcoma cells expressed PD-L1 (C), while PD-1 was negative in nearly all cases in the tumor cells but showed positivity in lymphocytes (D). Strong lymphocytic infiltration within the tumor with forming of lymphoid aggregates could nicely illustrated by CD45 immunohistochemical staining (E). All slides are at 40x magnification; all antibodie are stained with DAB.

First of all, we evaluated the lymphocytic infiltrate in the PS (LyPS: lymphocytes in peritumoral stroma) and the IS (LyIS: lymphocytes in intratumoral stroma). In the PS, we found two almost percentage equal categories, the low score category (scores 0 and 1) and the high score category (scores 2 and 3) with a slight superiority of the low score category. Similar results were found for the IS, for which the balance tilted slightly towards the high score category. Based on the CD45 expression we also noted the presence of LA. In our series, 46% of the tumors had LA at the periphery of the tumor while the rest did not.

In the majority of the cases we did not see IDO expression in the lymphocytes of the PS, the LA, the IS or in the tumor cells. In cases where IDO was positive, it was expressed with a low score (score 1). However, almost 5% of LyIS and 3% of tumor cells samples displayed a high IDO score (score 4).

Regarding CD70 expression, negative lymphocytes were found in one third of the cases in the PS, one third of the cases in the LA and one third of the cases in the IS. When positive, the percentage of positivity decreased as the score increased (from 1 to 4). On the opposite, CD70 expression on the tumor cells was high (scores 2, 3 and 4), reaching around 55%.

PD-L1 was variably expressed in the different compartments. It was usually negative and when positive, it was mostly a score 1 or 2. Interestingly, 1.5% of the samples, the tumor cells displayed a high score 4.

PD-1 was more often positive on the lymphocytes than PD-L1 and gave also slightly higher score percentages in these cases. Concerning the tumor cells, PD-1 was negative in in the vast majority of the samples.

c. Correlation of different markers with each other: T-cell exhaustion has a multivariate component

The different markers were correlated to find any statistical value. One of the interesting elements that arose is the fact that the lymphocytes in the PS frequently expressed PD-L1 ($p=0.0029$) and more importantly PD-1 ($p=0.0002$). The correlation coefficient between PD-L1 and PD-1 in the PS was $r=0.65$, $p<0.0001$.

Moreover, a strong correlation of PD-L1 on the tumor cells and PD-1 on the immune cells of the different compartments (PS, LA and IS) was noticed ($p<0.0001$, $p=0.0139$ and $p<0.0001$ respectively). These findings suggest a strong influence of the PD-L1 / PD-1 (exhaustion) pathway in the microenvironment of sarcomas. Strong correlation of CD70 and PD-L1 expression in the PS was seen in most of the cases ($p<0.0001$). Moreover, the presence of LyIS was strongly correlated with expression of IDO on the tumor cells ($p=0.0028$). Furthermore, IDO in the PS was correlated with the presence of PD-1 in the PS ($p=0.0367$) as well as PD-1 and PD-L1 in the LA located at the periphery ($p=0.0038$ and $p=0.0008$, respectively). These findings suggest that multiple pathways involved in T-cell exhaustion are at play in sarcoma samples.

d. Immune checkpoint expression is correlated with different clinical and pathological parameters

i.Survival

Patients' survival status was divided into those patients that were still alive, those that died from the disease and those that died from any other cause (**table 1**). All patients that died from the disease (n=22) had IDO negative tumor cells. This was statistically significant when correlated to the patients that died from any other cause (n=5), all of which had a positive IDO score in the tumor cells ($p=0.001237$). None of the other IHC parameters as expressed in their numeric form was linked to death ($p>0.05$ in all cases).

We examined the role of patient's life status (dead or alive) with the histological type, the histological grade, the metastatic status, as well as the type of therapy. The histological type was not proven to have a role

in the life status of patients [Odds Ratio (OR): 0.6177] and even when we grouped the tumors into the larger categories of bone sarcomas and soft tissue sarcomas, no difference was detected [OR: 1.05, 95% Confidence Interval (CI): 0.3-3.2, p= 0.9319]. On the contrary, the histological grade was important. Namely, from the 27 patients that died, one had a grade I tumor (3.7%, a total of four patients had grade I), two had a grade II tumor (7.4%, a total of 17 patients had grade II) and 22 were grade III (88.9%, a total of 46 patients had grade III); the p-value of the statistical test was 0.0072. The OR for dying from a grade III tumor in comparison to grade I and II combined was 6.5 (95%CI: 1.7-25.3, p=0.0035) and the risk of death was 52%. In terms of therapy and risk of death, it was found that patients under chemotherapy had 6.9 higher odds for death in comparisons to those that did not receive chemotherapy (95%CI: 2.3-20.5, p=0.0004), but this was probably also due to the high grade of those tumors, while excision and radiotherapy did not achieve a statistically significant level of evidence for their role in patient survival (p= 0.8840 and 0.3004 respectively). Finally, the identification of an oncogenic driver mechanism was not found to have a role for patient survival (p= 0.6461, OR: 1.3, 95% CI: 0.4-3.7 for surviving when a driver alteration is identified). Additional analysis showed that metastasis and multiple metastasis were linked to patient death, specifically metastasis (irrelevant of the type: locally, single or multiple) leads to death, showing an OR of 6.6 (95%CI: 2.1-21.0, p=0.0010) and multiple metastasis is linked to a higher OR of 26.8 (95%CI: 3.2-225.2, p<0.0001). Finally, higher age had an important role (p=0.0237) as patients that eventually deceased had higher age than survivors [median (Q1-Q3): 63.7 (52.6-70.8) vs. 45.7 (35.8-60.8) respectively].

ii.Histological types

The Box and Whisker plots in **figure 4 (A and B)** represent the expression of the different parameters in each histological type separately, while **figure 5** displays the different IHC results in relation to the histological type. To start with, the distribution of the lymphocytes was investigated according to the tumor type. The presence of lymphocytes in the IS, in other words the presence of tumor infiltrating lymphocytes (TILs), was significantly correlated to different tumor types (p=0.0001). We examined the differences in the expression of

lymphocytes in the IS when grouping the tumors into two large categories: on the one hand the bone sarcomas (namely Ewing, chondrosarcoma and osteosarcoma) and on the other hand the soft tissue sarcomas (namely angiosarcoma/Kaposi sarcoma, sarcoma not otherwise specified *or* NOS, leiomyosarcoma, liposarcoma, synovial sarcoma, myxofibrosarcoma, rhabdomyosarcoma). The results are presented in the **figure 6**. Accordingly, soft tissue sarcomas display a higher expression of LyIS ($p<0.0001$); in more details the median value of lymphocytes in the IS was 2 for the soft sarcomas (Q1-Q3: 1-3) while for the bone sarcomas was 0 (Q1-Q3: 0-1). Among the soft tissue sarcomas, the highest score of LyIS was seen in liposarcoma and myxofibrosarcoma with a median of 3, followed by angiosarcoma (among which also Kaposi sarcoma), leiomyosarcoma, soft tissue sarcoma NOS, rhabdomyosarcoma and synovial sarcoma that displayed a median of 2. Among the bone sarcomas, osteosarcoma and Ewing sarcoma had a median score of LyIS of 1 while chondrosarcomas a median score of 0.

IDO expression on the tumor cells was seen in six cases. Although not statistically significant, four out of the six cases with IDO positive tumor cells were bone tumors, namely three chondrosarcomas and one osteosarcoma. Chondrosarcomas were the ones with the highest scores (score 4, n=2) and osteosarcoma had a score 3 (n=1). The three cases with a low score 1 were a chondrosarcoma, a liposarcoma and a soft tissue sarcoma NOS.

The expression of IDO in the LyPS was different for each histologic type ($p=0.0470$) (**figure 5**). Among the different tumor types, leiomyosarcoma tend to express the highest score with a median of 1.5. Myxofibrosarcomas, synovial sarcomas and osteosarcomas displayed the second highest score with a median of 1. On the other hand, Ewing sarcoma and rhabdomyosarcomas scored usually low with a median of 0.

Moreover, leiomyosarcomas and synovial sarcomas had also the highest scores of IDO expression on the LyIS with a median of 2 ($p=0.0197$) (**figure 4B and 4A** respectively).

Although CD70 and PD-L1 expression in the different compartments were not significantly associated to the histological types, we noticed that myxofibrosarcomas had almost always high scores for PD-L1 in all compartments especially in the LyIS (median 2.5) and in the tumor cells (median 1) (**figure 4A**). Myxofibrosarcoma was also the only tumor type that expressed CD70 in the LA with a high score (3 and 4) (**figure 4A**).

Still, the highest score of CD70 in the tumor cells was noticed in synovial sarcoma (median 4) (**figure 4A**) and to a less extent in leiomyosarcomas, liposarcomas, angiosarcomas (among which also Kaposi sarcomas) and soft tissue sarcoma NOS (median 3) (**figure 4B**).

On the other hand, the PD-1 expression in the different compartments of the tumor was significantly correlated to the histological type; namely the p value regarding the expression in the LyPS was 0.0004, in the LAs 0.0347 and in the LyIS 0.0074 (**figure 5**). Regarding the LyPS, leiomyosarcomas and angiosarcomas gave the highest score with a median of 2. Leiomyosarcomas together with soft tissue tumors NOS showed strong expression for PD-1 in the LA with scores 2.5 and 3 respectively. High levels of PD-1 in the LyIS were mostly observed in angiosarcoma, leiomyosarcoma and myxofibrosarcoma, all of which with a median of 2.

Notably rhabdomyosarcomas and Ewing sarcomas were of the tumors with the lowest expression of the immune checkpoint in general (**figure 4A and 4B** respectively).

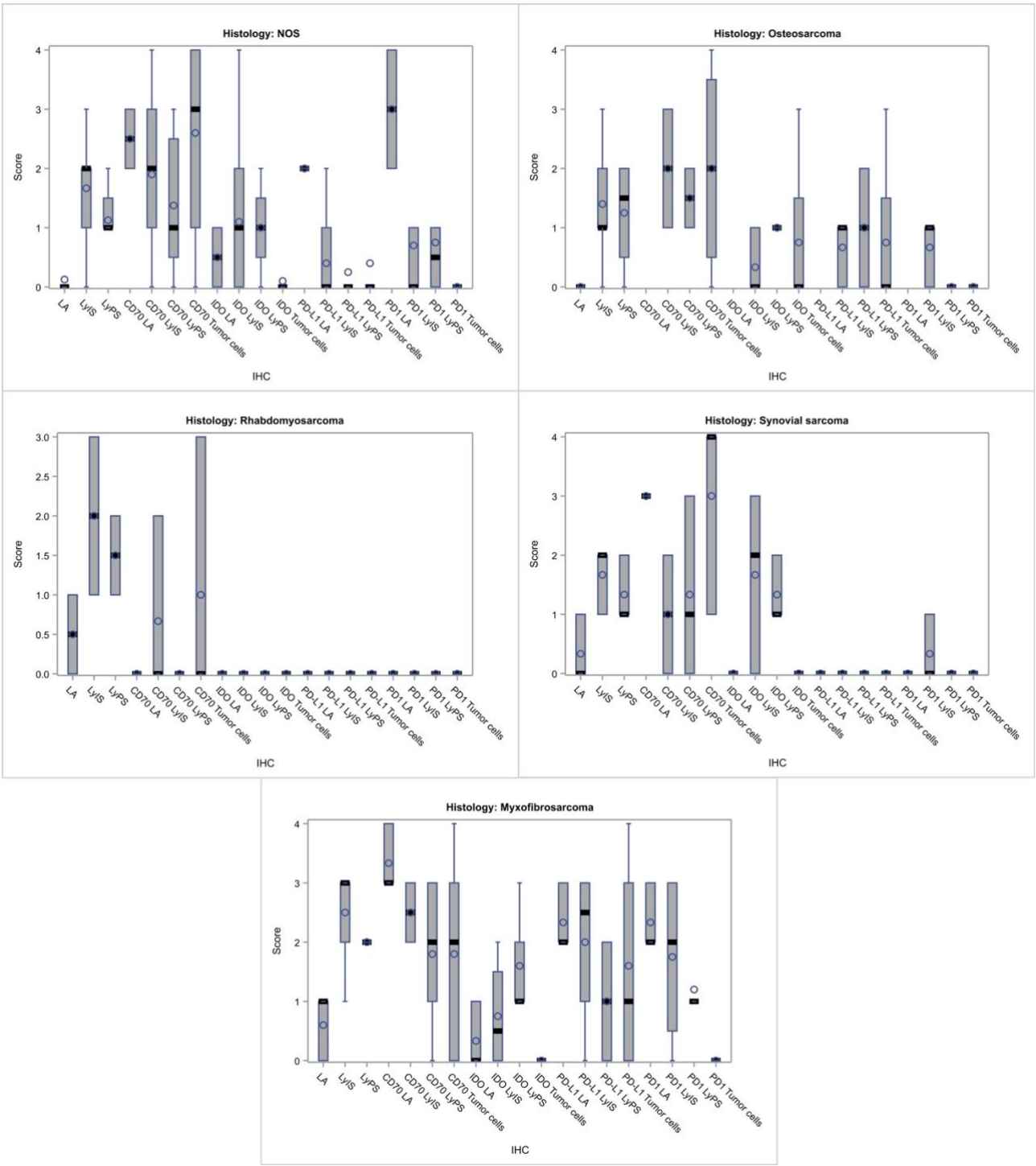

Figure 4A (for description see following page)

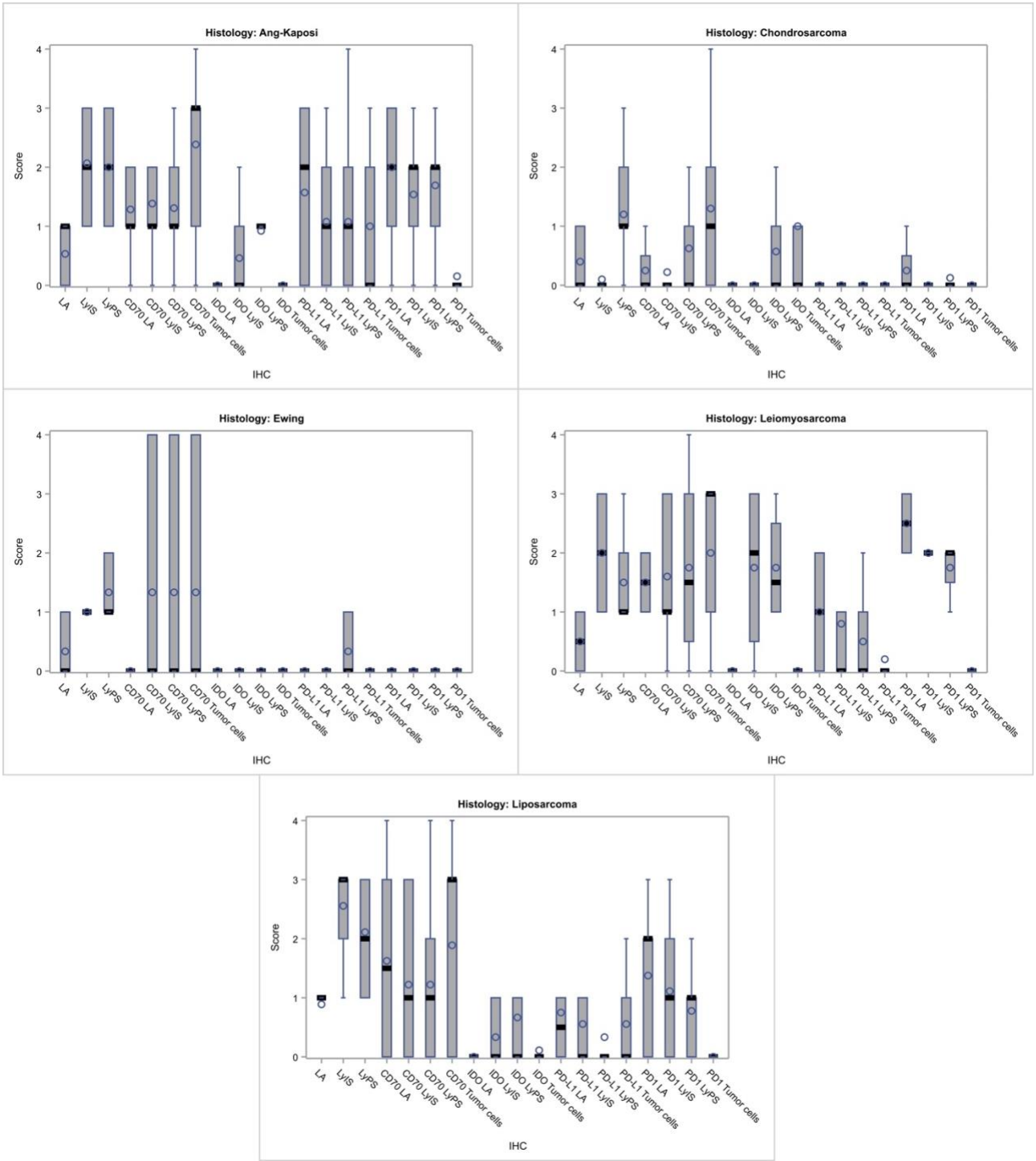

Figure 4B (for description see below)

Figure 4A and 4B: These figures represent the expression of the different parameters in each histological type separately. In the Box and whisker plots the lower and upper part of the gray box indicate quartiles 1 and 3 respectively, the bold solid line within the box indicates the median value, the circle the mean values, while the lower and upper part of the whiskers the minimum and maximum value after excluding outliers (not shown). *Abbreviations*: IHC; Immunohistochemistry, Ang; Angiosarcoma, NOS; (sarcoma) Not Otherwise Specified; LyPS; Lymphocytes in Peritumoral Strom; LyIS; Lymphocytes in Intratumoral Stroma; LA; Lymphoid aggregates

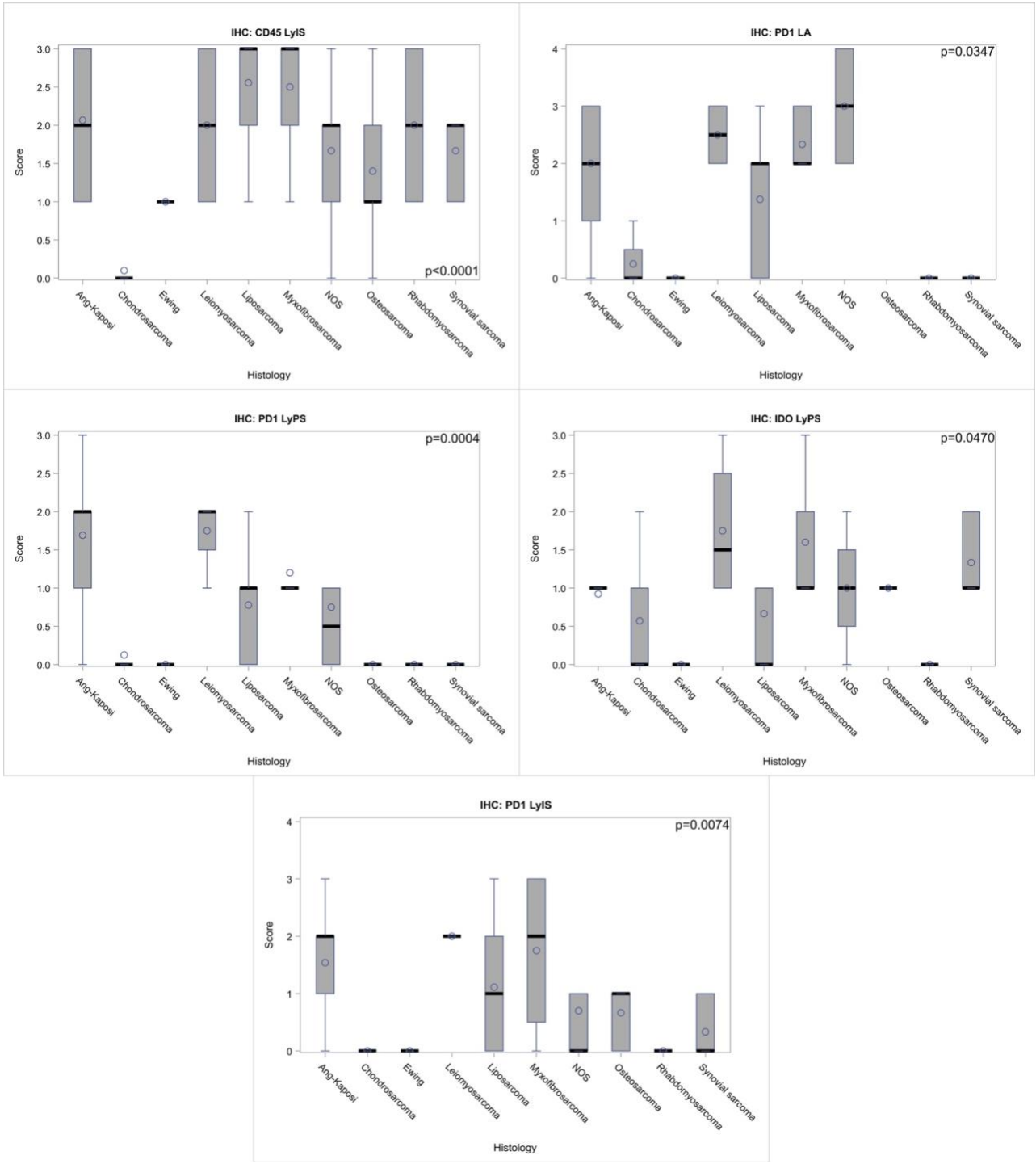

Figure 5: This graphics displays the different IHC results in relation to the histology type. In the Box and whisker plots the lower and upper part of the

gray box indicate quartiles 1 and 3 respectively, the bold solid line within the box indicates the median value, the circle the mean values, while the lower and upper part of the whiskers the minimum and maximum value after excluding outliers (not shown).
Abbreviations : IHC ; immunohistochemistry, Ang ; angiosarcoma, NOS; not otherwise spicified, TME; tumor microenvironment, TIL; tumor infiltrating lymphocytes, LA; lymphoid aggregates.

