Diplomarbeit

Comprehensive analysis of tumor microenvironment to predict clinical outcome in soft tissue sarcoma patients after curative resection

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Mark Goda

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Priv.-Doz. Dr. med. univ. Joanna Szkandera

Dr. med. univ. Maria Anna Smolle

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Eidesstattliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwendet habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Graz, am 4. April 2021 *Mark Goda eh*

Vorwort

Die Diagnose „Krebs“ ist ein schwerer Schock – für manche steht sie am Anfang einer schwierigen Lebensphase, für andere beginnt damit der letzte Lebensweg. Dementsprechend stellt die medizinische Begleitung von onkologischen Patienten und Patientinnen eine große Herausforderung dar, bei der Therapie-Leitlinien aus umfangreichen Studien auf die Situation des Einzelnen angepasst werden müssen. Dazu bedarf es auf der einen Seite viel Einfühlsamkeit und zahlreiche Gespräche, um auf eine sorgfältig abgewogene, gemeinsame Behandlungsentscheidung zu kommen. Andererseits sind eine intensive Forschung und gründliche klinische Beobachtung notwendig, um die bestmögliche therapeutische Strategie zu entwickeln. Weichteilsarkome stellen in dieser Hinsicht eine besondere Gruppe von bösartigen Tumoren dar, weil sie recht selten und doch wahnsinnig vielfältig sind. Das erschwert die Durchführung von aussagekräftigen Studien mit vielen Patientinnen und Patienten und damit die optimale Behandlung.

All das hat mich bewogen, mich dieser Herausforderung ein Stück weit zu stellen und einen kleinen Beitrag in der Erforschung dieser durchaus spannenden Tumor-Entität zu leisten.

Danksagung

An erster Stelle gilt ein riesengroßer Dank meinen beiden Betreuerinnen Priv.-Doz. Dr. med. univ. Joanna Szkandera und Dr. med. univ. Maria Anna Smolle für die großartige, fachlich versierte, motivierende und geduldige Anleitung und Unterstützung bei der Diplomarbeit. Meine zahlreichen, noch so banalen Fragen wurden immer sofort beantwortet und sie hatten für jedes meiner Probleme eine Lösung parat – ich hätte mir keine bessere Betreuung vorstellen können.

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Contents

Abbreviations and Glossary

AIDS	Acquired immunodeficiency syndrome
ASPS	Alveolar soft part sarcoma
CAF	Cancer-associated fibroblast
CCL2	CC-chemokine ligand
CD	Cluster of differentiation
CDK4	Cyclin dependent kinase 4
CHOP	C/EBP homologous protein (alternatively DNA Damage-Inducible Transcript 3 [DDIT3])
COL1A1	Collagen type 1A1
CSF1	Colony-stimulating factor 1 (also known as Macrophage colony-stimulating factor [M-CSF])
CT	Computer tomography
CTX	Chemotherapy
DAMPs	Damage-associated molecular patterns
DAPI	4′,6-Diamidino-2-phenylindole
DFS	Disease-free survival
DFSP	Dermatofibrosarcoma protuberans
DM	Distant metastasis
DNA	Deoxyribonucleic acid
DSS	Disease-specific survival
EBV	Epstein-Barr virus
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMA	Epithelial membrane antigen
FAK	Focal adhesion kinases
FDG-PET	Fluorodeoxyglucose positron emission tomography
FISH	Fluorescence in situ hybridization
FNCLCC	French Federation of Cancer Centers Sarcoma Group

FOXP3	Forkhead box protein 3
FPCRRMs	Flexible parametric competing risk regression models
FUS	Fused in sarcoma (gene and protein)
GIST	Gastrointestinal stroma tumor
GIST	Gastrointestinal stromal tumor
GM-CSF	Granulocyte–macrophage colony-stimulating factor (also known as colony-stimulating factor 2 [CSF2])
Gy	Gray (unit)
HE	Hematoxylin and eosin stain
HHV-8	Human herpes virus 8
HMGB1	High mobility group protein B1
HR	Hazard ratio
HU	Hounsfield Units
IFNγ	Interferon-gamma
IHC	Immunohistochemistry
IL	Interleukin
ILP	Isolated hyperthermic limb perfusion
iNOS	Inducible nitric oxide synthase
IQR	Interquartile range
LGFMS	Low-grade fibromyxoid sarcoma
LR	Local recurrence
MDM2	Mouse double minute 2 homolog
MDT	Multidisciplinary team
MERK-R	Mer tyrosine protein kinase receptor
MFH	Malignant fibrous histiocytoma
MP	Mean percentage
MPNST	Malignant peripheral nerve sheath tumor
MRI	Magnet resonance imaging
MSI	Microsatellite instability
MSKCC	Memorial Sloan Kettering Cancer Center
NA	Not applicable

NCCN	National Comprehensive Cancer Network
NF 1	Neurofibromatosis type I
NGS	Next generation sequencing of the genome
NK cells	Natural killer cells
NOS	Not otherwise specified
OS	Overall survival
PD	Progressive disease
PDGFB	Platelet-derived growth factor β
PD-L1	Programmed death ligand 1
PERSARC	Sarculator and Personalised Sarcoma Care
PET	Positron emission tomography
PI3Kγ	Phosphoinositide 3-kinase gamma
PR	Partial response
R0	Negative resection margins: ≥1mm healthy tissue between tumor and surface
R1	Marginal resection margins: <1mm healthy tissue between tumor and surface
R2	Microscopically positive resection margins: no healthy tissue between tumor and surface
RB1	Retinoblastoma 1 gene
RECIST	Response Evaluation Criteria In Solid Tumors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
RTX	Radiation therapy
SD	Stable disease
SD	Standard deviations
SFS	Sclerosing epithelioid fibrosarcoma
SMA	α-Smooth muscle actin
STS	Soft tissue sarcoma
SUVmax	Standardized uptake value, maximum

TAM	Tumor-associated macrophages
TGFβ	Transforming growth factor-beta
TH1/2	Helper T-cell type 1/2
TIIC	Tumor-infiltrating immune cells
TLR4	Toll-like receptor 4
TMA	Tissue microarrays
TME	Tumor microenvironment
TNF	Tumor necrosis factor
TSA	Tyramide-signal-amplification
UICC	Union Internationale Contre le Cancer
UPS	Undifferentiated pleomorphic sarcoma
US	Ultrasound
VACM1	Vascular cell adhesion molecule 1
VEGF-A	Vascular endothelial growth factor A
WHO	World Health Organization

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Kurzfassung

Einleitung: Die mikroskopische Umgebung eines Tumors (engl. *tumor microenvironment* [TME]) spielt eine entscheidende Rolle im biologischen Verhalten der Tumore und in der Wirksamkeit von Therapien. Bei Weichteilsarkomen (engl. *soft tissue sarcoma*, STS) jedoch ist der Einfluss der mikroskopischen Tumorumgebung noch nicht ausreichend erforscht worden. Diese Diplomarbeit hat deshalb zum Ziel, den Zusammenhang zwischen Tumor-infiltrierenden Immunzellen (engl. *tumor-infiltrating immune cells* [TIIC]) und dem Krankheitsverlauf von PatientInnen mit STS zu untersuchen.

Material und Methoden: In der retrospektiven Analyse wurden Kleingewebsproben von insgesamt 188 STS-PatientInnen mit Multiplex-Immunhistochemie gefärbt und sieben Immunzellphänotypen anhand der Multispektralbilder automatische gezählt: T-Zellen (CD3+), T-Helferzellen (CD3+, CD4+), zytotoxische T-Zellen (CD3+, CD8+), T-Gedächtniszellen (CD3+, CD4+. CD45RO+), B-Zellen (CD20+) und Makrophagen (CD68+).

Ergebnisse: Die zwei häufigsten Tumor-infiltrierenden Zellen waren CD68+ Makrophagen (medianer Prozentanteil [MP] 2,93%) und CD3+ T-Zellen (MP 2,65%). In der multivariaten Analyse anhand des Fine & Gray Regressionsmodells mit Tod als konkurrierendes Ereignis wurde eine signifikante, positive Korrelation zwischen der Infiltration durch CD68+ Makrophagen und dem Risiko für ein Lokalrezidiv (p-Wert 0.014) unabhängig von Alter, den Resektionsrändern und der Anzahl der B-Zellen gefunden. Zudem war die Anzahl der Makrophagen bei Patienten über dem medianen Alter von 62,5 Jahren signifikant höher (p-Wert 0.002), während die Zahl der B-Zellen signifikant niedriger in der älteren Patientengruppe war (p-Wert 0.013). Bezüglich der histologischen Subtypen zeigte sich die Gesamtanzahl von Tumor-infiltrierenden Immunzellen sowie die Anzahl der Makrophagen vor allem in undifferenzierten pleomorphen Sarkomen (UPS) und in Myxofibrosarkomen erhöht.

Schlussfolgerung: Die Ergebnisse bestätigen, dass eine vermehrte Infiltration von CD68+ Makrophagen in Weichteilsarkomen einen negativen, prognostischen Faktor hinsichtlich des Risikos für Lokalrezidive darstellt. Aufgrund dieser Erkenntnis könnten ausgewählte PatientInnengruppen mit Weichteilsarkomen von einer Makrophagen-gezielten Therapie profitieren.

Abstract

Introduction: The tumor microenvironment (TME) plays a crucial role in tumor behavior and therapeutic efficacy. However, in soft tissue sarcoma (STS), the influence of TME has not been investigated sufficiently. Thus, this diploma thesis aims to examine the correlation between tumor-infiltrating immune cells (TIIC) and patient outcome.

Material and methods: In this retrospective analysis, tissue microarrays of one-hundred-eighty-eight STS-patients were stained with multiplex immunohistochemistry and seven immune cell phenotypes were counted automatically on multispectral images: T-cells (CD3+), helper T-cells (CD3+, CD4+), cytotoxic T-cells (CD3+, CD8+), helper memory T-cells (CD3+, CD4+. CD45RO+), B-cells (CD20+), and macrophages (CD68+).

Results: The two most common TIIC were CD68+ macrophages (median percentage [MP] 2.93%) and CD3+ T-cells (MP 2.65%). In the multivariate analysis using a Fine & Gray risk-regression model with death as competing event, a significant positive correlation between CD68+ macrophages infiltration and risk of local recurrence (p-value 0.014) independently of age, resection margins, and presence of B-cells, was found. Moreover, the abundance of macrophages was significantly higher in patients older than the median age of 62.5 years (p-value 0.002), whilst B-cell counts were significantly lower (p-value 0.013) in the older patient cohort. Regarding histological subtypes, overall high counts of TIIC in general, and macrophages in particular, were observed in undifferentiated pleomorphic sarcoma (UPS) and myxofibrosarcoma.

Conclusion: The data confirm high levels of CD68+ macrophages in STS as a negative prognostic factor regarding local recurrence. Based on these findings, selected STS patient groups might benefit from macrophage-targeting therapies.

Indication of Publications

Smolle MA, Herbsthofer L, Goda M, Granegger B, Brcic I, Bergovec M et al. Influence of tumor-infiltrating immune cells on local control rate, distant metastasis, and survival in patients with soft tissue sarcoma. Oncoimmunology 2021; 10(1):1896658.

I Introduction

1) General Approach

The term Soft Tissue Sarcoma (STS) describes a rare, diverse group of mesenchymal malignant tumors. The heterogeneity of sarcoma, the complexity of the pathological diagnosis, and the challenges of an optimal treatment always require a multidisciplinary approach and should be referred to a reference center for soft tissue sarcoma. (1)

Due to their specific localization, pathophysiology, and treatment, gastrointestinal stromal tumors (GIST), Kaposi sarcoma, and soft tissue sarcoma of the uterus and of the mamma will not be discussed in this diploma thesis.

a) Epidemiology

The overall annual incidence of STS ranges in different epidemiological databases from 3 to 6 per 100,000 persons with slightly but significantly higher rates in males. (2–4) Although the absolute number of STS is higher in adults over 70 years than in children younger than 10 years (18.2/100,000 vs. 0.9/100,000) – primarily because malignancies in general are more common at higher age for the aged genome is more vulnerable to malignant mutations – the proportional incidence of STS among pediatric malignancies (7.4%) is larger compared to the percentage of STS in malignant tumors of adults (1.5%). However, certain entities show a specific age pattern. Rhabdomyosarcoma, for example, occurs typically in children and adolescents with a median age of 15 years, whereas liposarcoma is a STS subtype of patients above the age of 60 years. (3) Generally, translocation-related sarcomas are – compared to STS with complex karyotype – more common in younger patients and associated with higher rates of metastasis at presentation. (5)

Two peaks stand out regarding the age distribution among STS in general: The first one between the age of 5 to 15 years, explained by the significantly higher incidence of pediatric soft tissue

sarcoma like embryonal rhabdomyosarcoma, and the second peak with an increased number of patients with liposarcoma, leiomyosarcoma, and undifferentiated pleomorphic sarcoma at the age of 70. (3, 5)

b) Etiology and pathophysiology

In contrast to other tumor entities, soft tissue sarcomas are primarily not a malignant transformation of a preexisting benign tumor but develop mainly de novo. On the molecular level, two main patterns have been identified:

(A) On the one hand, there are specific genetic changes with simple karyotype: Single point mutations in oncogene or tumor suppressive genes are rare (e.g. retinoblastoma 1 [RB1] gene), but translocations and alterations on chromosomal level generating new fusion proteins are very common, e.g. translocation t(X;18)(p11;q11) leading to SS18/SSX fusion protein in synovial sarcoma or the specific nonreciprocal translocation t(17;p22)(q22;q13) causing Collagen type 1A1-Platelet-derived growth factor β (COL1A1-PDGFB) fusion transcripts in dermatofibrosarcoma protuberans (DFSP). (6–9) Simple karyotypes account for approximately one third of all STS and show usually a small range of atypia on histopathological level (well-differentiated, rather monomorphic tumor cells, mild nuclear atypia, low mitotic index). (10)

(B) On the other hand, complex, multifocal genetic alterations and unbalanced karyotypes with various functional conversions are characteristic for about two third of STS subtypes, for example leiomyosarcoma or undifferentiated pleomorphic sarcoma. P53 checkpoint pathway inactivation is suspected to be the pivotal mechanism for the initiation of this second type of STS with complex karyotype. (11, 10)

Although direct causalities are still uncertain, numerous risk factors for STS have been described and can be summarized in five different categories:

a) Genetic predispositions: Germ line mutations leading to STS are rare but can cause tumor development at an early age. The high risk of multiple malignancies over the course of life requires regular clinical follow-ups. Some examples for hereditary tumor syndromes regarding STS are Li Fraumeni syndrome (TP53

mutation), neurofibromatosis type I (neurofibromin 1 mutation), Gardner's syndrome, and retinoblastoma (RB1 mutation). (12)

b) Radiation therapy (RTX): One of the first associations between RTX and neoplasms was described in undifferentiated pleomorphic sarcoma (UPS). Also, angiosarcoma is associated with irradiation, particularly in women receiving RTX in case of breast cancer. Secondary STS due to RTX are more likely after therapy with high dosage (>40 Gray), simultaneous application of chemotherapeutics such as alkylating agents and anthracyclines, and at a low age, and show generally a poorer prognosis compared to primary STS. (13–15)

c) Chemicals: Different industrial chemicals are suspected to play a role in the development of STS, but only the connections between hepatic angiosarcoma and the exposure to arsenic or vinyl chloride has been established to be causal so far. (16, 17)

d) Chronic irritation: Some case reports suggests an association between chronic lymphedema (because of filarial lymphomatous infection or after axillary lymphadenectomy due to breast cancer) or foreign bodies (e.g. hydrocephalus shunt) and STS. (18–20) Although a traumatic event typically does not cause the tumor, but just draws the patient’s attention to the previously existing lesion, there is research on animals suggesting a causative link between injury and STS. (21, 22)

e) Viral infection and immunodeficiency: Human herpes virus 8 (HHV-8) as origin of Kaposi sarcoma, in patients with acquired immunodeficiency syndrome (AIDS) in particular, has been established in the meantime. (23) Moreover, Epstein-Barr virus (EBV) is associated with smooth-muscle tumors in immunocompromised hosts and organs. (24–26)

c) Clinical Presentation

Despite the rarity of malignant soft tissue tumors, general practitioners, orthopedics, dermatologists, and other physicians performing the initial examination of a patient presenting with a soft tissue lump should include STS in their differential diagnosis and be aware of signs

for malignancy, since early suspicion of malignant soft tissue tumors with transfer to specialized units may significantly increase patient outcome. (27, 28) In case of extremity STS and especially those growing superficially, patients initially present with concerns about constantly increasing swelling, usually painless and with low complaint. Specific symptoms are rarely present and arise from local effects of the growing mass, e.g. paresthesia, paralysis or edema by compressing nerves or lymph vessel. Constitutional and B symptoms, i.e. weight loss, fever, and night sweats, are uncommon presentations of patients with STS.

d) Diagnostic Evaluation

(1) Patient history

Inquiry of patient's history as first step in diagnostic algorithm is crucial for not missing important hints regarding the dignity. Recently discovered and constantly growing lesions without any bruising or skin rash should attract the attention to further evaluation. In case of recent trauma at the site of the lesion, hematoma should be excluded, especially in patients with anticoagulants. (29, 30)

STS are more common in the extremities but can arise at any anatomic site of the body. Thigh, groin, and buttock are the most affected localizations (together 46%), followed by the torso (18%). The upper extremity (13%), and the retroperitoneum (13%) are equally frequent sites of affection, whilst STS on head and neck (together 9%) are quite rare, according to American data of 4,550 adults with STS. (31)

(2) Clinical evaluation

Palpation and the attempt to move the tumor can help in differentiating tumor dignity. Superficial lesions with no change in shape or size over the years, smaller than a golf ball (approximate diameter 4cm) and easily moveable are most likely benign. On the other hand, barely moveable lumps located deeply under the fascia are indicative of STS and should be referred to a specialized tertiary tumor center.

The three major features of soft tissue swellings pointing to malignancy in clinical examination have been identified as: (a) recent growth in size, (b) subfascial, deep location, (c) and tumor

size larger than 4cm. Non-tenderness turned out to be an unreliable predictor in differentiation between benign and malignant soft tissue neoplasms. (29, 30, 32)

(3) Imaging

Every lesion with suspicion of sarcoma should be investigated by imaging prior to any biopsy or surgery. (33) Tumor size, tissue quality, tumor perfusion, and its relation to surrounding structures are the main interests in performing imaging, just as the assessment of the further procedure. Ultrasound (US) has turned out to be a reliable measurement to exclude hematoma, abscesses, cysts, and other pseudotumors. (34) Moreover, US displays size, shape and adjacent structures of the tumor and helps in estimating the tumor vascularization via Doppler method. Signs for a malignant tumor would be large centripetal vessels, scattered peripheral vascularization, and hypervascularity in general. However, US is not the method of choice to differentiate between benign and malignant soft tissue tumors.