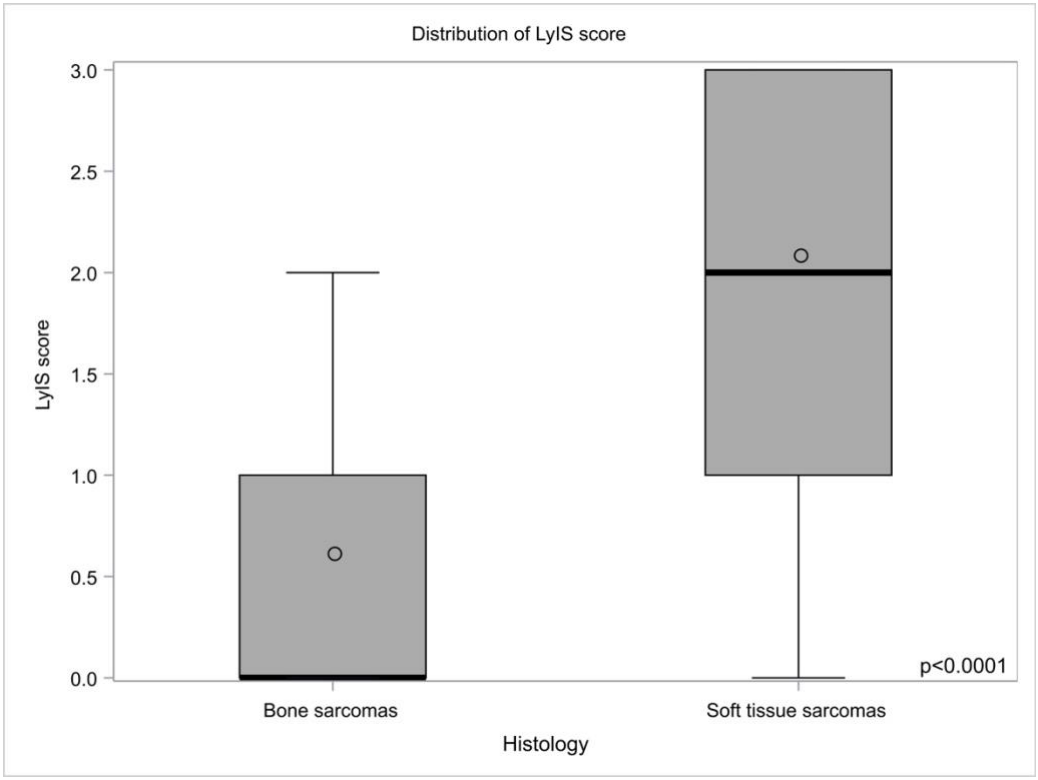

Figure 6: Distribution of the lymphocytes in the IS in relation to the histological group bone vs soft tissue sarcoma. In the Box and whisker plots the lower and upper part of the gray box indicate quartiles 1 and 3 respectively, the bold solid line within the box indicates the median value, the circle the mean values, while the lower and upper part of the whiskers the minimum and maximum value after excluding outliers (not shown).
Abbreviations: IS; Intratumoral Stroma, LyIS; Lymphocytes in Intratumoral Stroma

iii.Molecular status

We divided the tumors into those that are known from the literature to have an oncogenic driver mechanism (among them also sarcomas induced via an oncogenic virus) (n=22) and those with a complex karyotype without a known or a proven oncogenic driver alteration (n=46). In the case of tumors with a known oncogenic mechanism, this was confirmed by molecular techniques in our samples, in the context of initial diagnosis. We noticed that those with an oncogenic driver mechanism presented statistically significant more lymphoid aggregates and had more LyIS than the other category, median 1 vs 0 for the lymphoid aggregates and median 2 versus 1 for the LyIS (p=0.0104 and p=0.0181 respectively) (**figure 7**).

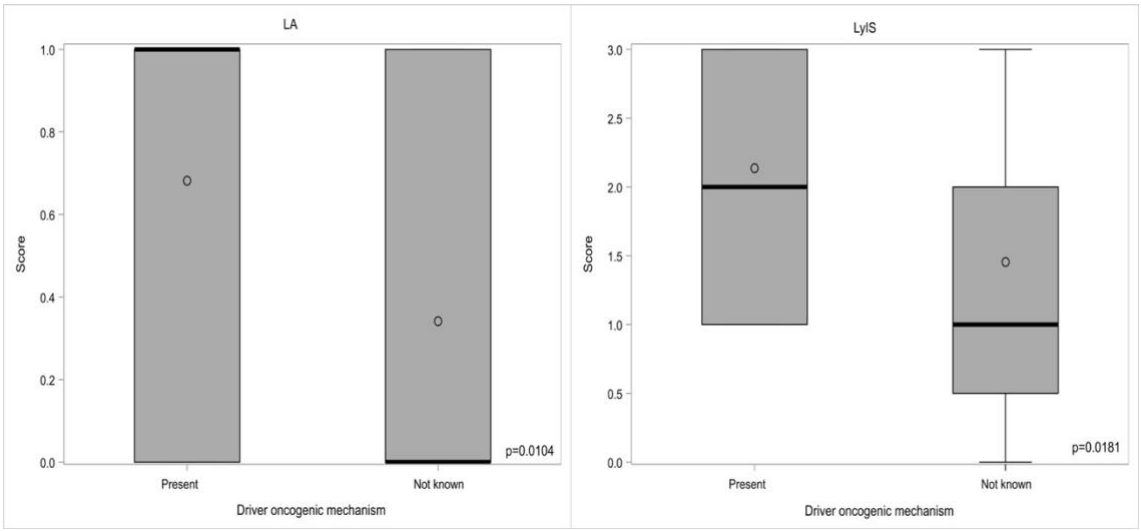

Figure 7. Presence of LA (left) and TILs (right), as evaluated by means of CD45 IHC staining, in relation to the presence or not (in this cases mentioned as not known) of driver oncogenic mechanism. Lower and upper boxes edges indicate Q1 and Q3 respectively bold horizontal lines correspond to the median values, upper and lower whisker edges indicate minimum and maximum observations and circles within the boxes indicate mean values, outliers are not presented. *Abbreviations:* LA; lymphoid aggregates, TIL; tumor infiltrating lymphocytes; IHC; immunohistochemistry

c. MSI

All cases were evaluated for MSI. Only one patient had an MSI-high tumor. The patient was a 45-year-old male with a history of lymphoma and HIV infection, diagnosed with non-metastatic skin Kaposi sarcoma, low grade according to the French Federation of Cancer Centers Sarcoma Group classification. This patient was treated with excision and was not reported to show recurrence or to die from the disease. The tissue sample showed a tumor with a presence of a strong immune response in the PS (score 2), presence of LA at the periphery, while the lymphocytes that infiltrate in the IS were rather limited (score 1). The tumor displayed a low score (score 1) for CD70 in the PS, in the IS as well as in the tumor cells, while the LA were negative for this marker. All other markers tested (IDO, PD-1 and PD-L1) were negative in all the tumor environmental sites that were examined.

5. Discussion

In this article we describe the expression profile of the immune checkpoints IDO, CD70, PD-1 and PD-L1 as well as the MSI status in tissue samples of diverse sarcoma types, both of the soft tissue and the bone. We investigated the expression of the immune checkpoints in the different compartments of the tumor, namely the PS, the LA at the periphery of the tumor, the IS as well as the tumor cells. MSI was investigated in the tumor cells.

The tumor and the surrounding microenvironment are closely related and interact constantly. The TME plays a very important role in tumor progression. One of its important elements are the immune cells. PD-1 is expressed by immune cells, mainly cytotoxic T lymphocytes. When it binds to its ligand PD-L1, a switch-off signal results in downregulation of the immune response [31,36]. In such cases the presence of PD-1 can indicate an exhausted phenotype. In our samples, we found a strong correlation between the expression of PD-1 and PD-L1 in the PS of the TME. This could mean that the PS in sarcomas might lead to tumor progression, as the tumor cells can escape the inactivated immune system. Moreover, the expression of PD-L1 in the tumor cells was correlated with expression of PD-1 in the immune cells of the PS, the LA and the IS, also suggesting an exhausted phenotype. Another feature

attributed to tumors is the inflammation signature. Tumors that display a strong immune infiltrate are called “hot” while those without one are called “cold” tumors [37]. The strong immune response seen in “hot” tumors may contribute to eradiation of tumor cells. Undifferentiated sarcomas have been described as “hot” tumors and this could explain the clinical activity of these tumors to pembrolizumab in clinical trials [38]. In our series, sarcomas NOS (i.e. undifferentiated sarcomas) showed also an inflamed phenotype. Still among our cases, the tumors with the strongest inflammation signature where myxofibrosarcomas and liposarcomas. Another very interesting conclusion that emerged from our observations is that there is a clear difference between soft tissue and bone tumors, as the latest are always “cold” tumors with very limited or no tumor immune infiltration.

In our series we describe that tumors with an oncogenic driver mechanism (among which also oncogenic viral mechanisms) correlate with more LA and more LyIS. We know that desmoid tumors for instance, which are known to harbor mutations of the CTNNB1 or the APC gene, present LA at the periphery of the tumor, indicating tertiary lymphoid organs [39]. The role of tertiary lymphoid organs is to recruit activated immune cells in the tumor, participating in anti-tumor immune response [40]. Still, as we already discussed, the lymphocytes in the LA can display an exhausted phenotype. This suggests immune suppression of these cells, indicating that immune cells in tertiary lymphoid organs are in a dynamic process, changing from activated to exhausted. In such cases a marker of exhaustion maybe interesting to be included when analyzing tissue samples. Recognition of the mechanism that drives this transformation could be of benefit for the patients with a sarcoma with a known oncogenic pathway.

As mentioned, PD-L1 expression was mainly observed in the lymphocytes of the PS and the LA. Few cases had a positive PD-L1 expression in the tumor cells. Among the different tumor types, myxofibrosarcoma was the one with the highest PD-L1 scores, followed by angiosarcoma, osteosarcoma and liposarcoma.

Notably, myxofibrosarcomas also showed high levels of LyIS as well as high levels of PD-1 in these LyIS. Although this was not statistically significant, it suggests that those cells represent an exhausted phenotype.

Myxofibrosarcomas are aggressive soft tissue tumors and chemotherapy and radiotherapy can be used in neo-adjuvant, adjuvant or metastatic setting [41]. Till now, not much is known about the use of immune checkpoint inhibitory therapy for the treatment of these tumors. In a case report of a patient with metastatic myxofibrosarcoma, the patient showed partial response for 16 months and overall survival for over 29 months with administration of nivolumab and bevacizumab [42]. In another case study, a patient in palliative setting receiving chemotherapy for metastatic myxofibrosarcoma showed a partial response with pembrolizumab for 14 cycles [43]. In a multicentric study with nivolumab plus ipilimumab in metastatic sarcomas, one patient with myxofibrosarcoma achieved complete response [44]. Although in these cases no correlation with PD-L1 expression in the tumor cells was reported, our data in combination with these reports, suggest that PD-L1 expression in myxofibrosarcoma may play a role in response to ICB. Further investigation is needed to evaluate if patients with this tumor type could benefit from this therapeutic approach.
Moreover, we found that myxofibrosarcomas displayed high levels of CD70 in the LyIS. Although statistically not significant, myxofibrosarcomas also showed high CD70 expression on tumor cells. The CD70 signaling pathway increases the cytotoxic T lymphocyte response and supports memory formation [45]. Transient CD70 expression is present on activated T and B cells and mature dendritic cells. On the other hand, persistent CD70 signaling leads to exhaustion of the T cells [14]. The presence of CD70 in myxofibrosarcomas has not been described previously, but given the consistent positivity in our cases, we suggest that those tumors need further investigation for the possibility of targeting with anti-CD70 antibodies.

Leiomyosarcomas expressed high levels of PD-1 in the lymphocytes of the PS, the LA as well as in the lymphocytes of the IS. Still, no expression of PD-L1 was noticed. Given the lack of PD-L1 in these cases, a possible inhibition of the immune system through the PD-L1 and PD-1 interaction seems less likely. Hence, the use of anti-PD-1 or anti-PD-L1 therapy may not have any value for this tumor type. In a phase II clinical trial with pembrolizumab and cyclophosphamide in advanced sarcomas, all of the leiomyosarcomas that were include in the study showed progressive disease [46].

IDO was mostly positive in the Ly PS. Few cases had IDO positivity in the LA and the Ly IS. Three cases had high expression of IDO in the tumor cells. Notably IDO expression in the tumor cells was mostly observed in the bone tumors, mainly the chondrosarcomas and osteosarcomas. Both chondrosarcomas and osteosarcomas presented IDO positivity also in the LyPS. Still the highest scores for IDO expression in the LyPS was noticed for the leiomyosarcomas. Studies of regulatory and effector pathways illuminate IDO as an inflammatory modifier [47]. Moreover, the IDO pathway is possibly an important mechanism for primary resistance to PD-1 inhibition[48]. Notably, PD-1/PD-L1 reverse signaling also induces IDO. Given that clinical trials of IDO and PD-1 inhibitors show promising results, co-inhibition of these two targets provides a rationale to evaluate additional combinations of immune checkpoint inhibitors with IDO inhibitors [47]. Although in our series limited IDO positivity was shown on the tumor cells, the statistical correlation with chondrosarcomas and osteosarcomas was strong.

We investigated the MSI in all our samples, which induces a hypermutated phenotype. MSI-H tumors generate numerous neoantigens and are highly sensitive to ICI therapy regardless of the tumor type and tissue of origin [32]. We found one case exhibiting an MSI. It was a patient with a low-grade Kaposi sarcoma through Human Immunodeficiency Virus (HIV) infection. The patient had a history of lymphoma, but he was not known with any other malignancies. To our knowledge, this is the first case of a Kaposi sarcoma showing an MSI. A case of body cavity based lymphoma (BCBL) associated with Kaposi sarcoma herpesvirus/ human herpesvirus type 8 infection, has been documented to display MSI [49]. These data suggest that MSI may be potentially involved in the pathogenesis of HIV-related lesions among which Kaposi sarcomas. Thus, evaluation of Microsatellite status is interesting in these cases, as ICB could be a promising therapy for disseminated disease.

6. Conclusions

In our study on different types of soft tissue and bone sarcomas we found that immune cells in general display an exhausted phenotype, still the driver mechanism is not always known.

In our series we found differences in the expression of immune checkpoints in distinct tumor types. Among the different tumor types, myxofibrosarcomas where the ones that expressed more PD-L1 tumor cell positivity. This, in correlation to studies that presented good response rates in treatment with PD-1 or PD-L1 inhibitors, make myxofibrosarcomas realistic candidates for ICI therapy. Moreover, expression of PD-L1 could be investigated as possible prognostic biomarker for those tumors.

Leiomyosarcomas have a strong infiltrate but no PD-L1 expression. IDO is expressed in the LyPS. While no anti-PD-L1 or anti-PD1 therapy is indicated, IDO inhibition or combination of IDO inhibitors with ICB could be an option for leiomyosarcomas.

We are the first to describe consistent IDO expression in the tumor cells in cases of chondrosarcomas and osteosarcoma. The biological relevance is unclear, but these tumor types could be investigated as possible candidates for IDO-inhibition.

We are also the first to describe a case of a Kaposi sarcoma that is MSI-high, among the 8 cases of Kaposi sarcoma in our series. We believe that Kaposi sarcomas needs further investigation, as people with disseminated disease may benefit from ICI therapy.

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CHAPTER 6

NTRK FUSIONS IN SARCOMAS: DIAGNOSTIC CHALLENGES AND CLINICAL ASPECTS.

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1. Abstract

Tropomyosin receptor kinase (TRK) is encoded by the neurotrophic tyrosine receptor kinase genes (*NTRK*) 1, 2, and 3, whose activation plays an important role in cell cycle proliferation and survival. Fusions of one of these genes can lead to constitutive activation of TRK, which can potentially be oncogenic. *NTRK* fusions are commonly present in rare histologic tumor types. Among sarcomas, infantile fibrosarcoma shows *NTRK* fusion in more than 90% of the cases. Many other sarcoma types are also investigated for *NTRK* fusions. These fusions are druggable alteration of the agnostic type, meaning that all *NTRK* fused tumors can be treated with NTRK-inhibitors regardless of tumor type or tissue of origin. TRK-inhibitors have shown good response rates, with durable effects and limited side effects. Resistance to therapy will eventually occur in some cases, wherefore the next-generation TRK-inhibitors are introduced. The diagnosis of *NTRK* fused tumors, among them sarcomas, remains an issue, as many algorithms but no guidelines exist to date. Given the importance of this diagnosis, in this paper we aim to 1) analyze the histopathological features of sarcomas that correlate more often with *NTRK* fusions, 2) give an overview of the TRK-inhibitors and the problems that arise from resistance to the therapy, and 3) discuss the diagnostic algorithms of *NTRK* fused tumors with emphasis on sarcomas.

2. Introduction

a. The Role of NTRK in Oncogenesis

Tropomyosin receptor kinase (TRK) is a member of the tyrosine kinase family, predominantly expressed in neuronal tissue[1]. There are three receptors (TRK A, B, and C) encoded by the three neurotrophic tyrosine receptor kinase genes (*NTRK*), *NTRK* 1, 2, and 3, respectively. Activation of one of these three genes initiates a downstream signaling that impacts cell proliferation, differentiation, and survival. Normal/physiological TRK signaling is ligand dependent and plays a critical role in the nervous system development. TRK is expressed in some mature neural cells, such as ganglion cells. Due to intra- and inter-chromosomal translocations, *NTRK* can undergo chromosomal rearrangements resulting in gene fusions. This leads to ligand

independent constitutive activation of the TRK pathway which can potentially be oncogenic[2].

NTRK fusions were first discovered in colon carcinoma in 1982[3]. Regarding sarcomas, the *ETV6-NTRK3* fusion was described in congenital fibrosarcomas back in 1998[4]. Nowadays, we recognize two major tumor categories based on the *NTRK* fusion frequency. In the first category, there are rare tumors with *NTRK* fusions in almost more than 90% of the cases[1], such as infantile fibrosarcoma, mammary analogue secretory carcinoma, secretory breast carcinoma, and congenital nephroblastic nephroma. The second category represents more common tumors with a low frequency for *NTRK* fusions, such as lung cancers, gastrointestinal cancers, melanoma, and thyroid cancers[1,2,5]. So far, more than 60 different fusion partners have been identified and many more novel fusions continue to emerge. *NTRK1* has got more fusion partners than *NTRK2* and *NTRK3* genes[6,7]. In general, though, *NTRK* fusions remain rare genetic events found in tumors. These gene fusions can represent targetable alterations. Targeting gene fusions is a paradigm of a tumor agnostic treatment, where the same drug is used for tumors with the same genetic alteration, regardless of the tumors site of origin or histologic type[8]. It is very important that all fusions can be targeted by the same class of drugs.

The Food and Drug Administration (FDA) approved the first-generation TRK-inhibitors, such as LOXO-101 (larotrectinib)[9] and entrectinib[10], for the treatment of tumors that harbor *NTRK* fusions. Nevertheless, additional mutations lead in some cases to resistance. Fortunately, the next-generation TRK-inhibitors, such as LOXO-195[11] and TPX-0005[12], have recently been introduced to overcome the resistance. TRK-inhibitors are in general well-tolerated drugs with good response rates in *NTRK* fused neoplasms. Hence, identification of patients that can benefit from this targeted therapy is of outmost importance. Till today, there are no pathognomonic morphological characteristics that correlate with the presence of *NTRK* fusions to be used as a diagnostic criterion. Nevertheless, in the various cases that have been published so far, some common features are described that could help us identify which tumors demand further investigation. The diagnosis still relies on the identification of *NTRK* fusion. Therefore, many algorithms have been introduced but no guidelines exist to date.

This review gives an overview of the diagnostic challenges and the clinical importance of *NTRK* fusions in tumors, with special emphasis on sarcomas.

3. Histopathology of Sarcomas Showing NTRK Fusions

Sarcomas show a variety of morphological features, ranging from spindle cells to small round cells, epithelioid cells, histiocytoid cells etc. While reviewing the literature we found a range of case studies describing *NTRK* fused sarcomas. Among these, spindle cell tumors are the ones described to present *NTRK* fusions more often than other morphological types. Different growth patterns of *NTRK* fused spindle cell tumors have been analyzed in the literature, while researchers have often focused on the role of CD34 and S100 in these cases. Here we give an overview of the case studies found in the literature. **Table 1** depicts all the fusion partners from the studies on soft tissue tumors listed below in correlation with the histomorphology.

One of the growth patterns that was described was the lipofibromatosis-like pattern. Lipofibromatosis (LPF) is known as a special type of benign mesenchymal tumors mostly arising in the pediatric population. Spindle cell tumors with an infiltrating growth pattern reminiscent of LPF, showing mild cytological atypia have been described to present recurrent *NTRK* fusions, especially of *NTRK1*[13,14]. Interestingly, because of—partial—CD34 and S100 protein immunohistochemical positivity, these cases were initially diagnosed as neural neoplasms. This tumor mostly affects the extremities of children and young adults. It is usually a low-grade, locally aggressive neoplasm with recurrences in cases of incomplete excision. Metastasis has only been reported in one case, but no fatalities have been recorded so far.

A case study describing a spindle cell neoplasm with myxoid features, low-grade morphology, and CD34 and partly S100 protein immunohistochemical positivity, had a rather aggressive course[15]. Some years after the initial diagnosis, the patient presented with metastatic disease in the lung and lymph nodes. An *LMNA*-*NTRK1* fusion was detected and a complete remission of the metastatic foci was achieved after one year of TRK-inhibitory therapy.

Fusions of the *NTRK1* gene also occur in low-grade sarcomas with a distinctive myopericytoma- or hemangiopericytoma-like morphology[16]. CD34 immunohistochemical positivity was not a consistent finding in these cases, while the S100 protein was not described. The tumors had a benign clinical course. The investigators of this study hypothesize that the presence of spindle cells with this distinctive growth pattern likely represents a novel morphologically and genetically defined soft tissue sarcoma (STS) entity with *NTRK1* fusions.

Spindle cell tumors of the gynecological tract, morphologically resembling fibrosarcomas can also harbor fusions of the *NTRK*[17-19], mainly of the *NTRK 1* and *3*. They are usually reported in younger patients, with the cervix being the predilection site. Largely varying morphological features and clinical outcomes are described. On the one hand, some of the tumors showed mild to moderate cytological atypia and in some instances a high mitotic activity. They all were CD34 and S100 protein positive. The tumors had a more aggressive course, nevertheless death from disease was not reported[17]. On the other hand, the tumors with high-grade morphology correspond to an aggressive disease that may, in some cases, be fatal. Interestingly, these tumor cells were negative for CD34 and only occasionally showed S100 protein positivity[18,19]

Table 1. Correlation between the different neurotrophic tyrosine receptor kinase genes (NTRK) fusions and main histo-morphological features of the soft tissue tumors as described in different publications.