Plain radiography may be helpful to exclude bone involvement and detect calcified structures like in synovial sarcoma. Thus, it is usually part of the common work up, also, because it is cheap and can be performed quickly. Nevertheless, no sure distinction can be drawn in tumor dignity or entity from plain radiograph images. (35)

The method of choice is magnet resonance imaging (MRI), preferably including gadolinium-based contrast enhancement (36, 37) Although MRI does not allow histological characterization, malignancy can be highly suspected by showing the tumor's expansion and heterogeneous signaling. Tumor size and its relation to deep fascia, nerves, vessels, bones, and joints can be equally measured by computer tomography (CT), however, the superiority of MRI to CT regarding muscle involvement and display of the anatomic compartment is discussed controversially. (36, 38–41)

Fluorodeoxyglucose positron emission tomography in combination with CT (FDG-PET-CT) can be useful in differentiating between benign lesions with generally low relative uptake and malignant tumors with increased uptake, and positive correlations between prognosis and maximum standardized uptake value (SUVmax) have been described. (42) In particular, FDG-PET-CT is essential for the distinction between benign neurofibroma and malignant peripheral nerve sheath tumor (MPNST) in patients with neurofibromatosis type I (NF 1). (43–45)

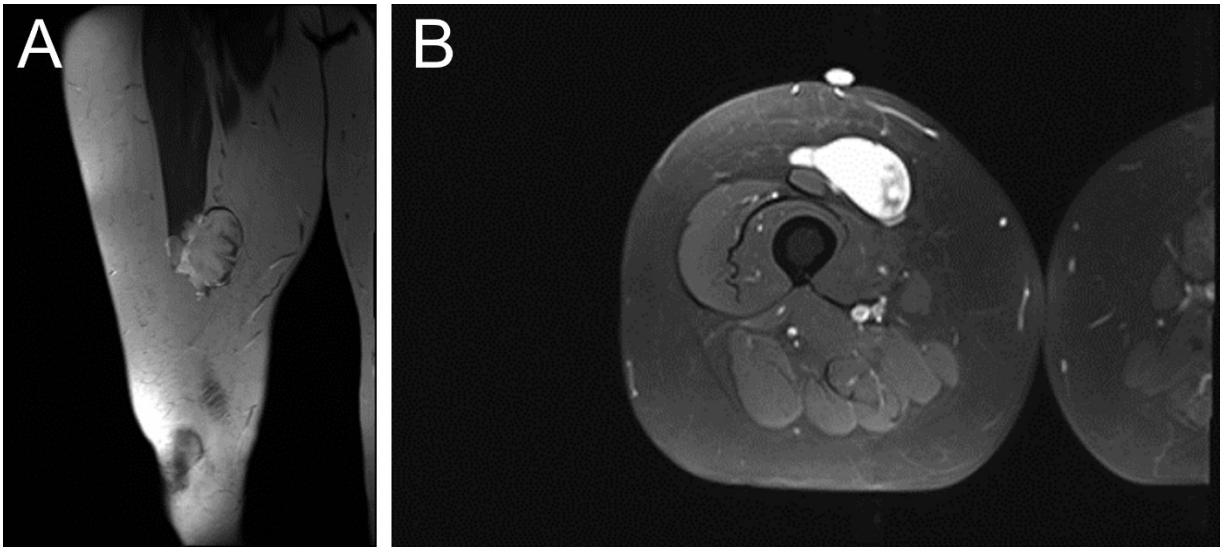

Figure 1 Magnet resonance imaging of a 57 year old patient with myxoid liposarcoma grade 1 on the right femur.
(A) Coronary T1 sequence with contrast agent (turbo spin echo). (B) In the transversal T2 sequence after fat suppression (turbo spin echo) the lesion appears as lobulated, hyperintense mass with inhomogeneous contrast agent uptake due to its myxoid structure.

(4) Biopsy

Although attempts were made for relying in certain cases for diagnosis on MRI only, pathological findings from a biopsy are currently indispensable for diagnosis of STS. In case imaging was not carried out in a reference center for STS, the biopsy should be inevitably performed by sarcoma specialists, since method and procedure of the biopsy determines the following surgery and the degree of required resection. All possibly contaminated tissue, like the entire biopsy channel, has to be excised. Hence, a careful planning of the shortest pathway through one compartment only, avoiding nerves, blood vessels, and joints, and based on reliable imaging in consultation with the radiologist is required in view of eventual resection.(32)

The four main types of biopsies, listed by increasing invasion, are: (a) fine needle biopsy under local anesthesia, but with little gain of tissue; (b) open biopsy, the most common method, performed under general or plexus anesthesia; (c) core needle biopsy, usually imaging-guided (US, CT) for hardly accessible lesions (d) and excision biopsy for tumors smaller than 2cm in diameter on MRI. The amount of required sample for morphological evaluation, grading, and cytogenetic or molecular examination should be discussed with the pathologist specialized in STS. In general, core needle biopsy is recommended. Although, open biopsy shows a higher

diagnostic accuracy than core needle biopsy, the complication rate is lower after core needle biopsy, whereas fine needle biopsy is limited regarding subtyping and grading and is not approved for the initial evaluation. (46–50) Figure 2 shows the usual algorithm of the initial evaluation. (32)

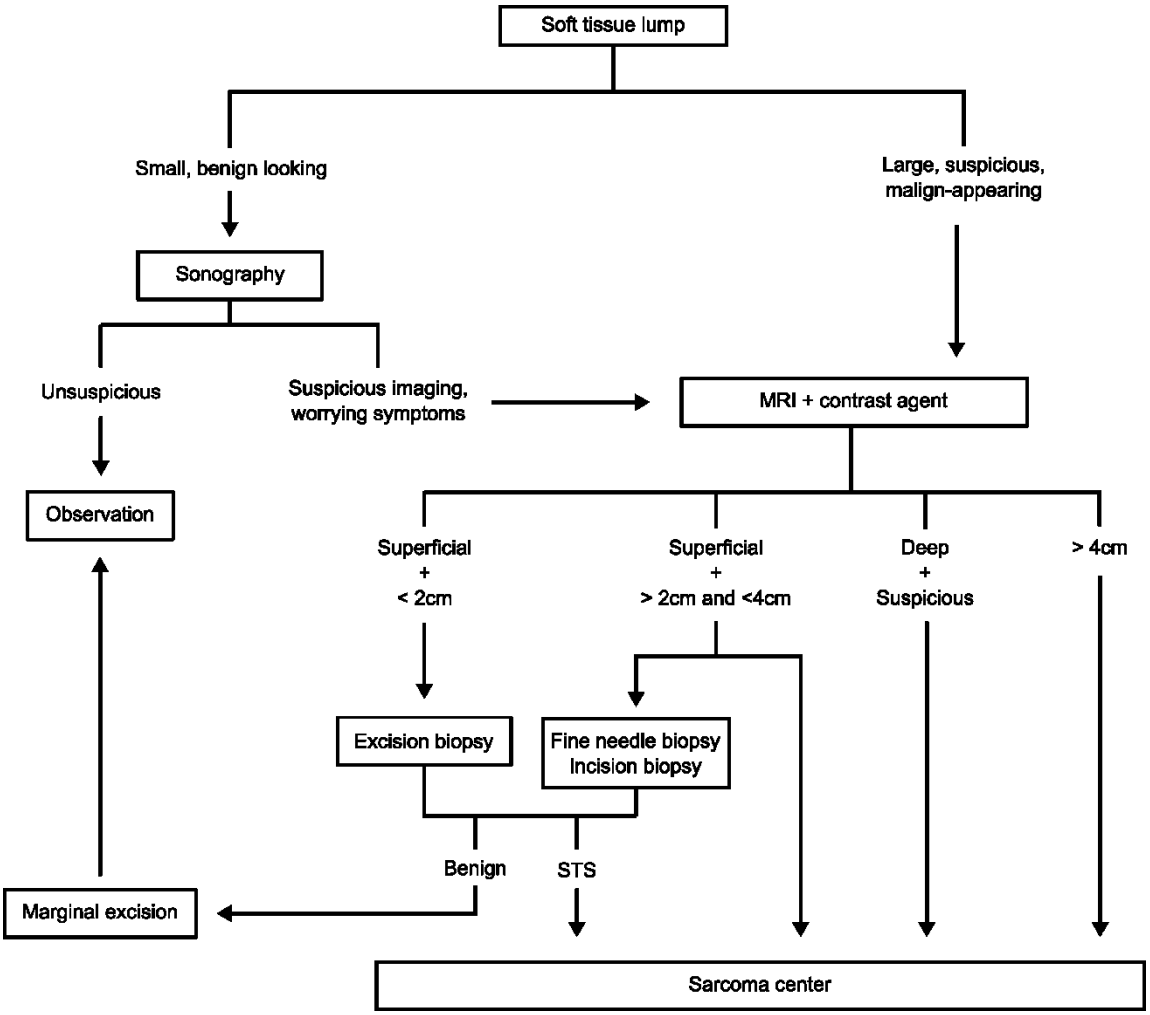

Figure 2 Suggested algorithm for the evaluation process. Adapted from Smolle et al. (32)
MRI, magnet resonance imaging. STS, soft tissue sarcoma.

e) Pathological evaluation and Grading

The diagnosis, and grading of soft tissue sarcoma always relies on the pathological findings of the lesion and is essential for the therapy and prognosis. (51) The definitive diagnosis from pathological side can be complex and requires almost always immunohistochemistry in addition to histopathology. Molecular evaluation is recommended in all cases with doubtful or uncertain

diagnosis, uncommon clinical presentation, and if molecular information is needed for prognostic or predictive assessment. In contrast, ultrastructural examination via electron microscope is rarely beneficial and hardly used in the meantime. Generally, the pathological diagnosis of STS results from the combination of histopathological, immunohistochemical, and molecular features. Due to the great variety of STS and relatively small incidence, pathological diagnosis should only be made by reference centers for STS, according to the actual ESMO guidelines. (1)

(1) Histopathology

Regarding the histopathological evaluation, a pattern-based approach is recommended and depends mainly on the identification of the dominant cells of the neoplasm, the underlying matrix, and specific structures of blood vessels or other special features. Hornick suggests six basic patterns as guidance for possible differential diagnosis (52):

1. Spindle cell: e.g. myxofibrosarcoma, dermatofibrosarcoma protuberans
2. Epithelioid: e.g. epithelioid sarcoma
3. Pleomorphic: e.g. undifferentiated pleomorphic sarcoma
4. Round cell: e.g. alveolar rhabdomyosarcoma, Ewing sarcoma
5. Biphasic or mixed: e.g. biphasic synovial sarcoma
6. Myxoid e.g. myxoid liposarcoma

Soft tissue sarcomas commonly have a pseudocapsule. The growth pattern might be fascicular, storiform, lobulated, plexiform, nested, or trabecular and the underlying matrix can contain myxoid stroma, collagenous stroma, calcifications, and prominent inflammatory cells, for example.

(2) Immunohistochemistry

The concept of immunohistochemistry (IHC) relies on the detection of expressed proteins in tumor tissue, targeted by antibodies that are made visible in different ways. Obviously, the more specific proteins for a group or subtype of STS are, the more IHC might help in confirming the diagnosis. The antigens can be for instance intermediate filament proteins (e.g. keratin), myogenic markers (e.g. desmin, α-smooth muscle actin), endothelial cell surface molecules (e.g.

CD34, CD31), and specific markers discovered by gene express profiling (e.g. transducin-like enhancer of split 1 for synovial sarcoma, STAT 6 for solitary fibrous tumor). (52)

Vimentin as general mesenchymal marker has lost its diagnostic relevance, since it is expressed universally in STS and does not help in distinguishing different subtypes. Moreover, vimentin is not even limited to mesenchymal tumors only, but is present in certain subsets of carcinomas and melanomas as well. (52)

(3) Molecular diagnostics

Generally, three main molecular techniques for analyzing genetic alterations and fusion genes are available: (a) cytogenetic methods like the visual detection of fusion genes via fluorescence in situ hybridization (FISH, cf. Figure 3); (b) detection of fusion genes by genetic multiplication with reverse transcriptase polymerase chain reaction (RT-PCR), and (c) direct sequencing of the genome with next generation sequencing (NGS) that even reveal simple alterations of the desoxyribonucleic acid (DNA) or ribonucleic acid (RNA). Especially in subtypes without specific chromosomal changes but characteristic protein amplification, like mouse double minute 2 homolog (MDM2) and cyclin dependent kinase 4 (CDK4) expression in well-differentiated liposarcoma, FISH is an indispensable method to approve the histological assumption.

An advanced method to scan for multiple specific translocations in shorter time is for example the “Archer Fusion Plex Sarcoma Panel”. Gene-specific primers are applied to amplify and then detect 26 RNA fusion genes distinctive for certain STS subtypes at the same time. (53)

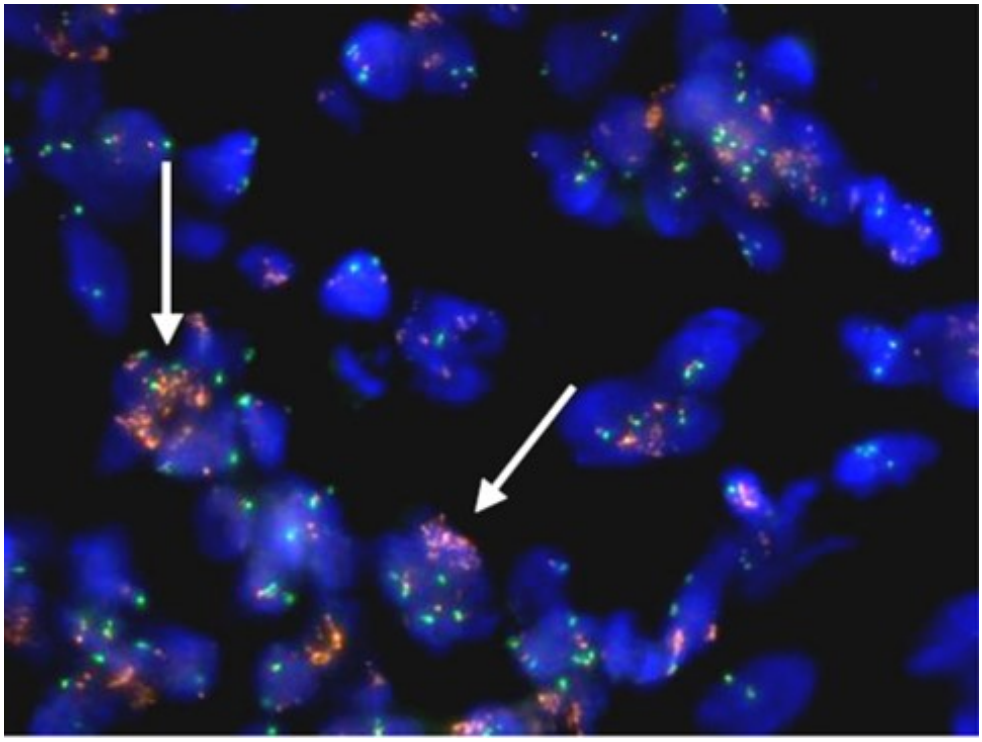

Figure 3 Fluorescence in situ hybridization (FISH) of mouse double minute 2 homolog (MDM2) gene on chromosome 12 in well-differentiated liposarcoma.
Red signals demonstrate the amplified MDM2 locus. The picture was provided courtesy of Univ.-Prof. Priv.-Doz. Dr.med.univ. Bernadette Liegl-Atzwanger, Diagnostic and Research Institute of Pathology, Medical University of Graz, Austria.

The enormous, recent development and rapidly growing ability of genetic testing led to an extensive informational gain about genetic profiles of STS. Some subtypes turned out to have distinctive gene alterations – mainly chromosomal translocations – causing an increased expression of characteristic proteins. For example, in myxoid and round cell liposarcoma the reciprocal translocation t(12;16)(q13;p11) generates a fusion of CHOP and FUS gene. Table 1 shows some examples of chromosomal translocations and related proteins specific for certain subtypes of STS.

Translocation	Genes	Type of fusion gene
Myxoid liposarcoma		
t(12;16)(q13;p11)	FUS-CHOP (FUS-DDIT3)	Transcription factor
t(12;22)(q13;q12)	EWSR1-CHOP (EWSR1-DDIT3)	Transcription factor
Synovial sarcoma		
t(X;18)(p11;q11)	SS18-SSX1, SSX2, or SSX4	Remodels chromatin to alter transcription
Clear cell sarcoma		
t(12;22)(q13;q12)	EWSR1-ATF1	Transcription factor
Alveolar soft part sarcoma		
t(X;17)(p11.2;q25)	ASPL-TFE3	Transcription factor
Dermatofibrosarcoma protuberans		
t(17;22)(q22;q13)	COL1A1-PDGFB	Growth factor
Desmoplastic small round cell tumor of the abdomen		
t(11;22)(p13;q12)	EWSR1-WT1	Transcription factor
Solitary fibrous tumor		
Inversion 12q13	NAB2-STAT6	Transcription factor

Table 1 Selected translocations associated with STS.
Adapted from Helman LJ, Meltzer P (54)

Even more, genetic research on non-neoplastic soft tissue lesions have revealed characteristic mutations, like MYH9-USP6 fusion gene in nodular fasciitis, and displayed their tumorous rather than traumatic etiology. (55)

However, molecular diagnostics alone without histopathology and immunochemistry is not sufficient: alpha-thalassemia/mental retardation syndrome X-linked (ATRX) gene, for example, may be altered in both, leiomyosarcoma and osteosarcoma, however, the morphological appearance of this two subtypes varies fairly as they are derived from different tissue origins. (56, 57) Moreover, specific genetic fusions of sarcoma have been described in carcinomas as

well, such as the unbalanced translocation der(17)t(X;17)(p11.2;q25), associated with alveolar soft part sarcoma, but also found in pediatric renal cell carcinomas. (58)

(4) Grading

Histological grading is always required and represents an independent risk factor for distant metastasis and death. (59–61) Different grading systems have been established in order to improve the prognostic value of histological findings. The one most used was developed by the French Federation of Cancer Centers Sarcoma Group (FNCLCC) and is a three-tier system (low grade [G1], intermediate grade [G2], high grade [G3]) based on tumor cell differentiation, mitotic activity, and necrosis dimension. (62, 63) Thus, high grade tumors are more likely to metastasize.