NTRK	**FUSION PARTNER**	**DIFFERENT PUBLICATION DESCRIBING HISTO-MORPHOLOGICAL FEATURES IN SOFT TISSUE SARCOMAS, IN REGARD TO DIFFERENT FUSION PARTNERS**														
		Kao et al.[13]	Aga-ram et al.[14]	So et al.[15]	Haller et al.[16]	Croce et al.[17]	Chiang et al.[18]	Rabban et al.[19]	Shi et al.[20] Brenca et al.[21]	Atiq et al.[22]	Alassiri et al.[23] Yamamoto etal.[24]	Olson et al[25]	Yama-zaki et al.[26]	Davis et al.[27]	Suurmeijer et al.[28]	Suurmeijer et al.[29]
NTRK1	*TPR*	LPF-like	LC SCT				Uterine/ cervix; FS-like	Cervix; HC SCT Adenosarcoma-like		«Wild type» GIST: HG				IFS-like	LC SCT	
NTRK1	*TPM3*	LPF-like	LC SCT HC SCT		MFS-like MPC-like	Cervix; FS-like	Uterine/ cervix; FS-like	Cervix; HC SCT adenosarcoma-like		«Wild type» GIST: IFS-like or HG				IFS, LG SCT, Unclassified, Inflammatory SCT/ RCT, Inflammatory fibroid polyp	LC SCT SCT with increased cellularity	
NTRK1	*LMNA*	LPF-like	LC SCT HC SCT	SCT with myxoid features	Infantile HPC-like		Uterine/ cervix; FS-like			«Wild type» GIST DFSP-like				Myxoid DFSP-, IFS-, Cellular schwannoma-like	LC SCT SCT with increased cellularity	

NTRK1	*MIR584F 1*												IFS-like		
NTRK2	*SQSTM1*												Unclassif ied		
NTRK3 NTRK2	*STRN*											FS-like (NTRK3)	Unclassif ied (NTRK2)		
NTRK3	*SPECC1 L*	LPF-like (NTRK2)	LC SCT (NTR K2)				Cervix; HC SCT adenosarc oma-like (NTRK3)		«Wild type» GIST: IFS-like appearan ce (NTRK3)					LC SCT: perivascula r thick collagen & LPS-like component (NTRK2)	
NTRK3	*TFG*	LPF-like											LG SCT		IG SCT
NTRK3	*EML4*				Cervix; FS-like						DFSP-like				HG SCT
NTRK3	*RBPMS*					Uterine/ cervix; FS-like							Unclassif ied, SCT, RCT, IFS, LG SCT		
NTRK3	*ETV6*							«Wild type» GIST-like	«Wild type» GIST: IFS-like	IMT-like					IG SCT
NTRK3	*STRN3*											FS-like			
NTRK3	*TMP4*														HG SCT

Abbreviations: DFSP; dermatofibrosarcoma protuberans, FS; fibrosarcoma, GIST; gastrointestinal stromal tumor, HC; high cellular/high cellularity, HG; high grade, HPC; hemangiopericytoma, IFS; infantile fibrosarcoma, IG; intermediate grade, IMT; inflammatory myofibroblastic tumor, MFS; myofibrosarcoma, MPC; myopericytoma, LC; low cellular/low cellularity, LPF; lipofibromatosis, LG; low grade, RCT; round cell tumor, SCT; spindle cell tumor.

The majority of gastrointestinal stromal tumors (GISTs) are known to carry an oncogenic driver mutation, like KIT, PDGFRa, SDH, and BRAF mutations, which in most cases can be druggable. GISTs without one of those mutations are called “wild-type”. The investigators of the NCT02576431 trial performed a comprehensive genomic profile analysis of almost 190 “wild-type” GISTs, investigating genes that correspond to more than 300 somatic alterations[20]. An *ETV6-NTRK3* fusion was detected in two of the patients. Both were middle aged, males, with tumors located in the small and large intestine, respectively. The tumors showed no response when a conventional therapy was used. One of the patients was treated with larotrectinib and demonstrated ongoing partial response (PR) four months after initiation of the treatment. Other studies on “wild-type” spindle cell tumors of the gastrointestinal tract confirmed this finding[21,22]. A very interesting approach for the diagnosis of the *NTRK* fused gastrointestinal sarcomas is suggested by Atiq et al.[22]. They divided these tumors into two categories. The first category of *NTRK3* fused tumors includes high-grade infantile fibrosarcoma-like tumors. The second category of *NTRK1* fused tumors was further subdivided into low-grade CD34 and S100 protein positive tumors and into high-grade unclassified sarcomas.

NTRK fusions are also investigated in neoplasms with morphology resembling that of inflammatory myofibroblastic tumors (IMTs). Typically, IMT shows a characteristic spindle cell morphology with a prominent inflammatory infiltrate. Fifty percent of these cases display a clonal rearrangement involving the anaplastic lymphoma kinase (ALK) gene[30]. In addition, fusions of the ROS1 and PDGF-beta genes also occur[31]. Recently, *ETV6-NTRK3* fusions have been described in three cases with IMT morphology that did not harbor an ALK rearrangement[23,24]. These tumors presented a striking plasma cell infiltrate. No CD34 and/or S100 protein positivity was described. One of the patients developed an advanced disease.

An *EML4-NTRK3* fusion was detected in a tumor with morphology suggestive of a dermatofibrosarcoma protuberans (DFSP)[25]. The tumor cells were positive for CD34, which is usually the case for DFSPs. In contrast, a COL1A1-PDGF-bèta fusion, typical of DFSPs, was not demonstrated in this case.

Another series presented two *NTRK3* fused fibrosarcoma-like spindle cell tumors of the extremities, one of which was located in the bone. The tumor cells were positive for CD34. Both cases were aggressive with the patients developing lung metastasis[26].

One of the largest studies investigating *NTRK* fusions in pediatric sarcomas included thirty patients[27]. An *ETV6-NTRK3* fusion was found in 40% of the cases, while the rest showed variable *NTRK* fusion partners. Morphologically, many different cytomorphological and architectural patterns were observed. All but two tumors showed a primitive spindle cell pattern and/or a fascicular/herringbone pattern. Myoid, IMT-like, and infiltrative/fibromatosis patterns were also described. Features included blood vessels with a hemangiopericytoma-like morphology as well as perivascular hyalinosis. Mitotic activity ranged from low to abundant while some tumors displayed necrosis. CD34 and/or S100 protein positivity was not detected in all cases. Recurrence rates were slightly higher for the non *ETV6*-positive tumors but the investigators mentioned that this was mainly due to incomplete excision of the lesions. Metastasis and death from disease sporadically occurred.

An attempt was made to divide the *NTRK1* fused spindle cell tumors into those with low and high cellular neoplasms[28]. CD34 and S100 protein was consistently expressed in both groups. Some of the low cellular neoplasms had keloidal stromal collagen deposition and perivascular hyalinized rings, or scattered pleomorphic and multinucleate tumor cells, or had an LPF-like morphology. This category of low cellular *NTRK1* fused tumors exhibited favorable prognosis. On the other hand, highly cellular tumors presented with pronounced atypia and showed a clear malignant behavior.

Similarly, the histopathological spectrum of *NTRK3* fused spindle cell sarcomas has been extensively analyzed[29] and investigators divided them in two major categories. The first category contained tumors with low to intermediate cytologic atypia; mitotic activity could be brisk. Stromal hyalinization and perivascular collagen rings of an LPF-like morphology were described. The second category contained tumors reminiscent of fibrosarcoma or high-grade malignant peripheral nerve sheath tumor (MPNST). They exhibited high-grade cytomorphology

with abundant mitotic features and sometimes necrosis. Notably, CD34 was positive in all cases as was the S100 protein in the majority of the cases. The investigators stress the fact that, in contrast to the low cellular *NTRK1* fused sarcomas, the low-intermediate grade *NTRK3* fused sarcomas can be aggressive neoplasms giving rise to metastatic disease.

In general, *NTRK1* fusions may be indolent, while *NTRK3* fusions mostly occur in aggressive tumors. Different histologic types have been analyzed, some of them presented more frequently. All tumors displayed a spindle cell morphology and the most common architectural patterns were LPF-like and fibrosarcoma-like. In addition, hemangiopericytoma-like and myxoid patterns were also described. Until now, no correlation between the fusion partner and the morphology or clinical outcome can be obtained.

Although a lot of research is done on soft tissue tumors, little is known about the role of *NTRK* fusions in bone sarcomas. A very interesting study revealed three osteosarcoma samples with non-functional *NTRK* fusions[32]. In the first case, an *NTRK2-UFD1* fusion was identified. This fusion led to a frame shift of the *NTRK2* resulting in a stop codon. Similarly, a premature stop codon occurred in the second case with an *NTRK3-VPS18* fusion. In the last case, the *NTRK3-RALGPS2* fusion displayed an in-frame start codon but this could not induce transcription of the functional domain of *NTRK3*. No morphology correlation was evaluated in these cases.

4. TRK-Inhibitory Therapy

a. Activity and Safety of the TRK Inhibitors in Sarcomas

Larotrectinib (LOXO-101) is one of the first FDA approved drugs for targeting tumors with *NTRK* fusions[9]. A multi-centric phase I study at the MD Anderson Cancer Center (NCT02122913)[33] investigated the dose escalation in seventy adult patients with locally advanced or metastatic solid tumor refractory to other standard treatment options. Among these were nine patients with STS and two patients with GIST. The patients were divided in six categories according to a standard 3 + 3 dose escalation scheme. Patients in cohort 1 received 50 mg larotrectinib once daily, in cohort 2100 mg once daily, in cohort 3 100 mg twice daily, in cohort 3a 200 mg once daily, in cohort 4150 mg twice

daily and in cohort 5 they received 200 mg larotrectinib twice daily, in cycles of 28 days. The primary endpoint was drug safety and maximum tolerated dose (MTD) while secondary endpoints were pharmacokinetics, objective response (OR), and duration of response (DoR). The drug was generally well tolerated with sporadic grade 3 treatment-related adverse events (trAEs) but no grade 5 or 6 trAEs were seen. Investigators concluded that a dose of 100 mg twice daily correlates with the best MTD and duration of response.

Eight patients in this trial had an *NTRK* fusion proven by next-generation sequencing (NGS); six involved *NTRK3* and two *NTRK1*. An objective response rate (ORR) was seen in seven out of eight patients with *NTRK* fusions. Among the patients with fusions there was one patient with STS who showed PR and two patients with GIST, one with PR, and the other with complete response (CR). An acquired gatekeeper mutation (TRKC F617L) was detected in a patient with GIST after initial response to the treatment.

Another multi-centric, phase I study (NCT02637687)[34] aimed to investigate the safety of larotrectinib in pediatric tumors. Twenty-four children, adolescents, and young adults, age ranging from 1 month to 21 years, with locally advanced and metastatic disease were included. Patients were divided in three cohorts. In cohorts 1 and 2 doses were dependent on both age and bodyweight to achieve an area under the curve equivalent to the adult doses of 100 mg twice daily (cohort 1) and 150 mg twice daily (cohort 2). Cohort 3 included a dose of 100 mg/m^2 twice daily (maximum 100 mg per dose); in contrast to the previous cohorts, dosage in this cohort was not dependent on age. Seventeen tumors were *NTRK* fused, among them fifteen sarcomas. Patients were divided in three cohorts and received different doses of larotrectinib. The secondary endpoints were MTD, ORR, progression free survival (PFS), and overall survival (OS). Patients tolerated larotrectinib well with only minor grade 3 trAEs. No grade 4 or 5 trAEs were observed. The recommended phase II dose was 100 mg/m^2 body surface area twice daily in cycles of 28 days.

Among the twenty-four enrolled patients, twenty-two were available for response evaluation. Sixty-four percent of them showed an ORR, all of them displaying an *NTRK* fusion. Among the *NTRK* fused patients the

ORR reached 93%, two with CR and twelve with PR. One patient with an acquired G623R solvent-front mutation developed resistance to the treatment.

Additional to its durable effects, larotrectinib demonstrated a rapid response onset. A newborn with an infantile fibrosarcoma of the tongue and extensive lymph node metastasis was treated with 100 mg/m^2 body surface area of larotrectinib. Only two months after initiation of the therapy the patient showed a CR ongoing sixteen months, with negligible toxicity and no safety concerns[35].

Most of the clinical trials investigate TRK-inhibitors in locally advanced or metastatic solid tumors that are refractory to the standard treatment options. Larotrectinib has also been investigated in a neoadjuvant setting as a selective therapy targeting *NTRK* fusions to facilitate surgical resection in children with sarcoma[36]. This treatment is very promising especially in bulky, locally aggressive tumors since surgery can be mutilating.

Another important question that arises is whether TRK-inhibitory therapy should be given as a first line therapy or should be kept for cases that do not respond to the standard treatment. Given the general poor response of unclassified STSs to the standard chemotherapy, some suggest the introduction of TRK-inhibitors as first line treatment whenever the drug is accessible [37].

On the other hand, patients that receive TRK-inhibitors will eventually develop resistance, as will be discussed below. Therefore, some clinicians prefer to keep this treatment option only for cases refractory to the classical treatment protocols.

b. Resistance to TRK-Inhibitory Therapy

Despite the durable effects of TRK-inhibitors, an acquired resistance will eventually develop in some patients with *NTRK* fusions.

This resistance is mostly related to kinase domain mutations, namely solvent front or xDFG substitutions that cause structural changes at the ATP-binding site of the *NTRK*, reducing the ability of larotrectinib to

adhere. A glycerin to arginine substitution in the solvent front of TRKA (G595R) and TRKC (G623R), as well as substitutions of the xDFG of TRKA (G667C) and TRKC (G696A), results in static clashes between the amino acids and components of larotrectinib. LOXO-195 and repotrectinib (TPX-0005), the two next-generation TRK-inhibitors, have been introduced in order to re-establish disease control and have already shown good results in patients with solvent front substitutions in clinical trials[11,12]. However, patients with acquired substitutions of the xDFG motif are more difficult to treat because they can show resistance to the next-generation TRK-inhibitors (type I). Very recently, a type II TRK-inhibitor was introduced, which along with the ATP pocket, also occupies the allosteric pocket that is accessible when the DFG motif is inactivated[38,39].

c. Non-Fusion NTRK Alterations and Their Role in Therapy

A variety of *NTRK* alterations, other than fusions, have been identified in several tumor types: point mutations, amplifications, deletions, and splice variants[40]. Nevertheless, to date very limited response has been established in tumors with non-fused *NTRK* alterations treated with larotrectinib. Hong et al. performed a meta-analysis of three clinical trials (NCT02122913, NCT02576431, NCT02637687) and showed that among the seventy-three patients that did not harbor an *NTRK* fusion, eight had an *NTRK* point mutation, seven an *NTRK* amplification, four an *NTRK* rearrangement, and one had an *NTRK* deletion[41]. Only one patient with an *NTRK* amplification presented a PR of short duration. None of the patients with a point mutation responded to the therapy with larotrectinib. In a case presentation, a metastatic esophageal squamous cell carcinoma with an *NTRK1* amplification that was treated with larotrectinib reported a PR of the primary and metastatic foci; yet, 3.5 months after the treatment initiation, the patient exhibited disease progression[42].

d. Additional Genetic Mechanisms in NTRK Sarcomas

According to the NGS data from the clinical trial of larotrectinib in *NTRK* fused tumors (NCT02122913), patients with such fusions showed no other actionable alterations within the same tumor[33]. Moreover, all tumors described previously harboring an *NTRK* fusion, lacked another

oncogenic driver mechanism. This implies that sarcomas with a known oncogenic driver event will not harbor *NTRK* fusions. Testing for *NTRK* fusions may not be applicable for specific histological types with a known or proven oncogenic driver mechanism, like synovial sarcoma or alveolar rhabdomyosarcoma.

In some cases of *NTRK1* and *NTRK3* fused tumors, an additional secondary cyclin-dependent kinase inhibitor 2A (CDKN2A) deletion has been described[16,17,26,29,43]. The CDKN2A tumor-suppressor locus on chromosome band 9p21 can be inactivated, and this locus is known to be prone to homozygous deletions in a wide range of human cancers[44]. The significance of this finding is unclear. However, it is suggested that especially for the *NTRK1* fusions an additional genetic mechanism is needed in order to induce carcinogenesis[16]. One of the cases with CDKN2A deletion also showed a simultaneous p53 mutation[29]. Finally, non-random gains in one or multiple chromosomes (8, 11, 17, and 20) previously associated with infantile fibrosarcoma have also been mentioned[27]. A rare case with an *NTRK1* fusion showed amplification of the fusion gene locus[16].

5. Diagnostic Algorithms

a. Techniques to Identify NTRK Fused Sarcomas

As illustrated, patients with sarcomas that harbor *NTRK* fusion are good candidates for TRK-inhibitory therapy in adjuvant, neoadjuvant, or metastatic setting. The response rates are durable and the drugs are well tolerated, even in younger ages. Resistance to the first-generation inhibitors can be largely tackled by introducing the new generation inhibitors. Therefore, identification of the *NTRK* fused malignancies is of outmost importance.

Morphology can augment in selecting those tumor types that are more likely to display an *NTRK* fusion. Immunohistochemistry reveals protein expression and has been proposed as a method to pre-screen for the presence of *NTRK* fusions[45]. **Figure 1** shows a spindle cell tumor with diffuse positivity for pan-TRK immunohistochemistry.

There are different pan-TRK immunohistochemical antibodies such as the EPR17341 from Roche and the A7H6R from Cell Signaling Technology. The sensitivity of this technique is not optimal, especially for detecting the *NTRK3*, which according to different studies does not exceed 79%[46,47]. It has been proven that samples from tumors with an amplification, mainly of the *NTRK1*, can show TRK immunohistochemical positivity in nearly 15% of the cases[48]. Hence, TRK immunohistochemical positivity can correlate also to other, non-fusion alterations of the *NTRK*. Although the technique lacks specified criteria for evaluation, a Belgian Ring Trial for pan-TRK immunohistochemistry showed excellent concordance between the participating laboratories compared to the referral centrum[49].

Confirmation of the fusion comes through molecular techniques. This can happen through Fluorescence in Situ Hybridization (FISH), although it does require using the three FISH probes for *NTRK1, 2*, and *3* separately[50]. Real Time Polymerase Chain Reaction (RT-PCR) is also readily available and a low-cost procedure. However, as the fusion partner has to be known, it cannot detect any novel fusions[51,52]. DNA-based sequencing can be problematic because *NTRK*, especially *NTRK2* and *NTRK3*, have large intronic regions in which all breakpoints cannot be adequately covered[53]. Instead, RNA-based sequencing is more preferable, given that splicing out of introns simplifies the technical requirements[54]. For RNA-based NGS, the quality of the sample plays a critical role[50]. This is particularly crucial in cases of bone sarcomas, where fixation and demineralization procedures can result in RNA degradation[55].

b. Guidelines and Algorithms for Identification of NTRK Fused Sarcomas

For patients to benefit from a targeted therapy, it is essential to identify tumors with *NTRK* fusions. Until today, different testing algorithms have been proposed but no guidelines exist. There are three main algorithms published to date; those proposed by the ESMO[54], those from the Memorial Sloan Kettering Cancer Center[56], and those presented by Penault-Liorca et al.[57]. In addition, the Canadian Consensus has published its own data recently[37]. Despite the differences between these algorithms, the principle remains the same. Regarding sarcomas, in case

that the tumor is known to have a high prevalence of *NTRK* fusions (e.g., infantile fibrosarcoma), direct confirmation with molecular techniques is recommended. If not, one can choose to perform immunohistochemistry with a pan-TRK antibody as an enrichment strategy. In cases that immunohistochemistry reveals an NTRK protein expression, a confirmation with molecular techniques is necessary. The molecular technique favored in most cases is the NGS at RNA level.

To date, each laboratory uses its individual strategy for the identification of the *NTRK* fused sarcomas and other tumors, therefore no suggestion can be made about the effectiveness of each diagnostic algorithm. **Figure 2** suggests a diagnostic algorithm for detection of *NTRK* fusions in sarcomas, according to the data presented in this review.

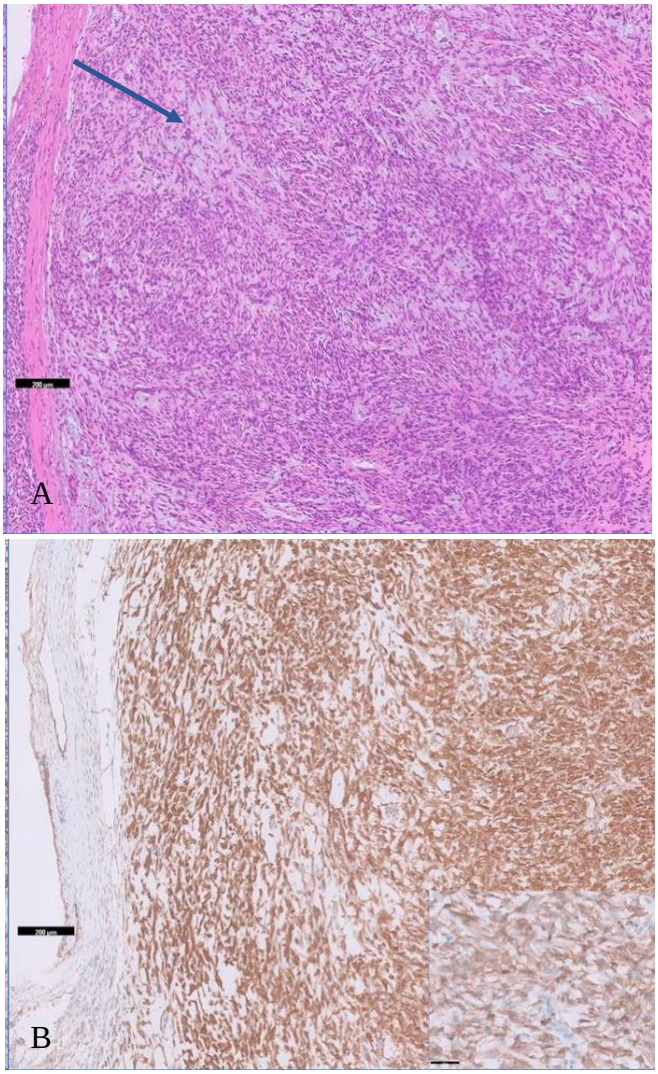

Figure 1. Spindle cell tumor on the deep soft tissue of the back with myxoid stroma (arrow) and diffuse cytoplasmatic positivity for pan-TRK IHC (**A**: Hematoxyline-Eosine, scale bar 200 μm, **B**: Antibody: EPR17341, Roche; Chromogene: DAB, scale bar 200 μm, insertion 50 μm).

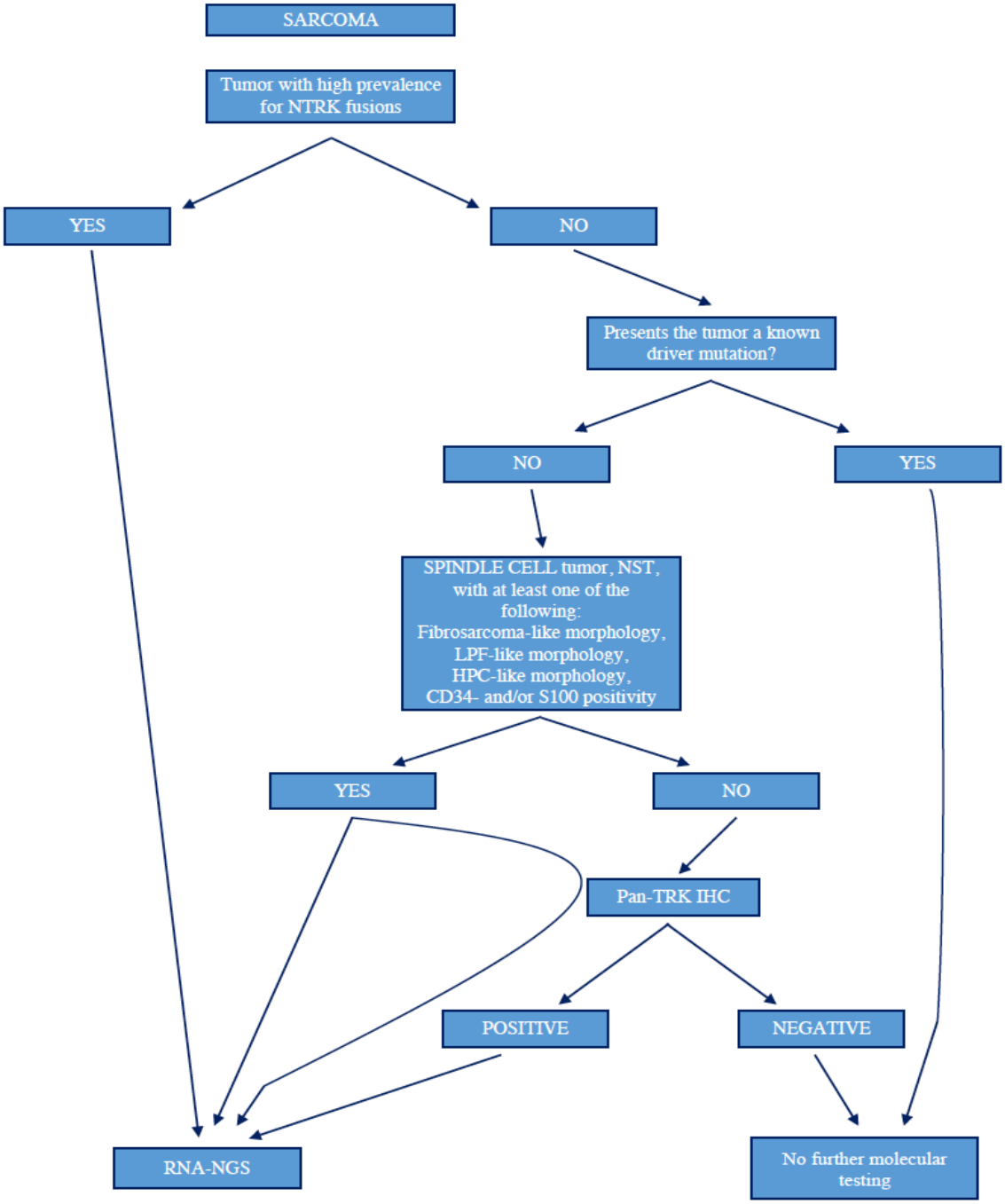

Figure 2. Diagnostic algorithm for the identification of NTRK fused sarcomas, according to the data presented in this review. In cases of a spindle

cell tumor NST with a histologic profile suggestive for a NTRK fusion, one can proceed directly to molecular testing or perform a pan-TRK IHC together with molecular testing, the latter in order to investigate the sensitivity of IHC especially in cases of NTRK3 gene fusions. The preferable molecular technique is RNA-NGS.
Abbreviations: HPC; hemangiopericytoma IHC; immunohistochemistry; LPF; lipofibromatosis, NGS; next generation sequencing, NST; non special type

6. Conclusions

The presence of an *NTRK* fusion is a rare genetic event arising in sarcomas. Inhibition of TRK is a promising and well-tolerated targeted therapy for selective tumors. An additional advantage of this treatment approach is that the same drug targets all three types of *NTRK* fusions. Today, most of the data show that all *NTRK* fused tumors are highly responsive to the available TRK-inhibitors. The efficacy of those drugs in sarcomas with *NTRK* alterations other than fusion remains questionable. Occasionally, patients with *NTRK* amplified tumors may respond to TRK-inhibitors, still this effect might not be durable. Hence, it is clear that standardization of the diagnostic algorithm is of outmost importance in order to select the patient population that is more likely to benefit from the therapy.

The histological picture seems to play an essential role in the identification of sarcomas that are good candidates for immunohistochemical and/or molecular testing. *NTRK* fusions are oncogenic driver alterations, therefore, tumors with a known driver alteration (mutation, translocation, amplification) are very unlikely to harbor *NTRK* fusions. As a consequence, molecular testing for those tumors may not be applicable. In summary, spindle cell tumors with a fibrosarcoma-, LPF-, myopericytoma/hemangiopericytoma-like or myxoid morphological pattern, positive for CD34 and/or S100 protein, but without a known driver oncogenic alteration, meet the suggested criteria for NTRK fusion testing. Confirmation warrants molecular techniques, with NGS testing at RNA level being the most preferable.

To date, *NTRK* fusions in osteosarcomas do not seem to be functional alterations. Nevertheless, given the limited data that are available, further investigation is needed.

Although TRK is a promising target for therapy with durable effects, resistance to first-generation drugs has already made its appearance. Next-generation drugs can re-establish disease control, still this is a considerable obstacle leading to uncertainty about the long-term effectiveness of this therapy.

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CHAPTER 7

RESULTS: NTRK FUSIONS IN SARCOMAS: EXPANDING THE MORPHOLOGICAL SPECTRUM AND ITS RELEVANCE TO THE CLINIC

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NTRK fusions in sarcomas: expanding the morphological spectrum and its relevance to the clinic
Manuscript submitted in Virchows Archiv (under review)

1. Abstract

Targeting molecular alterations has been proven to be an inflecting point in tumor treatment. Especially in recent years, inhibitors that target the tyrosine receptor kinase (TRK) show excellent response rates and durable effects in all kind of tumors that harbor fusions of one of the three neurotrophic tyrosine receptor kinase genes (*NTRK1*, *NTRK2* and *NTRK3*). Today, the therapeutic options in most metastatic sarcomas are rather limited. Therefore, identifying which sarcoma types are more likely to harbor these targetable *NTRK* fusions is of paramount importance. At the moment, identification of these fusions is solely based on immunohistochemistry and confirmed by molecular techniques. However, a first attempt has been made to describe the histomorphology of *NTRK*-fusion positive sarcomas, in order to pinpoint which of these tumors are the best candidates for testing.

In this study, we investigate the immunohistochemical expression of pan-TRK in 70 soft tissue and bone sarcomas. The pan-TRK positive cases were further investigated with molecular techniques for the presence of a *NTRK* fusion. The goal of this study is to expand the histomorphological spectrum of the *NTRK*-fused sarcomas, to identify their fusion partners and to correlate these parameters with the clinical outcome of the disease. In addition, we evaluated the immunohistochemical expression pattern of the pan-TRK and its correlation with the involved *NTRK* gene.

2. Introduction

Soft tissue and bone tumors are a very heterogenous group of neoplasms which makes prognosis difficult to assess. Moreover, given their rarity of occurrence, standardization of diagnostic criteria and precise treatment protocols is challenging. The majority of localized sarcomas are treated with excision mostly followed by adjuvant radiotherapy to control local recurrence [1,2]. Few sarcoma types are chemo-sensitive, such as rhabdomyosarcoma, osteosarcoma and Ewing sarcoma [2,3]. In the metastatic setting, the five-year overall survival rates do not exceed 20%, highlighting that effective treatment of advanced disease remains a challenge. There is clear need for new therapeutic options in advanced sarcomas [4].

Some of the molecular alterations that are found in tumors are druggable and this has been a significant turning point in cancer treatment. One of the most important representatives are tyrosine kinase inhibitors (TKI) that are used for the treatment of diverse tumor types, such as imatinib for gastrointestinal stromal tumors (GIST) [5]. Unfortunately, today most of the oncogenic driver alterations remain undruggable.

Very recently, it has been proven that tumors with fusions of one of the three neurotrophic tyrosine receptor kinase genes (*NTRK1*, *NTRK2* and *NTRK3*) can be treated with tyrosine receptor kinase (TRK) -inhibitors. The first generation TRK-inhibitors, larotrectinib and entrectinib [6], show excellent response rates and durable effects in tumors that harbor fusion of one of the *NTRK* genes, regardless of tumor type or site of origin. The adverse events are well tolerated. Still, a major obstacle for those inhibitors is the development of resistance. Therefore, new generation TRK-inhibitors are developed, such as LOXO-195 and repotrectinib [7,8].

Despite the promising results of TRK-inhibition, *NTRK* fusions are rare genetic events. Among sarcomas, infantile fibrosarcomas show fusions of the *NTRK3* gene in more than 90% of the cases [9]. The rates of *NTRK* fusions in the remaining sarcoma types are unfortunately very low. Moreover, no clear morphologic criteria are established for the recognition of the *NTRK*-fused sarcomas. A NTRK fusion has to be demonstrated by means of molecular analysis. Immunohistochemistry can be performed as an enrichment strategy to select tumors for subsequent molecular analysis In a previous publication, we suggested an algorithm for determining which sarcomas are more likely to carry this fusion [10].

In this study, we investigated the expression of the pan-TRK antibody in different types of soft tissue and bone tumors by immunohistochemistry and we correlated the expression of the positive cases with the presence of *NTRK* fusions. Furthermore, we summarized the clinicopathological characteristics, in an attempt to give more information about the identification of these tumors, in conjunction with the cases that are described in the literature.

3. Materials and methods

a. Tissue samples

Archival formalin-fixed paraffin embedded (FFPE) tissue samples from 70 patients with soft tissue and bone tumors were retrieved from the Department of Pathology at the Antwerp University Hospital. The tissue samples were collected from biopsy material as well as excision specimens. The material was not older than 5 years. The biopsies were fixed in 4% formaldehyde for up to 12 hours while the excision samples were fixed for up to 32 hours.
We received approval by the Ethics Committee of the Antwerp University Hospital/University of Antwerp (EC 18/45/517) to use historical samples. As it was a retrospective study on archival material, no informed consent of the patients could be obtained.

b. Immunohistochemistry

As a reference method we used the VENTANA pan-TRK assay (clone EPR17341) performed according to the instructions of the vendor on a Benchmark Ultra (Ventana Medical Systems, Tucson, AZ). This widely used EPR17341 clone is reactive with a conserved proprietary peptide sequence from the C-terminus of TRKA, TRKB and TRKC, and is therefore reactive with any of the oncogenic TRK proteins, although a lesser sensitivity for *NTRK3* fusions is described.

We looked at the expression of TRK in the tumor cells. Tumors were considered positive if $\geq$ 1% of tumor cells exhibit staining at any intensity above background [11,12]. In addition, the different subcellular staining patterns (cytoplasmic, membranous, nuclear and peri-nuclear) were all considered to be positive. Staining intensity was not taken into account. Evaluation of the slides was based on scanned slides at a Philips platform.

c. Molecular Techniques

The *NTRK* fusion status and possible fusion partner of the samples was confirmed by next generation sequencing (NGS). Targeted RNA-based NGS was conducted with the Oncomine Focus Assay (OFA) panel

(Thermo Fisher Scientific, San Francisco, CA) on an S5 instrument, according to the manufacturer's recommendations.

4. Results

a. Pan-TRK immunohistochemical expression in diverse tumor types and correlation with NTRK fusions

We investigated the immunohistochemical expression of TRK in diverse types of soft tissue and bone tumors. Tumor characteristics are summarized in **table 1**.

Among the different tumor types, seven tumors (10%) displayed immunohistochemical positivity for pan-TRK in the tumor cells. The remaining 62 tumors showed no pan-TRK expression.

Among the positive tumors there were two alveolar rhabdomyosarcomas, one epithelioid angiosarcoma, one malignant peripheral nerve sheath tumor (MPNST), one osteosarcoma and two spindle cell sarcomas, not otherwise specified (NOS). All but one were reported as high grade tumors. The low-grade tumor was a spindle cell sarcoma NOS.

NGS RNA analysis was performed in all positive cases. Two out of these (nearly 28,6% among the pan-TRK positive cases and 2,86% among all tumors included in this study) showed an *NTRK* fusion, while the rest did not. The characteristics of the tumors with the fusion are summarized in **table 2**.

Table 1. Tumor characteristics.

CHARACTERISTICS	FREQUENCY (n)
Gender	
Male	46
Female	24
Tumor location	
Bone	19
Deep soft tissue extremities	17
Deep soft tissue trunk and back	5
Deep soft tissue head and neck	4
Skin and subcutaneous fat tissue	12

Abdomen	6
Mediastinum	5
Retroperitoneum	2
Histological type	
Chondrosarcoma	10
Ewing sarcoma	3
Osteosarcoma	5
Angiosarcoma	7
Kaposi sarcoma	8
Leiomyosarcoma	5
Liposarcoma	9
Myxofibrosarcoma	5
Rhabdomyosarcoma	3
Synovial sarcoma	3
Sarcoma NOS	12
Grade	
High grade	48
Low grade	18
Not known	4

Abbreviations: n; number, NOS; not otherwise specified

Table 2. Summary of clinical, immunohistochemical, and molecular data.

Pt	**Age**	**Sex**	**Diagnosis**	**Location**	**Fusion**	**IHC**	
						Pattern	Intensity
1	10	M	Low grade spindle cell tumor	Skin, finger	*ETV6-NTRK3*	Nuclear	Weak
2	19	M	High grade spindle cell tumor	Deep soft tissue of the lower leg	*TFG-NTRK3*	Cytoplasmic	Strong

Abbreviations: IHC; immunohistochemistry, Pt; patient, M; male

b. A low-grade spindle cell tumor with an ETV6-NTRK3 fusion

The first case concerned a 10 year-old male with a skin lesion on his finger. The duration of the lesion could not be determined accurately but was estimated by the parents to be six to seven years. No previous operations or other therapies were mentioned.

Macroscopically, there was a polypod lesion that measured approximately 5 cm. Microscopy revealed a dermal spindle cell proliferation. The cells were arranged in a fascicular pattern. There was some variation in size and shape but there was no striking

pleiomorphism. Mitotic activity was present (5 mitosis / 10 high power fields), but no necrosis was perceived. There was local mucin deposition between tumor cells. Finally, some blood vessels within the tumor showed hyalinization and presence of multinucleated cells in vessel wall (**figure 1**). The tumor was located in the dermis with focal extension in to the subcutis. The lesion was completely removed but with narrow margins. Nuclear positivity of the tumor cells for pan-TRK was noticed on immunohistochemistry (**figure 2**). The NGS analysis revealed an *ETV6-NTRK3* fusion. The patient underwent positron emission tomography–computed tomography (PET-CT) but no metastatic lesions could be detected. No signs of local recurrence or disseminations were noticed six months after the diagnosis.

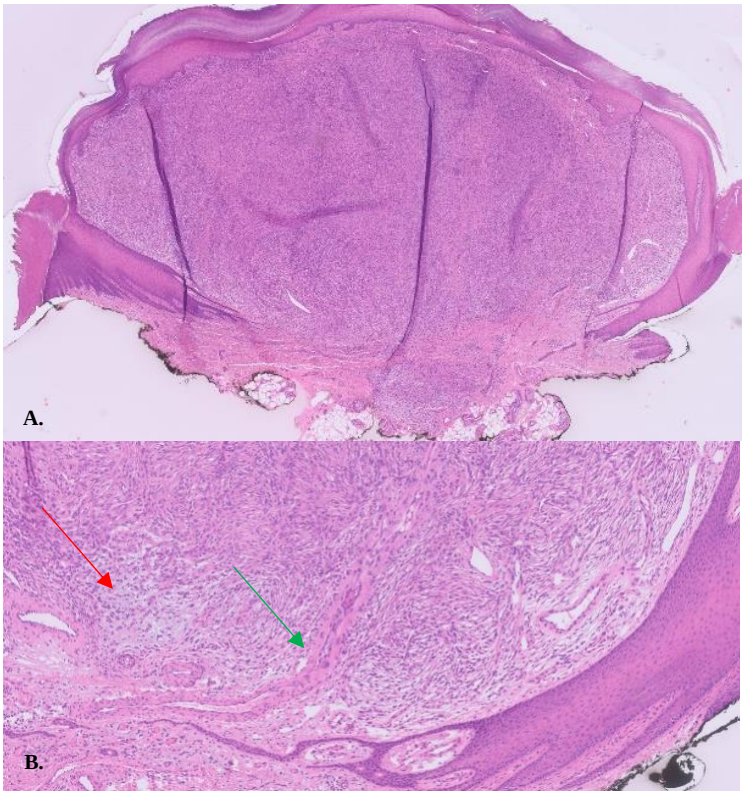

Figure 1. A (HE, 20x): polypoid dermal spindle cell proliferation. B HE, 100x): The tumors display a fascicular growth pattern. There are myxoid areas (red arrow), as well as vessel walls with presence of multinucleated cells (green arrow).

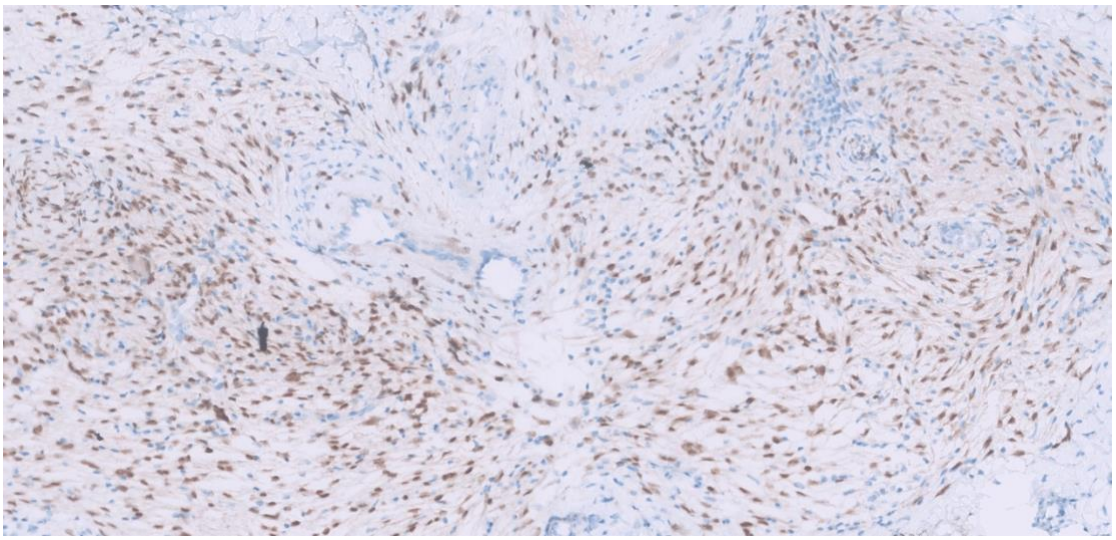

Figure 2. Pan-TRK nuclear positivity in the tumor cells. Pan-TRK assay (clone EPR17341), DAB, magnification 200X.

c. A high-grade spindle cell tumor with a TFG-NTRK3 fusion

The second case concerned a 19 years-old man. The patient presented with a tumor on the antero-external side of the right tibia dating for several months. The tumor was located intramuscularly with extension to the subcutaneous fat tissue. Three months after the first examination, the tumor was excised. Macroscopically, an almost 14 cm large mass was seen. Microscopy revealed a multinodular lesion that was partially surrounded by a thin capsule. Spindle-shaped tumor cells with a fascicular growth pattern was seen. Thickened collagen bundles were seen in between tumor cells, as well as areas with calcification and ossification (**figure 3**). There was high mitotic activity and also necrosis. After extensive immunohistochemical analysis, the tumor was classified as high-grade spindle cell sarcoma NOS. Despite the fact that the tumor was completely excised, given its high-grade, the patient received adjuvant radiotherapy. Four months after the excision the patient developed multiple lung metastases, which were treated with radiotherapy. Immunohistochemistry on the material from the first excision revealed a cytoplasmatic positivity for pan-TRK in the majority of the tumor cells (**figure 4**). NGS analysis showed the presence of a *TFG-NTRK3* fusion. The patient started with larotrectinib at a dose of

100mg twice daily. The patient was still alive a month after the initiation of treatment and was then lost from follow-up.

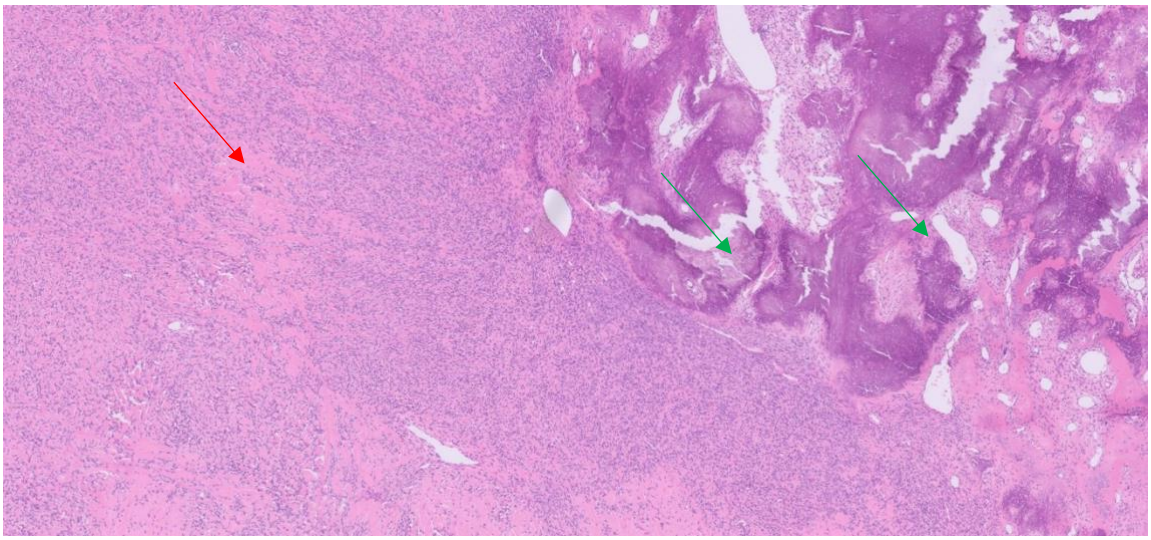

Figure 3. HE, 20x. Spindle cell proliferation. Between the tumor cells are thick collagen fibers (red arrow), as well as areas with calcification and ossification (green arrows).

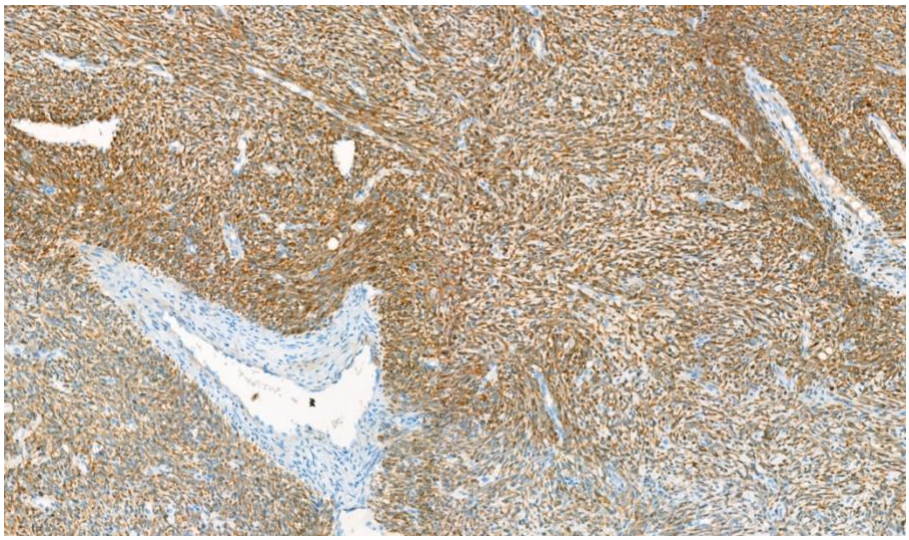

Figure 4. Pan-TRK nuclear positivity in the tumor cells. Pan-TRK assay (clone EPR17341), DAB, magnification 100X.