However, grading is restricted to untreated primary STS and should not be applied on alveolar soft part sarcoma, embryonal and alveolar rhabdomyosarcoma, clear cell sarcoma, angiosarcoma, and epithelioid sarcoma. (64, 65) Moreover, certain aggressive STS entities are always graded with G3, namely synovial sarcoma, pleomorphic rhabdomyosarcoma, and undifferentiated pleomorphic sarcoma, whereas well-differentiated liposarcoma is G1 per definition. (64, 63) Malignant peripheral nerve sheath tumors (MPNST) are mainly high grade, too, but grading for this subset shows no prognostic relevance. (66, 67)

f) Staging and pattern of metastasis

Once STS is confirmed, further evaluation regarding metastatic spread is essential for treatment and prognosis. The idea behind dividing tumor diseases into different stages is to allow prognostic assessments according to the progression of the disease. The four main pillars in staging STS are size and infiltration of the primary tumor (T), metastases in regional lymph nodes (N), distant metastases (M), and grading (G). The first three parameters correspond to the TNM system and staging especially according to tumor size varies depending on the affected region; there are slight differences between STS staging of (a) extremities and superficial trunk, (b) retroperitoneum, (c) and abdominal viscera and thorax (68, 69)

Overall, stadium T1 is assessed in case of tumors confined to the organ, whilst infiltration of tissues beyond the organ are defined as T2 with subcategorization into T2a (extension into

serosa or visceral peritoneum) and T2b (extension beyond serosa or mesentery). Stadium T3 describes invasion of other organs and T4 multifocal metastases with the subclassifications T4a to T4c according to the number of involved sites. Affection of regional lymph nodes (N) and presence of metastasis (M) are categorized dichotomously (N/M0 – not present; N/M1 – present).

The following stages are distinguishable based on the combination of TNM system and grading:

- Stage IA: T1, N0, M0, G1
- Stage IB: T2/T3/T4, N0, M0, G1/GX
- Stage II: T1, N0, M0, G2/G3
- Stage IIIA: T2, N0, M0, G2/G3
- Stage IIIB: T3/T4, N0, M0, G2/G3
- Stage IV: any T, N1, M0, any G

Mesenchymal tumors generally prefer hematogenous over lymphogenic spreading. In case of STS, lungs are most frequently affected. At time of initial STS diagnosis, distant metastases are already present in around 10% of the patients, mostly in the lungs (83%). (70) Risk factors for metastasis are large tumor size, deep localization, and high histological grade (71, 72, 60) Regional lymph node metastasis occurs in less than 3% and is predominantly associated with clear cell sarcoma, rhabdomyosarcoma, epithelioid sarcoma, synovial sarcoma, and vascular malignancies. (73) Lymphogenic spread shows overall a poorer prognosis than hematogenous metastasis in STS. Nevertheless, the benefit of sentinel lymph node biopsy and following lymphadenectomy in patients with STS subtypes with high frequency of lymph node metastasis has not been sufficiently investigated so far and is not generally recommended. (74–80)

Due to the high risk of pulmonary metastasis, the standard staging for all patients contains a chest imaging method – most of the guidelines recommend a spiral chest CT, but plain chest radiograph is also discussed as sufficient alternative for patients with low risk for metastasis (tumor size less than 5cm, superficially located, low grade histology). (1, 81–83) Positron emission tomography (PET) turned out to be less sensitive than CT in detecting lung involvement in patients with STS. (84–87)

Apart from pulmonary involvement, some extrapulmonary sites of spread are typical for certain entities. Thus, a subtype-based staging is suggested:

- Abdominal and pelvic CT are obligatory in case of round-cell and myxoid liposarcoma. The recommendation for a CT of abdomen and pelvis in patients with angiosarcoma, epithelioid sarcoma, and leiomyosarcoma by the National Comprehensive Cancer Network (NCCN) is controversial. (83, 88)
- Imaging of central nervous system is indicated for patients with alveolar soft part sarcoma and angiosarcoma. (83)
- Bone involvement in STS is rare in adults but might arise in patients with round cell or myxoid liposarcoma. In symptomatic cases of this entity, MRI of the bone is preferred over CT scan for its higher sensitivity for bone metastases. (89)

Despite the high sensitivity of PET to detect extrapulmonary metastatic disease, no PET is recommended in the standard staging work-up, since extrapulmonary spread is generally very uncommon in STS. (85)

2) The Variety of Soft Tissue Sarcoma

1 Histological subtypes

Depending on the entity, soft tissue sarcomas show a great variety in morphology and biological behavior. The classification is challenging and has been changed multiple times over the past decades. The current, 5th edition from 2020 of the classification by the World Health Organization (WHO) contains more than 50 subtypes. (65)

Adipocytic tumors

Benign

Lipoma and lipomatosis
Lipomatosis of nerve
Lipoblastoma and lipoblastomatosis
Angiolipoma
Myolipoma of soft parts
Chondroid lipoma
Spindle cell/pleomorphic lipoma
Atypical spindle cell/
pleomorphic atypical lipomatous tumor
Hibernoma

Intermediate (locally aggressive)

Atypical lipomatous tumor

Malignant

Well differentiated liposarcoma
(lipoma-like, sclerosing, inflammatory)
Dedifferentiated liposarcoma
Myxoid liposarcoma
Pleomorphic liposarcoma
Myxoid pleomorphic liposarcom

Fibroblastic/myofibroblastic tumors

Benign

Nodular fasciitis
Proliferative fasciitis and proliferative myositis
Myositis ossificans and fibro-osseous pseudotumor of digits
Ischaemic fasciitis
Elastofibroma
Fibrous hamartoma of infancy
Fibromatosis colli
Juvenile hyaline fibromatosis
Inclusion body fibromatosis
Fibroma of tendon sheath
Desmoplastic fibroblastoma
Myofibroblastoma
Mammary-type myofibroblastoma
Calcifying aponeurotic fibroma
EWSR1-SMAD3-positive fibroblastic tumor (emerging)
Angiomyofibroblastoma
Cellular angiofibroma
Angiofibroma NOS
Nuchal fibroma
Acral fibromyxoma
Gardner fibroma

Intermediate (locally aggressive)

Palmar/plantar-type fibromatosis
Desmoid-type fibromatosis
Lipofibromatosis
Giant cell fibroblastoma
Dermatofibrosarcoma protuberans

Intermediate (rarely metastasizing)

Dermatofibrosarcoma protuberans, fibrosarcomatous
Solitary fibrous tumor
Inflammatory myofibroblastic tumor
Low-grade myofibroblastic sarcoma
Superficial CD34-positive fibroblastic tumor
Myxoinflammatory fibroblastic sarcoma
Infantile fibrosarcoma

Malignant

Solitary fibrous tumor, malignant
Fibrosarcoma NOS
Myxofibrosarcoma
Low grade fibromyxoid sarcoma
Sclerosing epithelioid fibrosarcoma

So-called fibrohistiocytic tumors

Benign

Tenosynovial giant cell tumor
Deep benign fibrous histiocytoma

Intermediate (rarely metastasizing)

Plexiform fibrohistiocytic tumor
Giant cell tumor of soft parts NOS

Malignant

Malignant tenosynovial giant cell tumor

Vascular tumors

Benign

Synovial hemangioma
Intramuscular hemangioma
Arteriovenous malformation/hemangioma
Venous hemangioma
Anastomosing hemangioma
Epithelioid hemangioma
Lymphangioma and lymphangiomatosis
Acquired tufted hemangioma

Intermediate (locally aggressive)

Kaposiform hemangioendothelioma
Retiform hemangioendothelioma
Papillary intralymphatic angioendothelioma
Composite hemangioendothelioma
Kaposi sarcoma
Pseudomyogenic hemangioendothelioma

Malignant

Epithelioid hemangioendothelioma
Angiosarcoma

Pericytic (perivascular) tumors

Benign and intermediate

Glomus tumor NOS
Myopericytoma, including myofibroma
Angioleiomyoma

Malignant

Glomus tumor, malignant

Smooth muscle tumors

Benign

Leiomyoma

Intermediate

Smooth muscle tumor of uncertain malignant potential
EBV-associated smooth muscle tumor

Malignant

Inflammatory leiomyosarcoma
Leiomyosarcoma

Skeletal muscle tumors

Benign	Malignant
Rhabdomyoma	Embryonal rhabdomyosarcoma Alveolar rhabdomyosarcoma Pleomorphic rhabdomyosarcoma Spindle cell / sclerosing rhabdomyosarcoma Ectomesenchymoma

Gastrointestinal stromal tumor

Benign	Malignant
MicroGIST	Gastrointestinal stromal tumors

Chondro-osseous tumors

Benign	Malignant
Chondroma	Osteosarcoma, extraskeletal

Peripheral nerve sheath tumors

Benign	Malignant
Schwannoma Neurofibroma Perineurioma Granular cell tumor Nerve sheath myxoma Solitary circumscribed neuroma Meningioma Hybrid nerve sheath tumor	Malignant peripheral nerve sheath tumor Melanotic malignant nerve sheath tumor Granular cell tumor, malignant Perineurioma, malignant

Tumors of uncertain differentiation	
Benign	**Malignant**
Myxoma (cellular myxoma) Deep (aggressive) angiomyxoma Pleomorphic hyalinising angiectatic tumor Phosphaturic mesenchymal tumor Perivascular epithelioid tumor, benign Angiomyolipoma	Phosphaturic mesenchymal tumor, malignant NTRK-rearranged spindle cell neoplasm (emerging) Synovial sarcoma Epithelioid sarcoma: proximal and classic variant Alveolar soft part sarcoma Clear cell sarcoma Extraskeletal myxoid chondrosarcoma Desmoplastic small round cell tumor Rhabdoid tumor Perivascular epithelioid tumor, malignant Intimal sarcoma Ossifying fibromyxoid tumor, malignant Myoepithelial carcinoma Undifferentiated sarcoma Spindle cell sarcoma, undifferentiated Pleomorphic sarcoma, undifferentiated Round cell sarcoma, undifferentiated
Intermediate (locally aggressive)	
Hemosiderotic fibrolipomatous tumor Angiomyolipoma, epithelioid	
Intermediate (rarely metastasizing)	
Atypical fibroxanthoma Angiomatoid fibrous histiocytoma Ossifying fibromyxoid tumor Myoepithelioma	

Undifferentiated small round cell sarcomas of bone and soft tissue
Ewing sarcoma
Round cell sarcoma with EWSR1-non-ETS fusions CIC-rearranged sarcomas Sarcoma with BCOR genetic alterations

Table 2 Classification of soft tissue sarcoma according to World Health Organization, 5th edition.