5. Discussion

TRK is a member of the tyrosine kinase family, predominantly known for its role in neuronal cell differentiation. There are three receptors (TRK A, B, and C) encoded by the three *NTRK* genes, *NTRK 1*, *2*, and *3*, respectively [9]. TRK is a transmembrane receptor, that upon its binding with a ligand, undergoes dimerization. TRK dimerization leads to activation of three signaling pathways: the PI3K/AKT, the MAPK and the PLCγ pathway, all of which impact cell proliferation, cell growth, and cell survival [13] (**figure 5**). Fusion of one of the three *NTRK* genes leads to ligand independent constitutive activation of the TRK signaling pathway that can induce uncontrolled cell growth and proliferation, neo-angiogenesis and cell migration [14].

NTRK fusions are highly actionable driver alterations that are found across many different tumor types. TRK inhibitors are very active against tumors that harbor one of the *NTRK* fusions. These drugs have a high response rate and durable responses [13,14]. Hence, detection of *NTRK*-fused tumors can have important therapeutic consequences for the patient.

With regard to sarcomas, Infantile Fibrosarcoma (IF) shows the *ETV6-NTRK3* fusion in more than 90% of the cases [9]. No other specific sarcoma types are corelated with the presence of fusions of one of the *NTRK* genes. Moreover, sarcomas with an *NTRK* fusion show no other actionable alterations within the same tumor [15]. Thus, sarcomas with a known driver oncogenic alteration, as for instance alveolar rhabdomyosarcoma or synovial sarcoma, are less likely to harbor fusions of one of the three *NTRK* genes. This is in line with our results, where the two *NTRK*-fused tumors where not of a histologic type that carries a specific genetic alteration. Consequently, testing for *NTRK* fusions in sarcomas with a known driver oncogenic alteration might not have any diagnostic or therapeutic value. Specific sarcoma types could therefore be excluded from testing for *NTRK* fusions.

Currently, correct identification of *NTRK*-fused sarcomas is only possible by means of molecular techniques. However, recently there have been attempts to describe the morphological features of tumors carrying this molecular alteration. To date, various morphological

features have been described in *NTRK*-fused sarcomas, the most consistent being spindle cell morphology [10]. Growth patterns attributed to these tumors are summarized in our previous review [10] and include lipofibromatosis-like [16,17], hemangiopericytoma-like [18], fibrosarcoma-like [19-21], neoplasms resembling inflammatory myofibroblastic tumors [22,23] and dermatofibrosarcoma protuberans-like [24]. Moreover, spindle cell tumors with myxoid features [25] as well as characteristic blood vessels with perivascular thick collagen deposition [26] are also reported. On a molecular basis, *NTRK* fusions are the driving oncogenic mechanisms, with fusions of the *NTRK3* gene being the most frequent, followed by fusions of the *NTRK1* gene, while only a few show *NTRK2* fusions [10].

In our case, both tumors with *NTRK* fusion presented with a spindle cell morphology. None of the tumors showed a characteristic growth pattern like those previously mentioned. It is suggested that the majority of the *NTRK*-rearranged mesenchymal neoplasms display a combination of morphological patterns, which could be a helpful clue in tumor recognition [27]. Indeed, one of the tumors that we present also showed hyalinization of the blood vessels while the other displayed thickened collagen bundles between the tumor cells.

In many cases in literature, the tumors expressed CD34 and / or S100-protein on immunohistochemistry [16-21,24,26,28]. None of the *NTRK*-fused cases in our series were positive for these immunohistochemical markers. However, this could be a useful diagnostic tool in cases of a spindle cell tumor without a specific growth pattern, in order to pinpoint those neoplasms that need further investigation for the presence of the fusion.

Pan-TRK immunohistochemistry is a reliable screening method for the detection of *NTRK* fusions with sensitivity and specificity exceeding 95% [12,29]. In our samples, only two out of the seven immunohistochemical positive cases (nearly 28,6%) correlated with the presence of a *NTRK* fusion. However, the number of the investigated cases in our series is relatively small and these data should therefore be considered with caution. False-negative cases, with negative pan-TRK immunohistochemistry while the tumor was proven to harbor a *NTRK* fusion, are rarely described [27,29,30]. The staining pattern of the antibody

also varies in intensity and localization. Different subcellular staining patterns have been documented such as cytoplasmic, cell membranous, nuclear with or without peri-nuclear accentuation, all of which are considered as positive [20,29,31,32]. All these staining patterns suggest the presence of the fusion, and molecular testing is needed for confirmation. Subsequently, there has been an attempt to demonstrate a correlation between the staining pattern and the *NTRK* gene fusion. Hence, it has been shown that fusions of the *NTRK1* gene mostly correlate with a diffuse cytoplasmic staining, while nuclear and rather weak pan-TRK staining is frequently mentioned with *NTRK3* gene fusions [27]. Furthermore, nuclear positivity is correlated with *ETV6-NTRK3* fusion in different tumor types [29,33]. This is consistent with our findings, where one of the tumors showed pan-TRK weak nuclear positivity, and this tumor harbored an *ETV6-NTRK3* rearrangement.

The prognosis of *NTRK1*- and *NTRK3*- fused sarcomas in correlation with histomorphology, has also been a subject of investigation. Sarcomas with *NTRK1* gene fusions can present a low- or a high-grade histomorphology. While morphologically high-grade *NTRK1*-fused tumors display an aggressive course in the majority of the cases, morphologically low-grade *NTRK1*-fused sarcomas can have a favorable clinical course [10,34]. On the other hand, sarcomas with fusions of the *NTRK3* gene and especially those with *ETV6* fusion partner, are mainly aggressive neoplasms, even those with intermediate cytological atypia [10,35]. Low-grade cytomorphology is usually not a feature of these fusions. This is in contrast with our findings. We describe a tumor with *ETV6-NTRK3* fusion with a rather indolent course. The tumor was present for almost six to seven years prior to the diagnosis and showed no signs of recurrence or metastatic spread almost a year after the excision. Morphologically, the tumor showed a cellular spindle cell proliferation without pronounced cytological atypia; mitotic activity was apparent but rather limited, while no necrosis was seen. An interesting feature was the hyalinization and presence of multinucleated cells in the blood vessel wall.

In addition, we describe a *TFG-NTRK3* fused sarcoma with an aggressive course. From a histological point of view, the tumor was a high-grade neoplasm with marked cytological atypia, increased mitotic activity as well as areas of necrosis. No CD34 or S100-protein

expression was observed. The patient also developed disseminated disease. These findings are again in contrast with the features of the limited *TFG-NTRK3* fused mesenchymal tumors documented in the literature [16,35]. *TFG-NTRK3* sarcomas are amongst the rare *NTRK3*-fused sarcoma cases with a rather favorable histological and clinical picture. Opposite to our case, the tumors are known to display mostly an intermediate cytological grade or a lipofibromatosis-like morphology. Immunohistochemical positivity of the neoplastic cells with CD34 and S100-protein were documented, in contrast to our case. The prognosis of these tumors is reported favorable with no evidence of recurrence or metastasis in the (rather limited) follow up period.

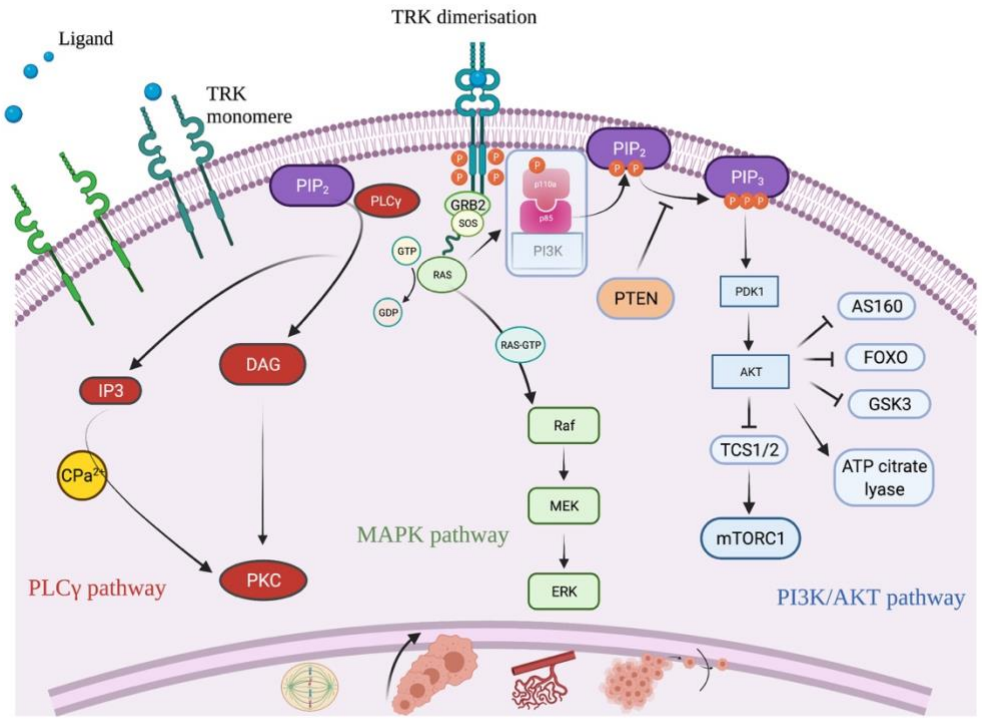

Figure 5. Schematic illustration of the TRK signaling pathway and its role to cell differentiation.
Created with BioRender.com

6. Conclusion

NTRK fusions are rare genetic events that can appear in a wide range of tumor types, including mesenchymal tumors. Identification of rearrangement of one of the three *NTRK* genes can lead to right treatment choices, as they show great benefit from TRK-inhibitory therapy. Nowadays, we start to recognize the histomorphology that in most cases correlates with the presence of the *NTRK* fusion in sarcomas. With our research, we aimed to broaden the diagnostic spectrum of this category and its correlation with the clinical and prognostic aspects. We described two soft tissue tumors with *NTRK3* fusions among 70 soft tissue and bone sarcomas. Both are spindle cell neoplasm with variable growth patterns, in accordance with prior publications. Pan-TRK immunohistochemistry was positive, and one of the cases displayed weak nuclear staining which correlated with the *ETV6* fusion partner. In contrast to what has been described in literature, none of the tumors we present showed CD34 or S100-protein expression on immunohistochemistry. Moreover, we investigated the correlation of morphology and clinical behavior of these tumors. Namely, the neoplasm with the *ETV6-NTRK3* fusion displayed an indolent course, in contrast to the published cases that display an aggressive behavior. Finally, we described a very rare *TFG-NTRK3* fusion in a sarcoma that developed metastatic disease.

To conclude, *NTRK*-fused sarcomas are spindle cell tumors with variable growth patterns. Pan-TRK immunohistochemistry can pinpoint the cases that need further investigation by means of molecular testing. Nuclear positivity correlates with *ETV6-NTRK3* fusion. According to our results, the presence of specific fusion partners do not seem to be a good surrogate marker to predict prognosis.

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CHAPTER 8

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

1. General discussion

a. Looking for predictive biomarkers for response to immunotherapy and possible targets to prevent immune evasion in sarcomas

The development of immunotherapy has reached an important inflection point in the treatment of various malignancies. Still, the notion of immunological mechanisms in resistance to disease has been around, in one form or another, for centuries. The first knowledge we have on the subject comes from ancient Greek writings. Thucydides (Θουκυδίδης) was an historian and a general, known for his evidence-gathering and analysis of cause and effect. After a devastating plague in Athens around 430 BC, Thucydides noticed that people who survived were not affected twice and he hypothesized that they developed an acquired resistance to the disease[1,2].

The idea of using infection as therapy to reduce tumor size is first recorded in the late 19th century. Specifically, Dr. William Coley, an American sarcoma surgeon, used erysipelas infection to treat unresectable sarcomas[3]. This is the first referral to immunotherapy used for sarcoma treatment and therefore Coley is recognized as the *Father of Cancer Immunotherapy*.

The idea behind immunotherapy lies in the use of the body's own resources to fight against a tumor. T cells patrol the body for non-self-cells. Host cells express certain proteins (antigens) on their surface that lead to their recognition by T cells. If the protein is not recognized, the T cells will attack the cell that carries the foreign antigen. Respectively, in case of malignancy, the immune cells will spot the foreign antigens on the surface of the tumor cells, and they will eventually attack the tumor. Tumor cells, in order to avoid elimination from the activated immune system, will express additional molecules, which by binding with their corresponding receptors on the immune cells, will lead to the inactivation of the latter. This way tumor cells can have a control on the activated immune system. These molecules and the corresponding receptors are therefore known as immune checkpoints (ICs). A well-known example of ICs is programmed death ligand-1 (PD-L1)

expressed on tumors cells that binds to programmed death-1 (PD-1) expressed on immune cells[4], resulting in exhaustion of the immune cells.

The past decade was a major turning point in cancer immunotherapy. Many tumor types nowadays benefit from this type of therapy with lymphoma, melanoma and lung carcinoma being the pioneers[5-8]. As sarcomas in advanced stage are difficult to treat more effective new therapeutic approaches are needed, and in this case immunotherapy seems to be a promising strategy. Still, in terms of sarcomas, no particular improvement has been made to date with regard to immunotherapy. The different therapeutic strategies that are used for sarcomas nowadays are shown in **figure 1**.

As we discussed in **chapter 3**, many ring trials already provided a rationale for the use of immune checkpoint blockade (ICB) to treat sarcomas. In these trials, different protocols using ICB (i.e. PD-1, PD-L1 an CTLA-4 inhibitors) as monotherapy or in combination with other types of therapy were investigated in diverse tumor types and different age groups. In general, the drugs were well tolerated with limited numbers of high-grade adverse events (AE). In a phase I ring trial (NCT01445379), the investigators noticed that patients with more immunotherapy related AE (irAE) responded better to the treatment9. In general, the ORR of ICB as mono- or combination therapy was limited in sarcomas. Yet, looking at the different sarcoma types individually, there are some positive conclusions for specific sarcomas. For instance, alveolar soft part sarcoma (ASPS) showed an objective response rate (ORR) that almost reached 40%10. This suggests that sarcomas should probably be investigated by type rather than as a group.

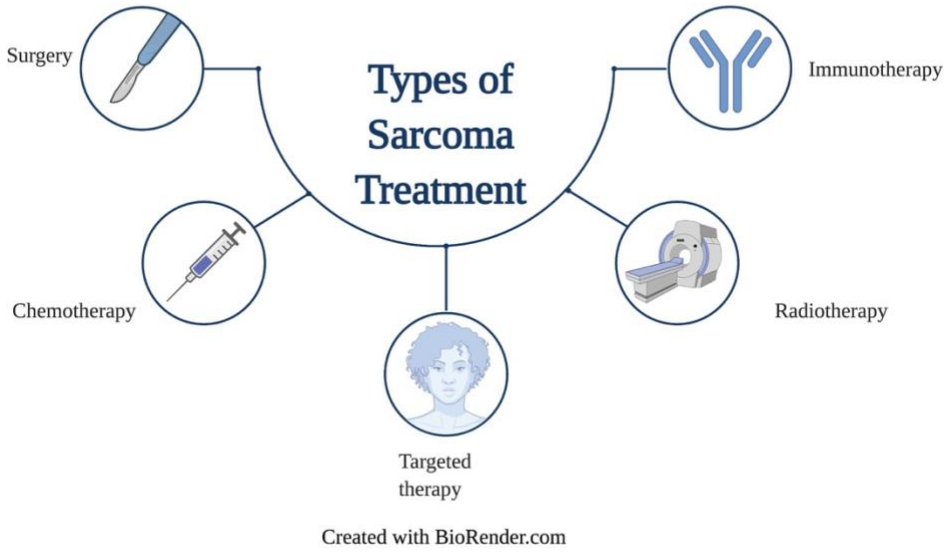

Figure 1. Therapeutic approaches in sarcomas.

Some biomarkers may play a predictive role in response to ICB. Among them, the expression of PD-L1 on tumor cells is being used as a surrogate to predict response to immunotherapy in specific cancers[11-14]. Only a few clinical trials investigated the expression of PD-L1 on sarcoma cells in tissue samples. No significant correlation between PD-L1 positivity and ORR could be demonstrated, still in few cases a prolonged progression free survival (PFS) was noticed in patients with PD-L1 positive tumors[15].

In some instances, an increased amount of CD4 positive T lymphocytes was seen after drug administration with anti-PD-1[16], while non-responders showed a higher percentage of CD4 positive T cells[10]. In other cases, responders had a higher tumor infiltrating lymphocyte (TIL) score, mostly CD3 positive and CD8 positive aggregates at the periphery of the tumor[17]. Thus, it seems that the tumor microenvironment (TME) also plays a role in the response to ICB.

Driven by the fact that the TME takes part in the response to ICB, we considered it necessary to investigate its composition in soft tissue tumors. Hence, in **chapter 4** we analyzed the TME in desmoid tumors (DTs). DTs are locally aggressive mesenchymal neoplasms that often present lymphoid aggregates at the tumor margins. We found that those lymphoid aggregates have a composition that strongly resembles tertiary lymphoid organs (TLOs). These organs are in general responsible for the recruitment of cells in the TME. We analyzed these structures in DTs and found a remarkable presence of B lymphocytes. T lymphocytes were also present, among which high percentages of cytotoxic CD8 positive T cells. In a recent study, sarcomas with TLOs that are rich in B cells and had also high levels of cytotoxic CD8+ T cells demonstrated improved survival and a high response rate to PD-1 blockade by pembrolizumab in a phase 2 clinical trial[18,19]. This suggest that the B cell-rich sarcoma group may be a good candidate for treatment with ICB, particularly with anti-PD-1. On the other hand, we know that specific mutations may manipulate the immune response. In particular, it is proven that activation of the β–catenin pathway gives rise to a T cell exclusion phenotype[20]. Patients with mutations of the CTNNB1 gene, such as DTs, are thus very unlikely to benefit from ICB, suggesting β–catenin as a possible negative predictive biomarker. It is therefore clear that the lymphoid aggregates and TLOs are of great importance for response to immunotherapy. However, they are not an independent predictive factor and other possible mechanisms should also be considered.

In **chapter 5** we focused on examining biomarkers that may prevent immune evasion in sarcomas. Hence, we investigated the expression of PD-1, PD-L1, Indoleamine-pyrrole 2,3-dioxygenase (IDO) and Cluster of Differentiation 70 (CD70) in almost 70 sarcoma samples from different patients. Although all these markers work in a different way, they have one thing in common: they modify the tumor immune response in such a way that it is no longer effective against the tumor. We examined the expression of each of these markers in the different compartments of the TME and the tumor. Our first interesting finding was that soft tissue tumors in most of the cases are “hot” or otherwise inflamed, meaning that they attract immune cells in the TME. In contrast, bone tumors almost always show lack or paucity of tumor immune cell infiltration, that characterizes the “cold” phenotype (**figure**

2). Many parameters have been implied as responsible for this phenotype, among others absence of T cell priming or activation, lack of tumor antigens and deficit of T cells homing to the tumor bed[21]. In osteosarcomas it is shown that immune cell infiltration is depended on the plasticity of the tumoral extracellular matrix as well as proteolysis of this matrix through metalloproteinases[22]. T cells may cross the blood vessels, but they cannot progress along the dense and tight fibers of the osteosarcoma extracellular matrix, thus remaining trapped within it[23]. Hence, it is suggested that induced proteolysis of the extracellular matrix could be the key of success of any immune cell therapy for osteosarcomas[24].
Regarding the bone sarcomas, we observed a higher expression of IDO on the bone sarcoma cells in contrast to the soft tissue sarcoma cells.

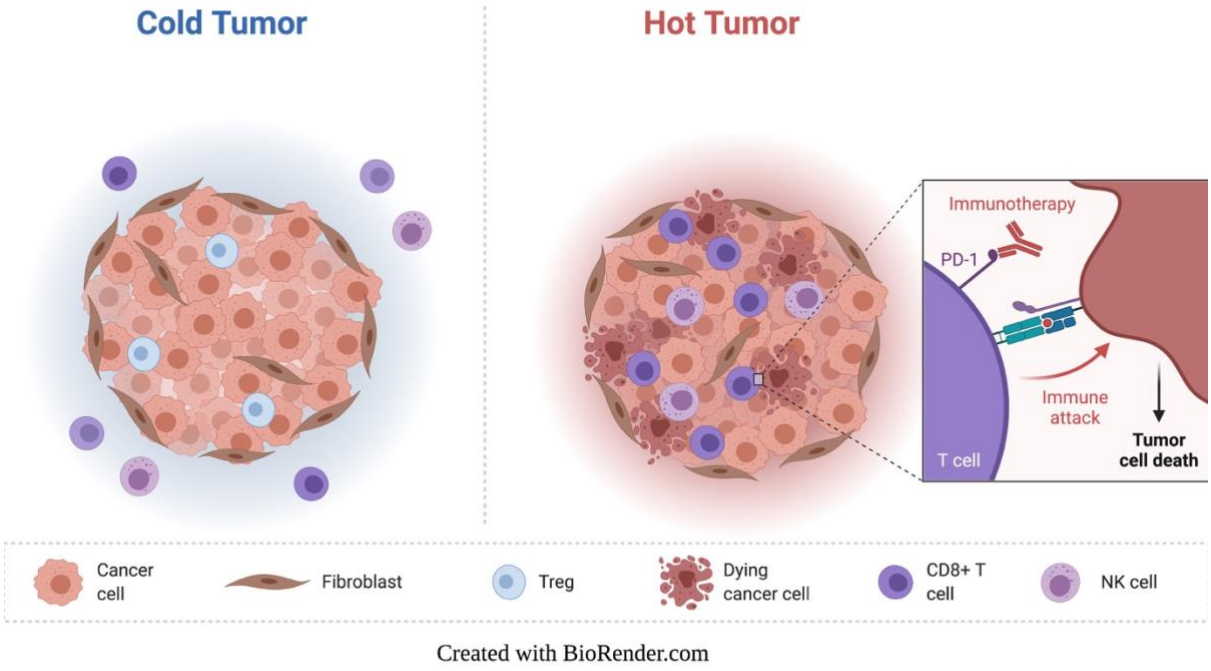

Figure 2 : Immune cell composition in “hot” and “cold” tumors

The clinical trials described in **chapter 3** that examined the levels of IDO in sarcoma patients suggested that the IDO pathway is possibly an important mechanism for primary resistance to
PD-1 inhibition[25] and that IDO suppression may potentially correlate with antitumor efficacy[26]. To our knowledge, we are the first to examine the expression of IDO in bone sarcomas. Therefore, the role of IDO expression in bone tumor cells remains to be elucidated. Moreover, in our study we found a high expression of IDO on the immune cells within the tumoral stroma, in cases of leiomyosarcoma. This is very important, especially in cases of metastatic disease, because the currently available therapeutic options for this tumor type are limited. Among the other ICs, PD-L1 was preferentially expressed in myxofibrosarcomas. Myxofibrosarcomas are often presented in clinical trials as responders to anti-PD-1 therapy with nivolumab or pembrolizumab[17,27], as discussed in **chapter 3**. PD-L1 expression was also described in some cases of myxofibrosarcomas in these clinical trials. Recent publications have highlighted the existence of alternative ICs, such as pro-apoptotic TIM-3 or anti-proliferative LAG-3, that contribute to T-cell exhaustion in myxofibrosarcomas[28]. Anti-TIM-3 antibodies are nowadays investigated in clinical trials for sarcomas (results not yet available) but have shown some promising results in murine models of sarcoma in preclinical studies[29]. Cluster of Differentiation 47 (CD47) is a cell membrane protein the can be overexpressed by tumor cells and by binding to signal-regulatory protein alpha (SIRPa) it inhibits macrophages' phagocytosis ("do not eat me" signal)[30]. Blocking the CD47/SIRPa has shown encouraging responses in advanced solid tumors[31]. Very recently, it is demonstrated high expression of SIRPa in tumor associated macrophages (TAMs) is correlated with worse prognosis by myxofibrosarcomas, among other tumor types[30]. Thus, one can conclude that immune evasion (through PD-L1, TIM3, LAG3 or CD47/SIRPa pathways) is at play in myxofibrosarcomas making these sarcomas good candidates for ICB.