On the one hand, nomenclature and categories are based on morphology or histological details. Insofar it is evident, the common denominator for subgroups is the presumptive original cell that a sarcoma is derived from, as it is with liposarcoma (adipocytes), rhabdomyosarcoma (striated muscle cells), leiomyosarcoma (smooth muscle cells), angiosarcoma (endothelium), or malignant peripheral nerve sheath tumor (nerve sheath cells). However, not every subtype is clearly relatable to its original tissue. In these cases, subtypes are named after their histological patterns, like alveolar sarcoma, epithelioid sarcoma, or clear cell sarcoma.

On the other hand, molecular analysis has made evident biological correlation between entities. In the past, many sarcomas were classified as undifferentiated pleomorphic sarcoma (UPS) or malignant fibrous histiocytomas (MFH) because histopathological and immunohistochemical analysis did not allow further in-depth differentiation. Today, however, molecular diagnostics enable differentiation of histologically almost identical tumors into specific categories, wherefore the name "malignant fibrous histiocytoma" has become historical. The tendency in classification is clearly heading towards grouping subtypes according to genetic aspects regardless their morphological presentation. (90)

Regarding overall distribution, the most frequent STS in adults are undifferentiated pleomorphic sarcoma (12 to 33%) and liposarcoma (18%), followed by leiomyosarcoma (12%). (5, 91) In the following section, the most common morphological subtypes are reviewed regarding morphology, biological characteristics, prognosis, and treatment.

a) Liposarcoma

Malignant soft tissue tumors arising from adipocytes are summarized under the term liposarcoma and among the four main subgroups well-differentiated, dedifferentiated, myxoid and round cell, and pleomorphic liposarcoma there is a great difference not only in morphology, but also in prognosis and treatment.

(1) Well-differentiated liposarcoma

Well-differentiated liposarcomas typically occur on extremities, show no risk of metastasis, and can be cured by resection only. Hence, different attempts were made to change the name to "atypical lipomatous tumor" in order to avoid unnecessary anxiety in patients receiving the diagnosis of a "sarcoma". (92) Nevertheless, in comparison with benign lipomas, the risk of local recurrence is high and should not be underestimated. (93) MDM2 and CDK4 amplification can be detected as dual color break apart in FISH (cf. Figure 3) or as positive markers in immunohistochemistry (cf. Figure 4). (52)

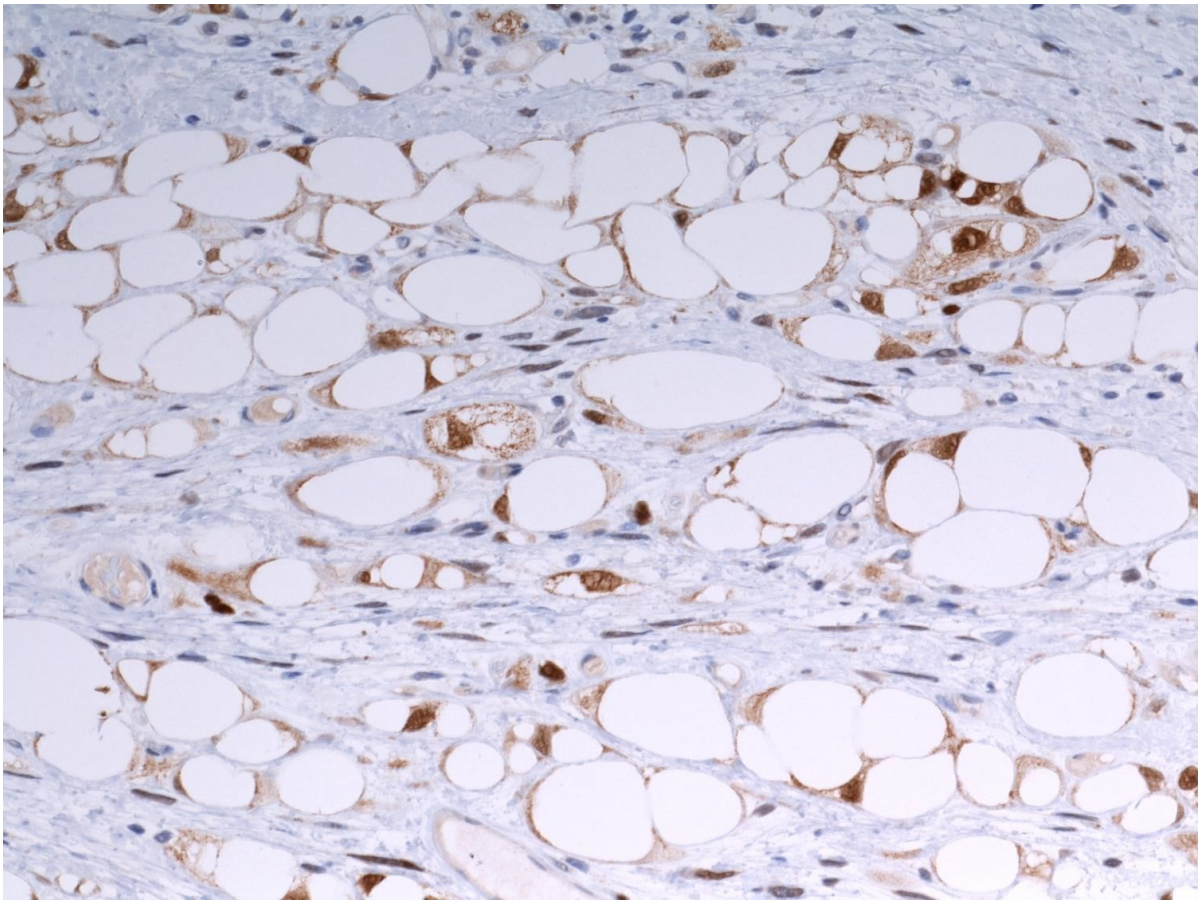

Figure 4 Well-differentiated liposarcoma with lipoblasts (lipoma-like subtype), positive for immunohistochemical stain with MDM2.

The picture was provided courtesy of Univ.-Prof. Priv.-Doz. Dr.med.univ. Bernadette Liegl-Atzwanger, Diagnostic and Research Institute of Pathology, Medical University of Graz, Austria.

(2) Dedifferentiated liposarcoma

Other than in extremities, a well-differentiated can turn into a dedifferentiated liposarcoma in retroperitoneum, mediastinum, and spermatic cord. Frequently, there are well-differentiated and dedifferentiated parts growing next to each other within one lesion. They are associated with a potential for metastasis and poorer prognosis compared to well-differentiated liposarcomas. Unusual for sarcomas, dedifferentiated liposarcomas metastasize to soft tissue and bones more frequently than to liver or lungs. (94) MDM2 and CDK4 are amplified as well and may be used for therapeutic interventions in the future. (95)

(3) Myxoid liposarcoma

Myxoid liposarcoma, the second most common liposarcoma, usually develop before the age of 40 years, often in children and adolescents. It is localized on the lower limb, mainly subfascial, and typically on the thigh. As slowly growing and indolent tumor, it is organized in lobules and on histopathological level dominated by myxoid stroma and a combination of round to oval shaped primitive mesenchymal cells and small, signet ring lipoblasts, preferably at the rim of the nodules (cf. Figure 5A). The intratumoral vessels remind of chicken-wires and are characteristic for myxoid liposarcoma (cf. Figure 5C). The mucus might accumulate to large pools with a pulmonary edema like appearance (cf. Figure 5B). In over 90%, the driver mutation for myxoid liposarcoma is the specific translocation t(12;16)(q13;p11) leading to a gene fusion of CHOP (C/EBP homologous protein; alternatively DNA Damage-Inducible Transcript 3 [DDIT3]) and FUS (fused in sarcoma; sometimes termed translocated in liposarcoma [TLS]). (96)