Another player in the field of immunotherapy is microsatellite instability (MSI), indicative of a deficient DNA mismatch repair (MMR) system. MSI-high (MSI-H) tumors generate numerous neoantigens increasing the possibility of tumor recognition by immune cells. By blocking the PD-1/PD-L1 interaction between tumor cells and the immune cells, the immune cells remain active against the tumor cells. It is proven that

MSI-H tumors respond well to ICB, therefore FDA gave approval for the use of pembrolizumab in tumors with deficient mismatch repair (MMR) system, regardless of the tissue of origin[32]. MSI profile in sarcomas has been a subject of research for many years. The rates of MSI-positive sarcomas range from 0% up to 100% in different studies, but these findings may relate more to generalized genomic instability than a deficient MMR system[33]. In fact MMR deficiency is a rare event occurring in not more than 1% of soft tissue and bone sarcomas[34]. The majority of sarcomas with MSI-H presented to date, show myogenic differentiation or are unclassified sarcomas. In **chapter 5** we described MSI-H in one out of 68 investigated cases. The case was a Kaposi sarcoma, to our knowledge being the first MSI Kaposi sarcoma reported in the literature. Although MMR deficiency is an uncommon event in sarcomas, it can rarely occurs and therefore screening focusing on MSI can give patients the opportunity for immunotherapy.

b. Agnostic-type treatment approach: treating sarcomas by gene fusion profile and not by location

Several actionable oncogenic somatic gene alterations are present in diverse tumor types, supporting the concept of agnostic-type treatment approach. Consequently, the same drug can be used for different malignancies bearing the same genetic alteration, independent the location or the tissue of origin. We already described that MSI-H and MMR deficient tumors can be treated with pembrolizumab. Concerning sarcomas, fusions of the Anaplastic Lymphoma Kinase (ALK) are known to occur in almost 50% of the inflammatory myofibroblastic tumors (IMTs)[35]. Beside IMTs, also low-grade sarcomas, leiomyosarcomas and *ALK*-positive non-Langerhans histiocytosis have been reported to harbor *ALK* fusions and have shown response to multiple ALK inhibitors[36-38] regardless site of origin.

In **chapter 6** we describe our extensive review on fusions of the Neurotrophic Tyrosine Receptor Kinase (*NTRK*) gene in sarcomas. Fusions of one of the *NTRK* genes (1, 2 and 3) are targetable events. Treating tumors with first or second generation TRK inhibitors showed excellent response rates and durable effects, while side effects were managed. The main purpose of this review was to describe the morphological features of the *NTRK* fused sarcomas, in an effort to

standardize histologic criteria to identify these tumors. The majority of the cases presented in the literature displayed an *NTRK1* or an *NTRK3* fusion. We noticed that the predominant histological pattern displayed spindle cells and showed frequently hyalinized blood vessel walls as well as myxoid degeneration. There seemed to be a difference between grade and *NTRK* gene, specifically, low grade morphology can be a feature of *NTRK1*- but not of *NTRK3*-fused sarcomas, where the latter are always intermediate or high-grade tumors. Thereafter, we proposed an algorithm for the investigation of *NTRK* fusions in soft tissue tumors. With this algorithm we suggest morphology as the first steps to identify which tumors need further molecular investigation in order to demonstrate the fusion.

Given the importance of these fusions as targetable events of tumor agnostic-type, we investigated *NTRK* fusions by means of immunohistochemical and molecular techniques in 70 sarcomas of different types and locations in **chapter 7**. Among those, 2 cases out of 70 were proven to harbor *NTRK* fusion. A recent survey in a 494 mesenchymal tumors showed a prevalence of around 1%[39]. This difference obviously lies in the diverse number of samples. Our results are in line with what we already described in our review in **chapter 6**. The tumors were spindle cell with characteristic blood vessels and myxoid degeneration in the stroma. The mitotic activity and the grade were different: one of the tumors was long standing, growing very slowly, while the other was very aggressive eventually giving rise to metastatic disease. Both tumors presented a fusion of the *NTRK3* gene. In contrast to what we had already described in **chapter 6**, we presented an *NTRK3* fused sarcoma with an indolent course. Moreover, the high-grade tumor had a *TFG-NTRK3*, a very rare variant that in literature is mostly described in less aggressive tumors with an intermediate grade[40,41]. Thus, with our research we have broadened the spectrum of *NTRK3* fused sarcomas by describing novel tumor characteristics.

2. Future perspectives

Our research provides new insights in the biology of soft tissue and bone tumors, mostly in regard to their therapeutic approach. Although the data are limited, we believe that immunotherapy can play a role in the treatment of sarcomas. We described the presence of lymphoid

aggregates within the TME in the form of TLOs with high expression of B cells, which according to the literature seem to play a role as biomarker for response to ICB. A limitation of our study is the lack of characterization of regulatory cells in the TME. These regulatory cells can manipulate the TME in a way that immune cells will not be able to attack the tumor, regardless of their abundance. In a future study we aim to clarify the consistency of the TLOs in relation to immune regulatory cells, such as cells expressing TIM-3, LAG-3, CD47/SIRPa and FOXP3, by multiplex analysis. Furthermore, we describe specific tumor types that are likely to benefit more from ICB. A very promising candidate is myxofibrosarcoma. Investigation of their TME in primary and metastatic locations could give us some information on what mechanisms may take part in an aggressive setting. This might help in defining biomarkers of aggressiveness.

MSI-H and MMR deficiency is an infrequent event in sarcomas. Still, we believe that in cases of refractory sarcomas or in sarcomas without other treatment options, investigation the microsatellite status can dramatically change the patient's survival expectation. In the same line, gene fusions should further be investigated in sarcomas. We focused on *NTRK* gene fusions, but we know that other gene fusions are also at play in sarcomas, such as *ALK* and *ROS1*. Inhibitors for these fusions already exist and can be used in an agnostic way to all fused tumors, regardless location or tissue of origin. A molecular panel that investigates targetable fusions could be applied for sarcomas that do not present another driver oncogenic alteration.

To conclude, we believe that sarcomas offer a wide ground for further investigation with regard to immunotherapy, which will be the subject of our future research.

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CHAPTER 9

SUMMARY – SAMENVATTING

1. Summary

Soft tissue and bone tumors show some very important characteristics: (i) they are rare tumors, (ii) they display great heterogeneity and (iii) in many cases the line of differentiation is unclear. We often regard these tumors as one entity but in fact we have to deal with more than 200 different entities. This makes clear that: (i) standardization of diagnostic criteria, (ii) definition of prognostic and predictive parameters and (iii) delineation of effective treatment remains very challenging to date.

With my research I touched upon the aforementioned issues. In **chapter 1** I made an extensive analysis of the pathologist's role in handling soft tissue and bone tumors. From the point of receiving the material to the final diagnosis, there are different steps that require special management and specialized knowledge. Morphology, accompanied by additional immunohistochemical and molecular techniques and in combination with the clinical and radiological image, is the key to accurate diagnosis. This is also the reason why sarcomas should be discussed within a multidisciplinary team, where professionals from different specialties analyze each case extensively and decide together on the treatment that will be most effective for each patient.

Personalized medicine and novel therapeutic options became of great interest the last years. One of the pioneering breakthroughs is to use the body's own immune system to fight against cancer by the use of immunotherapy. Immune checkpoint blockade or ICB is introduced in the daily routine clinical practice for the treatment of different malignancies. However, still not for sarcomas. In **chapter 3** the clinical trials that presented results about the treatment of sarcomas with ICB as mono- or combination therapy are described. In general, sarcomas do not respond very well to immunotherapy. Nevertheless, some sarcoma types - among which alveolar soft part sarcoma and undifferentiated sarcoma not otherwise specified - seemed to respond well to this type of therapy. In the second part of this chapter, I analyzed all factors that can possibly play a role as predictive biomarkers to response to ICB in sarcomas. Expression of PD-L1, composition of the tumor microenvironment (TME), molecular status and tumor mutational burden (TMB), as well as microsatellite instability (MSI) are parameters that are proven to interfere with immune response. Knowing the status

of each of these parameters could help identifying those tumors that will better respond to ICB.

The composition of the TME is of great importance, mainly in correlation to immunotherapy. Very often tumors are characterized according to their inflammation signature. “Hot” tumors are strongly inflamed, while “cold” tumors are not. “Cold” tumors may present either with an excluded phenotype where immune cells arrive at the tumor but cannot reach within the tumor, or with a desert phenotype, where immune cells are not present, not at the periphery nor in the tumor.

In **chapter 4** I investigated the TME in desmoid tumors (DT), a low-grade, locally aggressive mesenchymal tumor. For this type of tumor I described a strong inflammatory response at the periphery with formation of lymphoid aggregates that strongly suggest tertiary lymphoid organs (TLOs). Those TLO-like structures were composed of CD20 positive B cells. Furthermore, I showed that these lymphoid aggregates contained many CD8 positive cytotoxic T cells. The lymphoid infiltrate within the tumor was rather limited, suggesting that these are “cold” tumor with an excluded phenotype. Regulatory T cells with an immune phenotype positive for CD4/FOXP3 as well as PD-1 expression were also noticed in the lymphoid aggregates, leading to the conclusion that the TME in DTs shows evidence of immune suppression. There was no PD-L1 expression on the tumor cells, implying that the immune suppression is not driven through the PD-1/PD-L1 pathway but other mechanisms are possibly at play. Thus, PD-1 or PD-L1 blockade may not be the best option for treating DTs but further characterization of the mechanisms leading to immune suppression could help in finding the best immunotherapeutic strategy for this tumor type.

It is apparent that sarcomas are not (yet) the best candidates for ICB. I hypothesize that different pathways of immune evasion play a role. Therefore, in **chapter 5** I analyzed three different pathways responsible for tumor immune evasion in a range of sarcoma types. I investigated the immunohistochemical expression of PD-1, PD-L1, CD70 and IDO in all compartments of the TME as well as on the tumor cells. I found that T cells often show an exhausted phenotype, which has a multifactorial background. I also described that although soft tissue

tumors are almost always “hot” tumors, the majority of bone tumors have a “cold” phenotype. Thus, for bone sarcomas overcoming T cell priming should be the first concern. Still, I found that in our series, IDO was preferentially expressed in bone tumors, namely osteosarcomas and chondrosarcomas, in comparison to the soft tissue tumor category. Therefore, I believe that bone tumors are good candidates for IDO inhibitory therapy in combination with anti-PD-1 therapy.
Among the soft tissue tumors, myxofibrosarcoma is a “hot” tumor that moreover expressed PD-L1. I concluded that myxofibrosarcomas are among the soft tissue tumors that show promising results and thus need further investigation. Finally, I did a molecular analysis for MSI in all tumors and I found one Kaposi sarcoma with mismatch repair (MMR) deficiency. I am the first to describe MSI in this tumor type. MMR deficient tumors generate numerous neoantigens that elicit immune cells in the TME. Therefore, FDA gave approval for anti-PD-1 treatment with pembrolizumab for MSI tumors, regardless of tumor type or site of origin. MSI is a very rare event in sarcomas. Still, in the case of a refractory tumor or in metastatic disease with no other available treatment option, testing for the microsatellite status may give patients the opportunity to overtake palliative care.

Treating MSI tumors with ICB independent of tumor type or tissue of origin is an example of an agnostic type of treatment. Another type of agnostic treatment targets gene fusions. Fusion of one of the three neurotrophic tyrosine receptor kinase (*NTRK*) genes are common events in rare tumor types, for instance in infantile fibrosarcoma, but rare in more common tumor types. Our review in **chapter 6** is an extensive analysis of the pathological and molecular characteristics as well as the clinical implications of *NTRK* fused sarcomas. I describe that these sarcomas frequently have a spindle cell immunophenotype with hyalinization of the blood vessel wall and that myxoid degeneration are a constant finding. Still, identification of these sarcomas requires molecular confirmation of the fusion. I finally proposed an algorithm for the diagnosis of *NTRK* fused sarcomas.

In a series of 70 soft tissue and bone tumors, I found two tumors with fusion of the *NTRK3* gene, that I discuss in **chapter 7**. Both tumors had a spindle cell morphology, as described in chapter 6. Nevertheless, I described some features that deviate from what is already known. One

of the tumors displayed a low-grade morphology and had a very indolent clinical course. In contrast, all *NTRK3* fused sarcomas presented in the literature have at least an intermediate grade and usually a more aggressive course. The second tumor was a high-grade sarcoma that gave rise to metastatic disease. This tumor presented with a *TFG-NTRK3* fusion. Sarcomas with this fusion display mostly an intermediate grade and a less aggressive phenotype. Hence, with our research we expanded the morphological spectrum of *NTRK* fused sarcomas as well as the prognostic value of specific fusions.

To conclude, application of the findings of molecular pathology and immune-oncology in sarcomas is still in its infancy. Further research towards this direction is urgently needed.

2. Samenvatting

Wekedelen- en bottumoren hebben enkele zeer belangrijke kenmerken: (i) ze zijn zeldzaam, (ii) ze vertonen een grote heterogeniteit en (iii) in veel gevallen is de differentiatiegraad onduidelijk. We beschouwen deze tumoren vaak als één tumorsoort, maar eigenlijk moeten we rekening houden met meer dan 200 verschillende soorten tumoren. Daarom blijven (i) de standaardisering van diagnostische criteria, (ii) de definitie van prognostische en voorspellende parameters en (iii) de uitlijning van een effectieve behandeling tot op vandaag erg uitdagend.

Met dit onderzoek probeer ik al deze onderwerpen te belichten. In **hoofdstuk 1** maak ik een uitgebreide analyse van de rol van de patholoog in het beschouwen van tumoren van de wekedelen- en bottumoren. Van de ontvangst van het materiaal tot de uiteindelijke diagnose zijn er verschillende stappen, die elk speciale handelingen en gespecialiseerde kennis nodig hebben. Morfologie, samen met bijkomende immunohistochemische en moleculaire technieken, in combinatie met klinische en radiologische beeldvorming, vormt de sleutel tot een accurate diagnose. Daarom moeten sarcomen ook besproken worden binnen een multidisciplinair team, waarin mensen met verschillende specialisaties elk geval uitgebreid analyseren en samen beslissen welke behandeling voor elke patiënt het meest effectief zal zijn.

De laatste jaren zijn gepersonaliseerde geneeskunde en nieuwe therapeutische opties opgekomen. Een van die doorbraken is het aanwenden van bronnen binnen het eigen lichaam, zoals het immuunsysteem, om kanker te bestrijden. Dit mechanisme wordt immuuntherapie genoemd. Immuuncheckpointblokkade of ICB werd geïntroduceerd in de dagdagelijkse klinische praktijk voor de behandeling van verschillende maligniteiten, waaronder melanomen, non-Hodgkin lymfomen, longkankers en nierkankers, maar nog niet voor sarcomen. Daarom bevat **hoofdstuk 3** een uitgebreid overzicht van de klinische studies die resultaten beschrijven van de behandeling van sarcomen met ICB als mono- of combinatietherapie. Over het algemeen hebben sarcomen niet zo een goede respons op immuuntherapie. Toch lijken enkele sarcoomtypes – waaronder alvaiolair soft part sarcomen en ongedifferentieerde sarcomen – positieve gevolgen te ondervinden van

dit soort therapie. In het tweede gedeelte van dit hoofdstuk analyseer ik alle factoren die mogelijk een rol spelen als biomarkers om een respons op ICB in sarcomen te kunnen voorspellen. Expressie van PD-L1, samenstelling van de tumormicro-omgeving (TME), moleculaire status en tumormutatielast (TMB), alsook de microsatellietinstabiliteit, zijn bewezen parameters die immuunrespons verzwakken. Kennis over de status van deze parameters kan dus bijdragen bij de identificatie van de tumoren die een betere respons tonen op ICB.

De samenstelling van de TME is erg belangrijk, zeker in verband met immuuntherapie. Vaak hebben tumoren een typische ontstekingsrespons. "Warme" tumoren zijn erg ontstoken, waar "koude" tumoren dat niet zijn. "Koude" tumoren komen voor met een *excluded phenotype*, waarbij immuuncellen bij de tumor geraken maar niet in de tumorcellen zelf, of een *desert phenotype*, waar immuuncellen gewoon niet aanwezig zijn, noch perifeer, noch in de tumor. In **hoofdstuk 4** onderzoek ik de TME in desmoïde tumoren (DT): laaggradige, lokaal agressieve mesenchymale tumoren. Voor dit type tumoren heb ik een sterke perifere ontstekingsrespons beschreven, met de vorming van lymfoïde aggregaten die erg suggestief zijn voor tertiaire lymfoïde organen (TLO's). Die TLO-achtige structuren bestaan uit CD20-positieve B-cellen. Daarenboven toon ik dat die lymfoïde aggregaten veel CD8-positieve cytotoxische T-cellen bevatten. Het lymfoïd infiltraat in de tumor is eerder beperkt, wat dus suggereert dat het "koude" tumoren zijn met een *excluded phenotype*. T-cellen met een CD4/FOXP3-positief immuunfenotype en PD-1-expressie werden ook gezien in de lymfoïde aggregaten, waaruit we concluderen dat de TME in DT's immunosuppressie veroorzaken. Er was geen PD-L1 expressie, wat impliceert dat de PD-1/PD-L1 pathway niet de drijvende kracht achter de immunosupressie is, maar dat er andere mechanismes aan het werk zijn. Blokkade van PD-1 of PD-L1 is dus waarschijnlijk niet de beste optie voor de behandeling van DT's, maar verdere bepaling van het mechanisme dat leidt tot immunosuppressie kan helpen in het vinden van de juiste immunotherapeutische optie voor dit tumortype.

Het is duidelijk dat sarcomen (nog) niet de beste kandidaten zijn voor ICB. Ik kwam tot de hypothese dat verschillende pathways van immuunresponsontwijking een rol spelen. Daarom analyseerde ik in **hoofdstuk 5** drie verschillende pathways die verantwoordelijk zijn voor

tumor immuunresponsontwijking in verschillende sarcoomtypes. Ik onderzocht de immunohistochemische expressie van PD-1, PD-L1, CD70 en IDO in alle delen van de TME en in de tumorcellen. Ik zag dat T-cellen vaak een uitgeput fenotype vertoonden, wat een multifactoriële achtergrond heeft. Ik beschreef ook dat, hoewel wekedelentumoren bijna altijd "warme" tumoren zijn, de meeste bottumoren het "koud" fenotype hebben. Voor botsarcomen moet het aanpakken van T cell priming de eerste zorg zijn. Toch merkte ik dat IDO vaker tot expressie komt in bottumoren, nl. osteosarcomen en chondrosarcomen, in vergelijking met wekedelentumoren. Daarom geloof ik dat bottumoren goede kandidaten zijn voor IDO inhibitietherapie in combinatie met anti-PD-1 therapie.

Myxofibrosarcomen zijn wekedelentumoren die tot de "warme" tumoren behoren en die bovendien PD-L1 expressie vertonen. Daarom zijn myxofibrosarcomen veelbelovende wekedelentumoren en is verder onderzoek noodzakelijk. Uiteindelijk deed ik een moleculaire analyse voor MSI in alle tumoren en vond ik één Kaposi sarcoom met een *mismatch repair* (MMR) tekort. Ik was de eerste die MSI beschreef in dit tumortype. Tumoren met MMR deficiëntie genereren grote hoeveelheden neoantigenen die immuuncellen aantrekken in de TME. Daarom heeft de FDA toestemming gegeven voor anti-PD-1 behandeling met pembrolizumab bij MSI tumoren, onafhankelijk van het tumortype of de plaats van oorsprong. MSI is erg zeldzaam bij sarcomen. Toch kan het testen op microsatellietinstabiliteit in geval van refractaire tumoren of in metastatische ziektes zonder andere behandelopties, een opportuniteit zijn voor patiënten in plaats van palliatieve zorg.

Het behandelen van MSI tumoren met ICB, onafhankelijk van het tumortype en de plaats van oorsprong is een voorbeeld van een agnostisch behandeltype. Een ander agnostisch behandeltype richt zich op genfusies. Fusies van een van de drie neurotrofische tyrosine receptor kinase (NTRK) genen komen geregeld voor bij zeldzame tumortypes zoals infantiel fibrosarcoom, maar zijn zeldzamer bij vaker voorkomende tumortypes. Ons overzicht in **hoofdstuk 6** is een uitgebreide analyse van de pathologische en moleculaire kenmerken, alsook de klinische implicaties van sarcomen met NTRK fusies. Deze sarcomen vertonen vaak een spinocellulair immuunfenotype met

hyalinisatie van de bloedbaanwand en myxoïde degeneratie. Toch is voor de identificatie van deze sarcomen moleculaire bevestiging van de fusie noodzakelijk. Uiteindelijk introduceerde ik een algoritme voor de diagnose van NTRK gefuseerde sarcomen.

Onder 70 wekedelen- en bottumoren vond ik twee tumoren met een fusie in het *NTRK3* gen, die ik bespreek in **hoofdstuk 7**. Beide tumoren hadden een spinocellulaire morfologie, zoals beschreven in **hoofdstuk 6**. Toch zijn er ook enkele kenmerken die afwijken van wat al bekend is. Eén van de tumoren vertoonde een laaggradige morfologie en had bovendien een traag klinisch verloop. Nochtans hebben alle *NTRK3* gefuseerde sarcomen in de literatuur minstens een gemiddelde graad en hebben ze meestal een agressiever verloop. De tweede tumor was een hooggradig sarcoom dat aanleiding gaf tot metastatische ziekte. Deze tumor vertoonde een *TFG-NTRK3* fusie. Sarcomen met deze fusie vertonen meestal een gemiddelde graad en hebben een minder agressief fenotype. Met ons onderzoek hebben we dus het morfologisch spectrum van de *NTRK* gefuseerde sarcomen uitgebreid, alsook de prognostische waarde van bepaalde fusies.

We kunnen concluderen dat de toepassing van de bevindingen van moleculaire pathologie en immuno-oncologie in sarcomen nog in haar kinderschoenen staat. Verder onderzoek in deze richting is dringend nodig.

CHAPTER 10

CURRICULUM VITAE

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EDUCATION

1998-2004	Medical School, University of Ioannina, Ioannina – Greece Graduation degree: 7.51/10
1994-1997	Greek High School of Wuppertal, Germany (1994-1995) Zosimaia High School of Ioannina, Greece (1995-1997)

EMPLOYMENT HISTORY

02/2013 - present	Pathologist (with special interest in Dermatopathology and Soft Tissue Pathology), University Hospital of Antwerp (UZA), Belgium
09/2012 - 02/2013	Resident in the Department of Pathology, University Hospital of Antwerp (UZA), Belgium
08/2007- 08/2012	Resident in the Department of Pathology/Cytology, University Hospital of Ioannina, Greece
03/2007- 08/2002	Full time employee in a Pharmacy, Ioannina, Greece
03/2005- 02/2007	General Practitioner and Primary Care Provider at General Hospital "Hatzikosta" of Ioannina, Greece, and at Medical "Health Center" of Delvinaki, Ioannina Greece

CONFERENCES – CONGRESSES – COURSES AND SYMPOSIA ATTENDED

09/2021	Virtual PD-L1 Testing (22C3) – Professional Expert Course in Eosophageal Carcinoma CPS Training – taking place online using GoToMeeting, Thursday 09th and Friday 10th of September 2021
06/2021	Virtual PD-L1 Testing (22C3) – Professional Expert Course Triple-Negative Breast Carcinomas (TNBC) CPS Training – taking place online using GoToMeeting, Tuesday 01th and Wednesday 02th of June 2021

05/2021	11th Dermatopathology Seminar of Munster, Cutaneous Lymphomas by Lorenzo Cerroni, Graz, Virtual Meeting, May 29, 2021
12/2020	Advanced Course PD-L1 in NSCLC, 11 December 2021, Nederlandse Vereniging van Artsen voor Longziekte en Tuberculose (NVALT): 5 pubnten Nderlandse Vereniging voor Pathologie (NVVP): 5 punten
12/2020	32th European Congress of Pathology, 06-08 December, Brussels (live streaming), Belgium (18 European CME credit)
09/2020	Virtual PD-L1 Testing (22C3) – Professional Expert Course Cross Histology CPS Training (CHCT) – taking place online using GoToMeeting, Tuesday 15th and Wednesday 16th of September 2020
09/2020	ESMO virtual congress 2020, 19-21/09/2020
01/2020	The Pathology of Melanoma: An International Course, Institute Curie, 16-17/01/2020, Paris, France
10/2019	The Belgian Week of Pathology 2019, organized by the Belgian Society of Pathology, 18-19/10/2019, Brussels, Belgium
09/2019	31th European Congress of Pathology, 07-11 September 2019, Nice, France, (20 Europen CME credit)
04/2019	2 day PD-L1 Testing (22C3) – Train the Trainer Expert Course in Head and Neck Carcinoma, Kassel, Germany
10/2018	1 day PD-L1 Testing (22C3) – Professional Expert Course in Urothelial Carcinoma, Maastricht, Netherlands
09/2018	Ventana PD-L1 (SP142) Assay Workshop for Pathologists for Urothelial Cancer in Brussels on September 21st, 2018
09/2018	30th European Congress of Pathology, 08-12 September 2018, Bilbao, Spain, (20 Europen CME credit)

02/2018	Workshop of Ethics & Economics, themed: Financing of Pathology: Present and Future, ForPath, Brussels, Belgium (Totaal Aantal Verworven CP 2,5)
02/2018	ESMO Sarcoma and GIST symposium 2018, Milan Italy (20 European CME credits and 24 ESMO-MORA category 1 credits)
11/2017	Masterclass – Damya Laoui: "Naar een lichaamseigen vaccin tegen kanker?", 15 November 2017, University of Antwerp, Campus Drie Eiken
10/2017	Diagnostic Histopathology of Soft Tissue Tumors 2017, 12th-13th October 2017, Treviso, Italy
09/2017	XXXVIII Symposium of the International Society of Dermatopathology by the Royal College of Pathologists of the UK, Glasgow, UK (27 CPD + 2,5 credits for the self-assessment cases)
09/2017	29th European Congress of Pathology, Amsterdam, Netherlands, 02/09/2017-06/09/2017, (20 European CME credit)
02/2017	06/02/2017-03/02/2017: One month observer for dermatopathology by Dr. Thomas Brenn, NHS Lothian University Hospitals Trust, Edinburgh, United Kingdom
10/2016	7th Belgian Week of Pathology, Belgian Society of Pathology, Ghent, Belgium
09/2016	2 day PD-L1 Testing (22C3), Train the trainer Expert Course in NSCLC, Tagros Molecular Pathology, Kassel, Germany
06/2016	15th Congress of Hellenic Society of Pathology, Ioannina, Greece
01/2016	Congress, "A Tour around bone and soft tissue tumors", Department of Pathology, Hospital del Mar de Barcelona, Barcelona, Spain (16 ECMEC)

11/2015	EscoP Nephropathology course: A Practical Workshop, Pathology Department, Ankara University Hospital, Ankara, Turkey
10/2015	6th Belgian Week of Pathology, Belgian Society of Pathology, Ghent, Belgium
07/2015	Basel Seminars in Pathology, Renal Transplant Pathology, Hands-on Course, Basel, Switzerland (22 Credits, Swiss Society of Pathology SGPath)
05/2015	Edinburgh Haematopathology Tutorial 2015, "Haematological Malignancy and the Skin, Edinburgh, Scotland (15 Credits, CPD)
05/2015	London Dermatopathology Symposium, Royal College of Pathologists, London, Great Britain (24 Credits, CPD)
11/2014	Postgraduate Course "Diagnostic Breast Pathology", EACCME, Linz, Austria (18 CME credits)
10/2014	5th Belgian Week of Pathology, Belgian Society of Pathology, Ghent, Belgium
10/2014	Workshop of Slide Seminar, Upgrade in Soft Tissue Tumors Prof. Dr. Christopher Fletcher, ForPath, Brussels, Belgium (Totaal Aantal Verworven CP 3)
09/2014	The Harvard Medical School, Dermatopathology Update, Boston Massachusetts (19.5 AMA PRA Category 1 Credits)
09/2014	Praktische Nefropathologie, De Boerhaave Commissie, LUMC, Leiden, The Netherlands (NVK 12 punten, Nederlandse Vereniging voor Pathologie 12 punten, NIV 12 punten, Nederlandse Vereniging voor Immuunologie 6 punten)
06/2014	International Course on Native and Transplant Renal Biopsy Interpretation, ESP Working Group on Nephropathology and Renal Pathology Society and Institute of Pathology Faculty of Medicine University of Ljubliana, Ljubliana, Slovenia

05/2014 Breast Pathology with Radiological Correlation, ESP tutorial, Brussels, Belgium

05/2014 London Dermatopathology Symposium, Royal College of Pathologists, London, Great Britain (24 Credits, CPD)

03/2014 "First International Renal Conference Brugge", The Division of Nephrology and Infectious diseases AZ Sint-Jan Brugge-Ostende, Brugge, Belgium (11 Credits, CPD)

09/2013 The Harvard Medical School, Dermatopathology Update, Boston Massachusetts (23.25 AMA PRA Category 1 Credits)

03/2013 London Diagnostic Dermatopathology Course, London, Britain (Credit Points 30)

06/2012 13th Hellenic Congress of the Hellenic Society of Pathology, Kalamata, Greece (18 credits, CME-CPD)

04/2012 Ioannina University Courses in Pathology (IUCP), on ''Liver Pathology-Oncology'', Ioannina, Greece (23 ½ credit hours excluding breaks, and social events)

04/2012 3d Belgian Week of Pathology, Belgian Society of Pathology, Ghent, Belgium

03/2012 4th ESMO Conference on Sarcomas & Gist, Milan, Italy (12 Credits, ECMEC's)

02/2012 Scientific Conference with subject: ''Exploring the endometrium: from curettage to radical hysterectomy'', Gynaecological Pathology Working Group of Hellenic Society of Pathology, Athens, Greece (9 Credits, CME-CPD)

02/2012 6th Educational Seminar with subject: ''Molecular Oncology and Targeted Therapy for the Clinical Oncologist'', Metsovo, Ioannina, Greece

01/2012	Postgraduate course with subject: ‘‘Head and Neck Tumors: Differential diagnosis according histological pattern and immunophenotype’’, A΄ Pathology Department, University of Athens, Greece
Academic year 2011-2012	Monthly Postgraduate courses (3nd Series) with subject: ‘‘Principals of molecular and cellular biology’’, Molecular Pathology Working Group of Hellenic Society of Pathology, Athens, Greece (20 Credits, CME-CPD)
12/2011	Scientific Conference with subject: ‘‘The role of Bone Marrow Biopsy in the diagnostic approach and surveillance of hematopoietic diseases’’, Hematopathology Working Group of Hellenic Society of Pathology, Athens, Greece (6 Credits, CME-CPD)
10/2011	GIST GOLS, Changing the Roadmap for GIST Survival, Athens, Greece (7,5 CPD Credits and 1CPD Credit for ‘‘Meet The Expert Session: Pathology in GIST’’)
08-09/2011	23rd European Congress of Pathology, Helsinki, Finland (27 European CME credits, ECMEC’s)
From 13-06 until 15-08/2011	Soft Tissue and Surgical Pathology Fellowship in the Pathology Department, University Hospital Antwerp, Belgium
06/2011	Workshop Soft Tissue Pathology, Maastricht, The Netherlands (12 Credits EACCME)
05/2011	Slide Seminar with subject: ‘‘Differential diagnosis: primary from metastatic lung tumor’’, Pulmonary Working Group of Hellenic Society of Pathology, Thessaloniki, Greece
04/2011	Scientific Conference with subject: ‘‘Differential diagnosis of Tumors of the Urinary System and Male Genital Organs, depending on histologic pattern and immunophenotype’’, Nephro-

	Uropathology Working Group of Hellenic Society of Pathology, Athens, Greece (15 Credit, CME-CPD)
02/2011	5th Educational Seminar with subject: ‘‘Molecular Oncology and Targeted Therapy for the Clinical Oncologist’’, Metsovo, Ioannina, Greece
01/2011	4th Melanoma Congress, Athens, Greece (15 Credit, CME-CPD, EACCME-UEMS)
Academic year 2010-2011	Monthly Postgraduate courses (2nd Series) with subject: ‘‘Principals of molecular and cellular biology’’, Molecular Pathology Working Group of Hellenic Society of Pathology, Athens, Greece (20 Credits, CME-CPD)
11/2010	8th Tumor Markers-Targeted Therapy Congress, Athens, Greece (17 Credits, CME-CPD, EACCME-UEMS)
09/2010	Ioannina University Courses in Pathology (IUCP), on ‘‘Kidney Pathology-Oncology’’, Ioannina, Greece (11 ½ credit hours excluding breaks, and social events)
09/2010	Ioannina University Courses in Pathology (IUCP), on ‘‘Kidneys and Adrenals Pathology-Oncology’’, Ioannina, Greece (11 ½ credit hours excluding breaks, and social events)
08-09/2010	Intercongress Meeting of the European Society of Pathology, Krakow, Poland (18 European CME credits, ECMEC’s)
06/2010	12th Hellenic Congress of the Hellenic Society of Pathology, Thessaloniki, Greece (19 credits, CME-CPD)
03/2010	Slide Seminar with subject:‘‘Unusual lung neoplasms’’, Pulmonary Working Group of Hellenic Society of Pathology, Thessaloniki, Greece

02/2010	4th Educational Seminar with subject: ‘‘Molecular Oncology and Targeted Therapy for the Clinical Oncologist’’, Metsovo, Ioannina, Greece
Academic year 2009-2010	Monthly Postgraduate courses (1st Series) with subject: ‘‘Principals of molecular and cellular biology’’, Molecular Pathology Working Group of Hellenic Society of Pathology, Athens, Greece (18 Credits, CME-CPD)
01-04/06/2009	Training in practice and use of P.A.L.M. Laser-MicroBeam System, “Biomedical Research Foundation”, Academy of Athens, Greece
05/2009	2nd Scientific Conference of Hellenic Society of Pathology, Pieria, Greece (9 credits, CME)
11/2008	2nd Educational Seminar on Colposcopy, University Hospital of Ioannina, Greece (5 credits on Obstetrics and Gynecology)
03/2008	2nd Educational Seminar with subject: ‘‘Molecular Oncology and Targeted Therapy for the Clinical Oncologist’’, Metsovo, Ioannina Greece
11/2006	1st International meeting on the treatment of human brucellosis, Ioannina, Greece (22 credits, CME)
09/2006	4th Continuing Education Seminar on Pathology, Ioannina, Greece (12 credits, CME)
05/2006	2nd Inter-Congress of the European Society of Pathology, Ioannina, Greece
03/2006	Postgraduate Seminar for Diabetes type 2 with subject: ‘‘Prevention of Cardiovascular Disease in Patients with Diabetes type 2’’, Ioannina, Greece (6 credits, CME)
11/2005	Scientific Conference of the Greek Society for the Study of Bone Metabolism with subject: ‘‘Clinical approach on metabolic Bone Diseases’’, Ioannina, Greece (6 credits, CME)
09/2005	3nd Seminar with Subject: ‘‘Internal Medicine’’, Ioannina, Greece
04/2005	3nd Scientific Conference of the General Hospital “Hatzikosta” of Ioannina, Ioannina, Greece

03/2003	9th Scientific Seminar, that was organized by the Greek Medical Students, Athens, Greece
12/2002	Seminar with subject: ‘‘New Aspects on Cardiovascular Risk’’, Ioannina, Greece

PUBLICATIONS

Book chapter

Vasiliki Siozopoulou, Patrick Pauwels
Chapter: Pathology, Genetics, and Molecular Biology of Soft Tissue Tumors
Springer, 2017, Imaging of Soft Tissue Tumors
Editors: Filip M. Vanhoenacker, Paul M. Parizel, Jan L. Gielen
ISBN: 978-3-319-46677-4 (Print) 978-3-319-46679-8 (Online)

Publications in peer-review journals

Aerts O, Dendooven E, Siozopoulou V.
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Van Acker SI, Van den Bogerd B, Haagdorens M, Siozopoulou V, Ní Dhubhghaill S, Pintelon I, Koppen C.
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Siozopoulou V, Vanhoenacker FM.
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Mauritz A, Vandendriessche A, Thiessen F, Tondu T, Wetzels K, Mangodt E, Siozopoulou V
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De Winne K, Sorber L, Lambin S, Siozopoulou V, Beniuga G, Dedeurwaerdere F, D'Haene N, Habran L, Libbrecht L, Van Huysse J, Weynand B, Wouters K, Pauwels P, Zwaenepoel K.
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Van Den Broucke S, Potters I, Van Esbroeck M, Cnops L, Siozopoulou V, Hammoud C, Huyse T, Bottieau E.
A Woman With Chronic Lower Abdominal Pain, Vaginal Discharge, and Infertility After a Stay in Mali.
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Pyl J, Aerts O, Siozopoulou V, Lambert J, Dendooven E, Poschet K, Dandelooy J.
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Siozopoulou V, Marcq E, Jacobs J, Zwaenepoel K, Hermans C, Brauns J, Pauwels S, Huysentruyt C, Lammens M, Somville J, Smits E, Pauwels P.
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Posadzy M, Vanhoenacker F, Siozopoulou V.
Juxta-Cortical Chondroma of the Phalanges: Is there a Role for Cone-Beam Computed Tomography in Diagnosis and Local Staging?: Main teaching point: Low-dose cone-beam computed tomography (CT) may be of additional value to radiographs and magnetic resonance imaging (MRI) in preoperative characterization and local staging of juxta-cortical chondroma.
J Belg Soc Radiol. 2019 Apr 4;103(1):22. Doi: 10.5334/jbsr.1657

Jacobs J, Deschoolmeester V, Zwaenepoel K, Flieswasser T, Deben C, Van den Bossche J, Hermans C, Rolfo C, Peeters M, De Wever O, Lardon F, Siozopoulou V, Smits E, Pauwels P.
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Oncoimmunology. 2018 Mar 19;7(7):e1440167. Doi: 10.1080/2162402X.2018.1440167. Unveiling a

De Hous N, Paelinck B, Van den Brande J, Siozopoulou V, Laga S.
Primary cardiac synovial sarcoma of the interatrial septum.
J Card Surg. 2018 Jul;33(7):391-392. Doi: 10.1111/jocs.13733.

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Pediatr Dermatol. 2018 May;35(3):e173-e177. Doi: 10.1111/pde.13445.

Zwaenepoel K, Jacobs J, De Meulenaere A, Silence K, Smits E, Siozopoulou V, Hauben E, Rolfo C, Rottey S, Pauwels P.
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Van den Kerckhove E, Roosens L, Siozopoulou V, Verbrugghe W, Aerts J, Huyskens J, Raemen H, Jorens PG.
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Michiel Eyselbergs, Inge Verslegers, Mireille Van Goethem, Xuan Bich Trinh, Vasiliki Siozopoulou and Paul Parizel
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Journal of the Belgian Society of Radiology, 101(1): 2, pp. 1–2, DOI: https://doi.org/10.5334/jbr-btr.1017

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Christophe Van Berckelaer, Manon Huizing, Mireille Van Goethem, Andrew Vervaecke, Konstantinos Papadimitriou, Inge Verslegers, Bich X. Trinh, Peter Van

Dam, Sevilay Altintas, Tim Van den Wyngaert, Ivan Huyghe, Vasiliki Siozopoulou, Wiebren A.A. Tjalma
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O. Aerts, V. Siozopoulou
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Georgieva LA, Gielis EM, Hellemans R, Van Craenenbroeck AH, Couttenye MM, Abramowicz D, Van Beeumen G, Siozopoulou V, Van Rosmalen M, Bracke B, Hartman V, De Greef K, Roeyen G, Chapelle T, Ysebaert D, Bosmans JL.
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Laura Wuyts, Julie Dandelooy, Vasiliki Siozopoulou, Julien Lambert, Olivier Aerts
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Dandelooy J, van Hal PT, Even P, Lechkar B, Siozopoulou V, Lambert J, Aerts O.
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J Eur Acad Dermatol Venereol. 2016 Jul;30(7):1223-4

Katsanos KH, Siozopoulou V, Sigounas D, Tsianos VE, Christodoulou D, Mitsi V, Tsianos EV
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Eur J Gastroenterol Hepatol. 2011 Oct;23(10):957-60

Siozopoulou V, Batistatou A, Kamina S, Malamou-Mitsi V
Alveolar soft part sarcoma. Case report and review of the literature
Hellenic Archives Pathol 2011, 25(1-3):44-48

G. Pappas, P. Papadimitriou, V. Siozopoulou, L. Christou, N. Akritidis
The globalization of leptospirosis: worldwide incidence trends
International Journal of Infectious Diseases, 2008 Jul;12(4):351-7

G. Pappas, V. Siozopoulou, N. Akritidis, M.E. Falagas
Journal of Infection, 2007 May;54(5):459-62
Doxycyclin-rifampicin: Physicians' inferior choice in brucellosis or how convenience reigns over science

G. Pappas, V. Siozopoulou, K. Saplaoura, A. Vasiliou, L. Christou, N. Akritidis, E. Tsianos
Health literacy in the field of infectious diseases: The paradigm of brucellosis
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Short Publications

A Domen, C Deben, C Hermans, H Lambrechts, V Siozopoulou, P Pauwels, M Van De Wiel, A Janssens, JMH Hendriks, PE van Schil, T Vandamme, H Prenen, M Peeters, F Lardon, A Wouters
Senescence signature affects overall survival in non-small cell lung cancer
Annals of Oncology, September 2021, Volume 32, pp S943, https://doi.org/10.1016/j.annonc.2021.08.1782

Maxime De Fré, Katrien Smets, Michal Ulicki, Veronique Verhoeven, Vasiliki Siozopoulou, Tine Strobbe, Specenier Pol, Olivier Aerts, Julien Lambert, Thierry Tondu, Filip E. F. Thiessen
Eccrine porocarcinoma of the scalp: diagnosis and importance of early surgical intervention
European Journal of Plastic Surgery, December 2019, Volume 42, Issue 6, pp 623–627

S. Sirimsi, J. Lambert, S. Declercq, V. Siozopoulou
Comparison of the pT1 primary cutaneous melanomas between the AJCC TNM 7th and 8th edition; a retrospective study in a single institute
Virchows Archiv (2019) (Volume 475, Supplement 1, S:111)

V Siozopoulou, K De Winne, E Smits, J Jacobs, K. Zwaenepol, P. Pauwels
A PD-L1 IHC 28-8 PharmDX ring trial on metastatic melanoma: practical aspects
Virchows Archiv (2019) (Volume 475, Supplement 1, S:28)

Sterkens A, Siozopoulou V, Mangodt V, Lambert J, Bervoets A. Generalized hyperpigmentation after pyrimethamine use.
Generalized hyperpigmentation after pyrimethamine generalized hyperpigmentation after pyrimethamine use
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V. Siozopoulou, K. Zwaenepoel, M. Baldewijns, K. De Winne, P. Pauwels. Participating in Quality Assurance schemes has a major impact in PD-L1 scoring trend in Belgium.
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J. Jacobs, V. Deschoolmeester, K. Zwaenepoel, C. Hermans, C. Rolfo, M. Peeters, F. Lardon, V. Siozopoulou, E. Smits, P. Pauwels. Blocking CD70+ cancer associated fibroblasts: Prognostic marker and therapeutic target.
Acta Gastro-enterologica Belgica, Volume 80, Number 1, R11, December 2017, Quarterly

J. Jacobs, V. Deschoolmeester, K. Zwaenepoel, C. Hermans, C. Rolfo, M. Peeters, F. Lardon, V. Siozopoulou, E. Smits and P. Pauwels. Blocking CD70+ cancer associated fibroblasts: Are we paving the way towards immunotherapy in colorectal cancer.
Tumor Biology, Abstract 958, July 2017

Gielis, E. M., Siozopoulou V., Van Rosmalen, M., Moorkens, G., Abramowicz, D., Bosmans, J. L. (2015). Donor-Transmitted Cancer: A Rare Event With A Tremendous Impact.
Acta Clinica Belgica, 70(S3), 27.

Vaerenberg, C., Even, P., Hajian, B., Aerts, O., Horst, N., Siozopoulou, V., De Backer, W. (2015). A Young Woman With Asthma And Eosinophilia: A Case Report Of Eosinophilic Granulomatosis With Polyangiitis (EGPA).

Acta Clinica Belgica, 70(S3), 11.