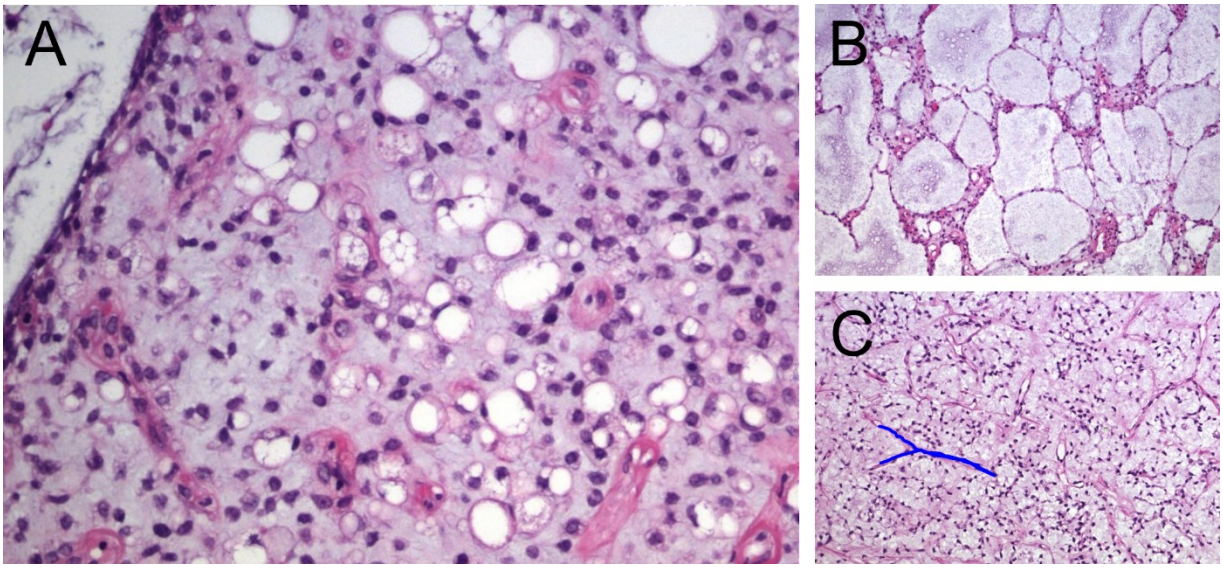

Figure 5 Myxoid liposarcoma.

(A) Small lipoblasts at the periphery of the tumor nodules. (B) Pulmonary edema like pattern caused by mucoid accumulation. (C) Prominent chicken-wire like vessels. The pictures were provided courtesy of Univ.-Prof. Priv.-Doz. Dr.med.univ. Bernadette Liegl-Atzwanger, Diagnostic and Research Institute of Pathology, Medical University of Graz, Austria.

(4) Pleomorphic liposarcoma

Pleomorphic liposarcomas are defined as high grad malignant lipoblasts without any characteristics for other types of liposarcomas and account for 5% of all liposarcomas. (97) As an aggressive neoplasm, it grows rapidly in deep soft tissue, usually of the trunk, and leads in 40 to 50% to death, predominantly due to metastases in the lungs. (98, 99) A mixture of pleomorphic spindle cells, round cells, multivacuolated lipoblasts, giant cells with multiple nuclei, and necrosis are typical. Like the heterogeneity in morphology, genetic alterations are various and complex, too, without any specific mutation. (94)

b) Fibrosarcoma

(1) Dermatofibrosarcoma protuberans

Listed as tumor entity in the WHO classification in 2013 for the first time, dermatofibrosarcoma protuberans (DFSP) shows typical features of intermediate dignity: it is locally aggressive with high local recurrence rates (mostly if not resected entirely), but metastases are rare. Primarily, trunk and extremities are affected in young adults. DFSP grows slowly and appears with red-bluish coloration and telangiectasises. (100) Previous plaques for several years on the place of tumor development are described. (101) Histopathology displays storiform arrangements and whorl patterns of homogenous slender spindle cells, infiltrating dermis and subcutis. The characteristic honeycomb appearance is a mixture of subcutaneous adipocytes and malignant spindle cells. However, necrosis, mitotic activity, and nuclear pleomorphisms are not common. CD34 is highly positive in immunohistochemistry. (52) The most frequent genetic alteration is a chromosomal translocation t(17;22)(q22;q13) and leads to an upregulated expression of platelet-derived growth factor β (PDGFB) under the control of collagen type 1A1 (COL1A1). Thus, DFSP can be treated with the receptor tyrosine kinase inhibitor imatinib. (102)

Distinction to giant cell fibroblastoma is difficult since both histological patterns can be found within one lesion and share the same immunohistochemical markers and cytogenetic mutations. Hence, classification of giant cell fibroblastoma as subtype of DFSP is discussed. (103, 9)

(2) Adult fibrosarcoma

Adult fibrosarcoma is characterized by malignant fibroblasts, collagen production, and herringbone architecture, but is mainly a diagnosis of exclusion and is not necessarily restricted to adults. Although the histology is similar to infantile fibrosarcoma, the genetic profile differs and metastatic potential in adult fibrosarcoma is higher with an overall survival of less than 50%. (104) It grows in deep soft tissue of extremities, trunk, neck, and head and is composed of relatively monomorphic spindle-cells in sweeping fascicles. No specific underlying genetic alteration has been found so far. (105)

(3) Sclerosing epithelioid fibrosarcoma (SFS)

In comparison to adult fibrosarcoma, the usual sites of involvement are the same, but the malignant epithelioid tumor cells in sclerosing epithelioid fibrosarcoma (SFS) are not arranged in herringbone pattern, but in nests and chords. It is a very rare entity and genetic relation to low-grade fibromyxoid sarcoma (LGFMS) is discussed. (106) Further investigations have shown that the overlap with LGFMS is compatible with MUC4 expression. FUS gene rearrangement in SFS might be used as sensitive markers for this STS subtype as well. (107) Biological behavior appears in SFS in a great variety and metastases to lungs are not uncommon.

(4) Myxofibrosarcoma

Among STS, myxofibrosarcoma represents about 5.6% of all cases and occurs mainly at the age of sixty to eighty years. (108, 5) Limbs and limb girdles of the lower extremities are the main sites of involvement, whereas an origin in trunk, head, neck, hands, and feet is rare. The lesion grows mostly on dermal or subcutaneous level. Histopathology shows the combination of malignant fibroblastic cells with myxoid stroma and multinodular growth divided by incomplete fibrous septa. Elongated, curvilinear vessels are typical for myxofibrosarcoma (cf. Figure 6). Highly complex cytogenetic aberrations with triploid and tetraploid karyotypes have been described; chromosome numbers vary within the tumor. (109)

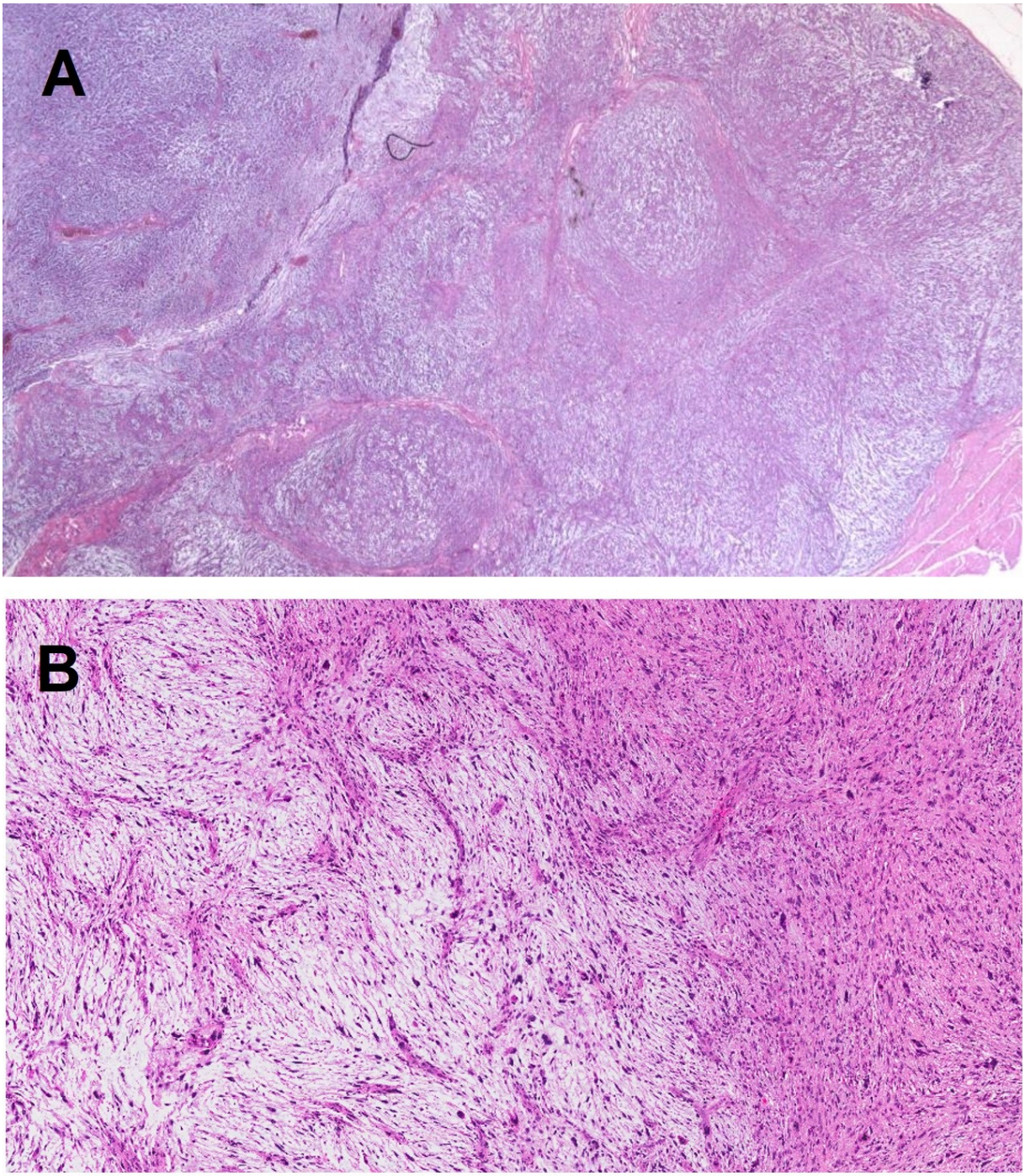

Figure 6 Morphological findings of myxofibrosarcoma.
(A) Multinodular growth with incomplete fibrous septa and myxoid stroma. (B) Myxoid stroma with curvilinear vessels on the left; fascicular growth pattern without myxoid stroma on the right side of the image. The pictures were provided courtesy of Univ.-Prof. Priv.-Doz. Dr.med.univ. Bernadette Liegl-Atzwanger, Diagnostic and Research Institute of Pathology, Medical University of Graz, Austria.