Jan F. Gielis, Vasiliki Siozopoulou, Patrick Lauwers, Jeroen Hendriks, Paul Van Schil
When Bone Starts Growing in the Lung: A Case Series of Pulmonary Ossifications
Journal of Thoracic Oncology Volume 10, Number 9, Supplement 2, September 2015

Seghers, C., Vlieghe, E., Siozopoulou, V., Bosmans, J. L. (2014, December).
Leptospirosis: An Atypical Presentation.
Acta Clinica Belgica (Vol. 69, Pp. S15-S16)

Jan F. Gielis, Vasiliki Siozopoulou, Patrick Lauwers, Jeroen Hendriks, Paul Van Schil
Pulmonary Ossifications: To Be Considered In The Differential Diagnosis Of Solitary Pulmonary Nodules
Journal of Thoracic Oncology Volume 8, Supplement 2, November 2013

Siozopoulou, V., Batistatou, A., Kamina, S., Simou, N., Zioga, A., Malamou-Mitsi, V. (2011, August). Immunohistochemical Expression Of Estrogan And Progesterone Receptors In Cartilagenous Tumors. Virchows Archiv (Vol. 459, Pp. S15-S15)

G Hardavella, V Siozopoulou, A Batistatou, P Galanis, Y Dalavanga, M Alchanatis, S. Constantopoulos, D. Stefanou
Lymphangiogenesis in small cell lung carcinomas (SCLC); new insights in prognosis
Lung Cancer Volume 71, Supplement 2, Page S27, February 2011

G Hardavella, E Tzortzaki, V Siozopoulou, P Galanis, E Vlachaki, D Stefanou, N Siafakas
Lymphangiogenesis in Chronic Obstructive Pulmonary Disease (COPD): New Insights in Airways Remodeling
Chest Volume 138, no. 4, Supplement 752A, October 2010

INVITED SPEAKER

"Certificate in Molecular Pathology", organized by the working group of Molecular Pathology of the BSP, 27 March 2021, Leuven, Belgium, topic: "Molecular skin tumor pathology"

"Soft Tissue Pathology-Oncology" IUCP 2020, organized by the Ioannina University Courses in Pathology (IUCP), 13-15 October 2020, Ioannina, Greece, topic: "Site Specific Soft Tissue Tumors"

"Move On" organized by the MSD Spain, 31 Jenuary-02 February 2020 in Sevilla, Spain, topic: "PD-L1 as a biomarker beyond Lung"

Lunch Event: A New Generation of Firstline Treatments for Head and Neck Cancers, organized by MSD Belgium, 30 January 2020, Leuven, Belgium, topic: "PD-L1 testing in HNSCC"

"Training PD-L1 testing met CPS scoring: UC, HNSCC and SC", organized by MSD
20 September 2019 in Antwerpen
24 September 2019 in Ghent
10 October 2019 in Hasselt

13th Belgian Symposium on the Integration of Molecular Biology Advances into Oncology Clinical Practice and Post-MASCC, topic: "Immunotherapy and predictive biomarkers", Organized by the Institute Jules Bordet and BSMO, Brussel, 23/11/2019

10th Belgian Week of Pathology, topic: "New Belgian guidelines on dysplastic nevi", Organized by the Belgian Society of Pathology, Brussels, 18/10/2019

"Training PD-L1 testing met CPS scoring: UC", organized by MSD
15 January 2019 in Antwerpen
18 January 2019 in Brussel

"Educational sessions on PD-L1 testing in melanoma", organized by BMS
17 December 2018 in Grimbergen
07 January 2019 in Luik

15th Congress of Hellenic Society of Pathology, organized by the Hellenic Society of Pathology, topic: "Case presentation of cutaneous soft tissue tumors", Ioannina, Greece, 06/2016

Invited speaker by the Nephrology Armenia Flanders (NAF) organization, topic: "Histology of the transplanted kidney", Yerevan, Armenia, 10/2015

Rijksinstituut Voor Ziekte-En Invaliditeitsverzekering, LOK Meeting, topic: "Cutaneous mesenchymal tumours, case studies", Antwerpen, Belgie (Totaal Aantal Verworven CP 2)

Master Class in Kidney Transplantation, topic: "Histology of the Transplated Kidney: interactive case presentation", University Hospital of Antwerp, Belgium

Slide Seminar, topic: ''Differential diagnosis: primary from metastatic lung tumor'', organized by the Pulmonary Working Group of Hellenic Society of Pathology, Thessaloniki, Greece

Slide Seminar, topic: ''Unusual lung neoplasms'', organized by the Pulmonary Working Group of Hellenic Society of Pathology, Thessaloniki, Greece

ORAL - POSTER PRESENTATION IN SCIENTIFIC MEETINGS

A Domen, C Deben, C Hermans, H Lambrechts, V Siozopoulou, P Pauwels, M Van De Wiel, A Janssens, JMH Hendriks, PE van Schil, T Vandamme, H Prenen, M Peeters, F Lardon, A Wouters
Poster: Senescence signature affects overall survival in non-small cell lung cancer, ESMO, Paris, France, 16-21 September 2021

V. Siozopoulou, K. De Winne, E. Smits, J. Jacobs, K. Zwaenepol, P. Pauwels
Oral Presentation: A PD-L1 IHC 28-8 PharmDX ring trial on metastatic melanoma: practical aspects, ECP, Nice, France, 7-11 September 2019

S. Sirimsi, J. Lambert, S. Declercq, V. Siozopoulou
Poster: Comparison of the pT1 primary cutaneous melanomas between the AJCC TNM 7th and 8th edition; a retrospective study in a single institute, ECP, Nice, France, 7-11 September 2019

V. Siozopoulou, K. Zwaenepoel, M. Baldewijns, K. De Winne, P. Pauwels.
Poster: Participating in Quality Assurance schemes has a major impact in PD-L1 scoring trend in Belgium. ECP, Bilbao Spain, 8-12 September 2018

Niels Horst, Olivier Aerts, Julien Lambert and Vasiliki Siozopoulou
Poster: Eosinophils in psoriasis: long time no see ?

XXXVIII Symposium of the International Society of Dermatopathology by the Royal College of Pathologists of the UK, Glasgow, UK, 09/2017

Jacobs Julie, Deschoolmeester Vanessa, Zwaenepoel Karen1, Hermans Christophe, Rolfo Christian, Lardon Filip, Siozopoulou Vasiliki, Smits Evelien, Pauwels Patrick
Poster: CD70-positive colorectal cancer associated fibroblasts: prognostic factor and immune escape mechanism
LKI Symposium "Tumor Immunology & Immunotherapy: harnessing the immune system to fight cancer" Leuven, Campus Gasthuisberg, Onderwijs en Navorsing II, 09/2016

Elly Marcq, Vasiliki Siozopoulou, Jorrit De Waele, Jonas Van Audenaerde, Karen Zwaenepoel, Eva Santermans, Niel Hens, Patrick Pauwels, Jan P van Meerbeeck, Evelien LJ Smits
Poster: Characterization of the tumor microenvironment and investigation of immune checkpoint expression in malignant pleural mesothelioma
LKI Symposium "Tumor Immunology & Immunotherapy: harnessing the immune system to fight cancer" Leuven, Campus Gasthuisberg, Onderwijs en Navorsing II, 09/2016

Vasiliki Siozopoulou, Elly Marcq, Karen Zwaenepoel, Christophe Hermans, Johan Somville, Evelien Smits, Patrick Pauwels
Poster: "Desmoid tumors: characterization of immune cell infiltration for the development of new treatment options", 14th CIMT Annual Meeting, Rheingoldhalle Congress Center Mainz, Germany, 05/2016

Elly Marcq, Vasiliki Siozopoulou, Jorrit De Waele, Jonas Van Audenaerde, Karen Zwaenepoel, Christophe Hermans, Niel Hens, Patrick Pauwels, Jan P van Meerbeeck, Evelien LJ Smits
Poster: "Into the deep: closer look at immune cells and immune checkpoint expression in human malignant pleural mesothelioma", 14th CIMT Annual Meeting, Rheingoldhalle Congress Center Mainz, Germany, 05/2016

Docx MKF., Helbert M., Siozopoulou V., Vande Walle J.
Poster: "Relapsing Nephrotic Syndrome as a conseqyuence of [immune-deposit-negative] non-IgA Mesangioproliferative Glomerulonephritis" in a 4 year old African Girl"
44e Jaarlijks Congres van de Belgische Vereniging voor Kindergeneeskunde, 03/2016

J. Dandelooy, E. Vermander, J. André, V. Siozopoulou, B. Blaumeiser, M. Schramme, J. Snauwaert, J. Lambert
Oral presentation: "Psoriasis met stip(s)", Belgian Dermatology days, Brussels, Belgium, 03/2015

N. Lauwers, V. De Groot, V. Siozopoulou, R.J.W. De Keizer
Poster: "An aggressive small choroidal melanoma. Or how optic disc swelling helped to suspect extraocular invasion", European Association for Vision and Eye Research 2014, Nice, France, 10/2014

Jan F. Gielis, Vasiliki Siozopoulou, Patrick Lauwers, Jeroen Hendriks, Paul Van Schil
Poster: ''Pulmonary Ossifications: To Be Considered In The Differential Diagnosis Of Solitary Pulmonary Nodules'', 18th Congress on Cardio-Thoracic Surgery, La Hulpe, Brussels, 10/2013

V Siozopoulou, A Goussia, A Stoyianni, G Pentheroudakis, A Cervantes, M Bobos, M Panagiotidis, A Papoudou-Bai, G Fountzilas, N Pavlidis, V Malamou -Mitsi
Oral presentation: ''*Epithelial-Mesenchymal Transition in cancer of unknown primary'',* 13th Hellenic Congress of the Hellenic Society of Pathology, Kalamata, Greece , 06/2012

A Batistatou, V Siozopoulou, S Kamina, A Zioga, N Simou, V Malamou-Mitsi
Oral presentation: ''Immunohistochemical expression of Estrogen and Progesterone Receptors in Cartilaginous Tumors'', 13th Hellenic Congress of the Hellenic Society of Pathology, Kalamata, Greece, 06/2012

V Siozopoulou, A Batistatou, E Lampri, D Stefanou
Poster: ''Immunohistochemical expression of matrix metalloproteinases 1,2 and 9 in small cell lung carcinoma'', 13th Hellenic Congress of the Hellenic Society of Pathology, Kalamata, Greece, 06/2012

A Goussia, V Siozopoulou, D Krikelis, G Pentheroudakis, D Petrakis, M Bobos, A Stoyianni, K Grepi, B Golfinopoulos, A Cervantes, T Ciuleanu, G Fountzilas, N Pavlidis, V Malamou -Mitsi
Poster: ''Intracellular signalling pathways *in cancer of unknown primary'',* 13th Hellenic Congress of the Hellenic Society of Pathology, Kalamata, Greece, 06/2012

M Papageorgiou, A Batistatou, A Papoudou-Bai, V Siozopoulou, D Stefanou
Poster: ‘‘Immunohistohemical expression of Mismatch Repair Genes in epithelial colon neoplasms*’’,* 13th Hellenic Congress of the Hellenic Society of Pathology, Kalamata, Greece, 06/2012

V Siozopoulou, A Papoudou-Bai, S Kamina, A Zioga
Poster: ‘‘Inflammatory Myofibroblatic Tumor of the Small Intestine*’’,* 13th Hellenic Congress of the Hellenic Society of Pathology, Kalamata, Greece, 06/2012

A Zioga, A Batistatou, V Siozopoulou, A Papoudou-Bai, V Malamou-Mitsi
Poster: ‘‘Histohemical examination of reticulin pattern for the differential diagnosis of melanocytic lessions *’’,* 13th Hellenic Congress of the Hellenic Society of Pathology, Kalamata, Greece, 06/2012

V Siozopoulou, A Batistatou, S Kamina, N Simou, A Zioga, V Malamou-Mitsi
Oral presentation: ‘‘Immunohistochemical expression of Estrogen and Progesterone Receptors in Cartilaginous Tumors’’, 23rd European Congress of Pathology, Helsinki, Finland, 08-09/2012

V Siozopoulou, A Goussia, A Stoyianni, G Pentheroudakis, I Nesseris, P Oikonomou, A Papoudou-Bai, N Pavlidis, V Malamou-Mitsi
Poster: ‘‘Epithelial-Mesenchymal Transition in Cancer of Unknown Primary’’, 23rd European Congress of Pathology, Helsinki, Finland, 08-09/2012

V Siozopoulou, A Batistatou, G Hardavella, D Stefanou
Poster: ‘‘Lymphangiogenesis in small cell lung carcinomas: new insights in prognosis’’, 23rd European Congress of Pathology, Helsinki, Finland, 08-09/2012

A Stoyianni, A Goussia, G Pentheroudakis, V Siozopoulou, A Cervantes, M Bobos, G Fountzilas, V Malamou-Mitsi, N Pavlidis
Poster: ‘‘Epithelial-Mesenchymal Transition in Cancer of Unknown Primary’’, European CanCer Organization (ECCO) Congress, Stockholm, Sweden, 09/2011

G Pentheroudakis, D Petrakis, A Goussia, V Siozopoulou, M Bobos, G Fountzilas, A Cervantes, V Malamou-Mitsi, T Ciuleanu, N Pavlidis
Poster: ‘‘Immunohistochemical Profiling of Signalling Pathways in Cancer of Unknown Primary (CUP)’’, European CanCer Organization (ECCO) Congress, Stockholm, Sweden, 09/2011

G Hardavella, V Siozopoulou, A Batistatou, P Galanis, Y Dalavanga, M Alchanatis, D Stefanou, S Constantopoulos
Poster: ‘‘Comparing LYVE-1 and D2-40 expression in small cell lung carcinomas (SCLC); association with clinical parameters and lymphatic invasion’’, ERS Annual Congress, Amsterdam, The Netherlands, 06/2011

Economou G, Siozopoulou V, Balasi E, Margariti P, Zikou A, Assimakopoulos D
Oral presentation: ‘‘Metastatic Breast Cancer to the Paranasal Sinuses: case report and review of the literature’’, 2nd International French-Hellenic Congress in Otorhinolaringologie and Head & Neck Surgery, Lefkada, Greece, 02/2011

G Hardavella, V Siozopoulou, A Batistatou, P Galanis, Y Dalavanga, M Alchanatis, S Constantopoulos, D Stefanou
Poster: ‘‘Lymphangiogenesis in small cell lung carcinomas (sclc): new insights in prognosis’’, European Multidisciplinary Conference in Thoracic Oncology (EMCTO), Lugano, Italy, 01/2011

A Zioga, V Siozopoulou, A Batistatou, M Doukas, E Lykoudis, V Malamou-Mitsi
Oral presentation: ‘‘The correlation of regression in primary melanoma with sentinel lymph node status’’, 4th Melanoma Congress, Athens, Greece, 11/2010

V. Siozopoulou, G. Hardavella, P. Galanis, A. Batistatou, D. Stefanou
Poster: ‘‘Lymphangiogenesis in small cell lung carcinoma (SCLC): new insight in prognosis’’, 8th Tumor Markers-Targeted Therapy Congress, Athens, Greece, 11/2010

V Siozopoulou, N Simou, A Goussia, D Stefanou
Poster: ‘‘ Giant cell lung carcinoma: report of a case’’, 8th Tumor Markers-Targeted Therapy Congress, Athens, Greece, 11/2010

G Hardavella, V Siozopoulou, E Arkoumani, Y Dalavanga, S Constantopoulos, D Stefanou
Oral Presentation: ‘‘Expression of D2-40 in small cell lung carcinomas (SCLC); Association with lymphatic invasion(L.I), clinical parameters and prognosis’’, ERS Annual Congress, Barcelona, Spain, 09/2010

A Batistatou, V Siozopoulou, S Kamina, E Balassi, V Malamou-Mitsi

Poster: ‘‘Osteoclast-like giant cells in pelvic leiomyosarcoma: characteristic immunophenotype and possible histologic origin’’, Intercongress Meeting of the European Society of Pathology, Krakow, Poland, 08-09-2010

A Batistatou, V Siozopoulou, S Kamina, E Balassi, V Malamou-Mitsi
Poster: ‘‘Osteoclast-like giant cells in pelvic leiomyosarcoma: characteristic immunophenotype and possible histologic origin’’, 12th Hellenic Congress of the Hellenic Society of Pathology, Thessaloniki, Greece, 06/2010

V Siozopoulou, G Hardavella, Ch Charalabidis, P Galanis, A Batistatou, D Stefanou
Poster: ‘‘Lymphangiogenesis in small cell lung carcinoma (SCLC): new insight in prognosis’’, 12th Hellenic Congress of the Hellenic Society of Pathology, Thessaloniki, Greece, 06/2010

V Siozopoulou, G Hardavella, P Galanis, A Batistatou, D Stefanou
Poster: ‘‘Lymphangiogenesis in Chronic Obstructive Pulmonary Disease (COPD)’’, 12th Hellenic Congress of the Hellenic Society of Pathology, Thessaloniki, Greece, 06/2010

I Zinovieva, O Skoufi, M Doukas, V Siozopoulou, E Ioachim
Poster: ‘‘Polyarteritis Nodosa in Small Intestine’’, 12th Hellenic Congress of the Hellenic Society of Pathology, Thessaloniki, Greece, 06/2010

V Siozopoulou, A Goussia, G Lazos, G Baltayannis, A Ntoulia, I Zinovieva, P Oikonomou, I Dosi, E Tsianos, V Malamou-Mitsi
Poster: ‘‘Immunohistochemical Expression of Transforming Growth Factor Beta-1 in Chronic Gastritis’’, 12th Hellenic Congress of the Hellenic Society of Pathology, Thessaloniki, Greece, 06/2010

A Goussia, I Nesseris, A Mouladaki, G Baltayannis, V Siozopoulou, A Ntoulia, Ch Zois, E Tsianos, V Malamou-Mitsi
Poster: ‘‘Immunohistochemical Expression of Transforming Growth Factor Beta-1 in Chronic Liver Diseases’’, 3th Hellenic-Jordanian Congress of Pathology, Cyprous, 10/2009

I Zinovieva, V Siozopoulou, M Doukas, M Doumas, E Ioachim
Poster: ‘‘Polyarteritis Nodosa in Small Intestine’’ 3th Hellenic-Jordanian Congress of Pathology, Cyprous, 10/2009

A Goussia, I Nesseris, A Mouladaki, G Baltayannis, V Siozopoulou, A Ntoulia, Ch Zois, E Tsianos, V Malamou-Mitsi
Poster: ‘‘Immunohistochemical Expression of Transforming Growth Factor Beta-1 in Chronic Liver Diseases’’ 22nd European Congress of Pathology, Florence, Italy, 09/2009

INVITED REVIEWER IN PEER-REVIEW JOURNALS

Journal: Oncotargets and therapy
Manuscript ID: 315676

Journal: Journal of Clinical Medicine
Manuscript ID: not publicizable data

Journal: Biomolecules
Manuscript ID Biomolecules - 621358

Journal: Oncoimmunology
Manuscript ID: ONCOIMM – 20190416

Journal: Oncotargets & Therapy
Manuscript ID: 231052

Journal: Cancers
Manuscript ID: cancers - 569158

Journal: Vaccines
Manuscript ID: vaccines – 701849

AWARDS AND CERTIFICATES

Award for the performance in exams of the postgraduate course with topic: ‘‘Head and Neck Tumors: Differential diagnosis according histological pattern and immunophenotype’’, A΄ Pathology Department, University of Athens, Greece, 01/2012

Poster award for the study: ‘‘Lymphangiogenesis in Chronic Obstructive Pulmonary Disease (COPD)’’, 12th Hellenic Congress of the Hellenic Society of Pathology, Thessaloniki, Greece, 06/2010

Award for the progress as student for the subject of Pathology, Medical School of Ioannina, Greece, 06/2003

ORGANIZATION OF MEETINGS - CONGRESS

Organization of Dermatopathology meetings with invited speakers and case presentations (“Capita Selecta uit de dermatopathologie”), monthly, accredited by the “Institute for Public Sickness insurance and handicaps” “Rijksinstituut voor Ziekte- en Invaliditeitsverzekering”

Organization of the Working Group of Dermatologists and Dermatopathologists for the Guidelines for the Dysplastic Nevi

Member of the Organizing Committee at the 1st International Meeting on the Treatment of Human Bucellosis, Ioannina, Greece, November 2006

SUPERVISION OF STUDENT PROJECTS

Co-promotor of one Master Thesis in Medicine of the University of Antwerp: Dr. Niels Horst, Dermatology UZA, title: The role of eosinophils in psoriasis,

Promotor of one Master Thesis in Medicine of the University of Antwerp: Dr. Sabriya Sirimsi,
Pathology UZA, title: Comparison of the pTNM primary cutaneous melanomas between the AJCC TNM 7th and 8th edition; a retrospective study in a single institute

ADDITIONAL INFORMATION

President of the dermato-working group of the Belgian Society of Pathology
Member of the Belgian Society of Pathology
Member of the College of Oncologist – group for the guidelines for diagnosis and treatment for melanoma
Member of the EORTC, groups of melanoma, soft tissue and bone tumors, cutaneous lymphomas
Member of the Soft Tissue Panel group in Eindhoven, the Netherlands

Member of the Reviewer Board of Vaccines (IF 4.086)
Participation in different Ring Trials On PD-L1 Scoring, organized in the UZA
Trainer for PD-L1 (CPS score, 22C3)
Member of the EQA/ESP for long tumors
Member of the International Society of Dermatopathology
Member of European Society of Pathology, ESP
Member of Hellenic Society of Pathology, HSAP
Member of the dermatopathology group and soft tissue group of the HSAP

Google scholar: h-index: 8 and i10-index: 8
Research gate: RG score: 30.75 and h-index: 10
Web of science: h-index: 9

LANGUAGES

Greek Native speaker
Dutch Professional level
English Professional level, Certificate of Proficiency in English, University of Michigan
German Professional level, Kleines Deutsches Sprachdiplom, Goethe Institut

CHAPTER 11

ACKNOWLEDGEMENTS

Last chapter in this journey: the acknowledgments, or otherwise "*het dankwoord*". Last but so important. Because great results follow hard effort only when you have people next to you to support and help you. In any way and at any cost.

Looking back in time, exactly 9 years passed since I arrived in Belgium. It was mid-June 2012 when the phone rang and on the other end of the line was prof. dr. **Pauwels**. Next day I was checking for tickets and started packing my suitcase. Begin September I was in Antwerp and I did not regret it for a moment. I hope neither do you, dear Professor Pauwels, my dearest colleague and friend. Thank you for this great gift, to be here, to work in this wonderful environment, to have as many opportunities as one can dream, to sit right now in front of my computer and write the last chapter of my PhD.

After a while I entered CORE and there I met **Evelien**, or prof. dr. Evelien Smits. Evelien, thank you so much for being honest and simple despite of your position and the duties that derive from this position, for giving me the time and even the space in your own office to work when I was at CORE, for your valuable advice, for your never fading smile.

Elly you have been given the impossible task to be my promotor. But nothing is impossible for you. You have incredible courage and patience! I must have been a very difficult student. I want to thank you especially, not only for your effort, your time, your patience, but also for the respect you showed in my effort. You are the reason, the driving force to finish my PhD. I still remember your checklists, I admired you (and still do) for keeping everything under control. I tried also, but we both know it, checklists were not a big success for me. Well, I don't give up; since then I have a small pad on my desk! **Jonas**, take care of her!

Meanwhile **Julie** you have been presented with the challenging task to guide me. Lucky you! For you everything was easy, I came with the most complex questions and you gave me the most complex answers which for you were self-evident! I must admit that this gave me courage, since you knew, there was no reason to be afraid. Julie, we miss you, we still have to arrange the dinner that we were planning before you left, with Laure and Elly!

At that point I would like to thank my second co-promotor em. prof. dr. **Somville** for his support to my work. Additionally, I would like to show my gratitude to the members of the internal jury prof. dr. **Specenier** and prof. dr. **Bogers**, for not only taking the time to read and approve my work but also for their excellent collaboration as colleagues. Prof. dr. **Batistatou** and prof. dr. **Dei Tos**, it is an honor to me to have you as members of the external jury.

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