c) Leiomyosarcoma

Leiomyosarcomas are sarcomas with smooth muscle differentiation. They can occur at any place of the body originating from vessels but not necessarily. Leiomyosarcomas are divided into uterine and nonuterine leiomyosarcoma, for the genetical pattern differ decisively between those two subgroups. Regarding biological behavior, cutaneous leiomyosarcomas tend to be less painful and have a lower metastatic potentiality compared to the subcutaneous and deep-seated subset. (110, 111) The diagnosis is based on positive smooth muscle markers like α-smooth muscle actin (SMA) and desmin. (52)

d) Rhabdomyosarcoma

(1) Embryonal rhabdomyosarcoma

As the most common soft tissue sarcoma among children, embryonal rhabdomyosarcoma typically occurs on head and neck or the genitourinary system in children under the age of ten years. Generally, heterogeneous rhabdomyoblasts with eosinophilic cytoplasm in diverse stages with compact and mucoid areas are characteristic. Stellate cells with a central, oval nucleus in the amphophilic cytoplasm can be found as well as “spider cells” with elongated shape and increasing cytoplasmatic eosinophilia as they progress. (94)

The three main types are spindle cell, botryoid, and anaplastic variants. Especially the botryoid type is worth mentioning since it grows in varying nodules and shows linear arrangement of tumor cells tight to mucosa. It affects mostly the urinary bladder, biliary tract, pharynx, auditory canal, and conjunctiva, and causes often symptoms of obstruction due to the rapid growth in those hallow organs. Depending on differentiation level, desmin, actin, myoglobin, myosin, and creatine kinase M are expressed immunohistochemical markers and antibodies against MyoD1 and myogenin have been shown to be useful for the diagnosis of rhabdomyosarcoma. (112) No specific genetic mutations have been detected; molecular analyses reveal consistently complex structural or numerical chromosomal changes. (94)

(2) Alveolar rhabdomyosarcoma

Alveolar rhabdomyosarcoma show small round cell morphology with skeletal muscle differentiation (cf. Figure 7). The tumor is rapidly growing and compared to embryonal rhabdomyosarcoma, it is more aggressive, arises more frequently in adolescents and young adults and there is no preferred site of involvement. The two main histopathological subtypes are tumor nests and fibrovascular septa surrounded by tumor cells in picket fence pattern on the one side, and solid pattern without fibrovascular stroma on the other side. Two translocations have been described as specific cytogenetic mutation in alveolar rhabdomyosarcoma, leading to fusion proteins PAX7/FKHR and PAX3/FKHR. (113, 114) Additionally, gene amplification – for example of CDK4, MDM2, and GLI – is common.

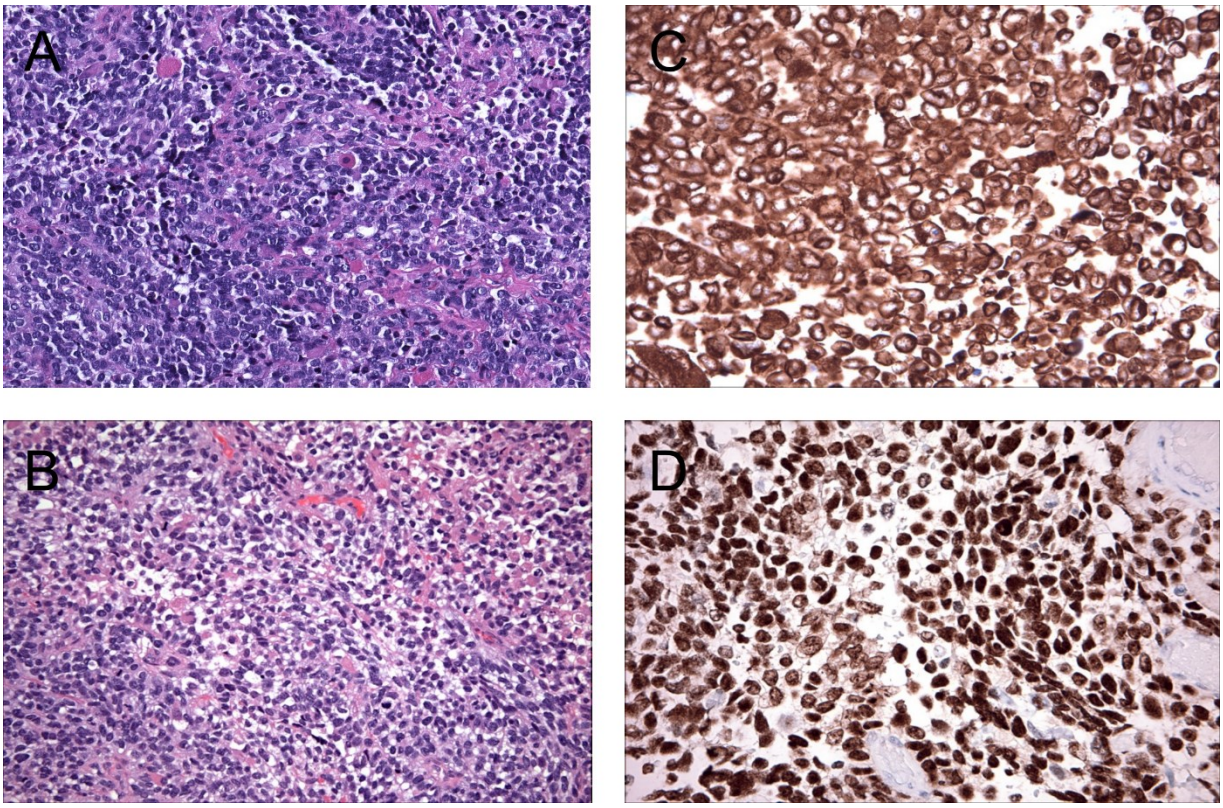

Figure 7 Morphological and immunohistochemical features of alveolar rhabdomyosarcoma.
(A) Small round blue cell morphology with individual rhabdomyoblasts. Hematoxylin and eosin stain. (B) Within the tumor nests, clear cell changes can occur and correspond to lipid or glycogen vacuoles in electron microscopy. (115) (C) Immunohistochemistry is positive for desmin (D) and MyoD1 antibodies. The pictures were provided courtesy of Univ.-Prof. Priv.-Doz. Dr.med.univ. Bernadette Liegl-Atzwanger, Diagnostic and Research Institute of Pathology, Medical University of Graz, Austria.

e) Angiosarcoma of soft tissue

Angiosarcomas of soft tissue are malignant mesenchymal tumors with endothelial differentiation. They are known to be highly aggressive with poor prognosis. Soft tissue angiosarcomas occur in the deep soft tissue of the extremities as well as in the head and neck area. The seventh decade marks a peak in the incidence distribution. The vascular tumor can cause anemia, coagulopathy, and persistent hematoma and may lead to cardiac output failure due to arteriovenous fistula. Angiosarcomas of soft tissue are associated with therapeutic radiation (especially in women after radiotherapy due to breast cancer), chronic lymphoedema, neurofibromatosis type I, synthetic vascular grafts, and rarely with benign hemangiomas in Klippel-Trenaunay or Maffacci syndrome. (116) Multinodular hemorrhages and rudimentary, infiltrating vascular malformations are distinctive and both, spindle and epithelioid cells, occur on light microscopy. Mitotic activity is usually high. Vascular antigens like CD31, CD34, and von Willebrand factor are specific in immunohistochemistry and essential for diagnosis. (94) Overall, the prognosis of angiosarcomas is poorer than for other STS subtypes.

f) Malignant peripheral nerve sheath tumor

Malignant peripheral nerve sheath tumor (MPNST) represents a special group of STS because of its neural origin from ectodermal layer and occurs to 50% in adolescents with neurofibromatosis type I. It is the result of malignant transformation of plexiform neurofibromas and, thus, arises from peripheral nerves accompanied with pain, preferably in the trunk, but also in head, neck, and extremities. A whorled pattern with epithelioid perivascular tumor cells and malignant features of Schwann cells are common histopathological signs. Protein S100 as histochemical marker of nerves is often expressed but can be lost in the process of dedifferentiation. (94) MPNST is highly aggressive; regular imaging to detect malignant transformation in patients with neurofibromatosis I is strongly recommended. (117)

g) Tumors of uncertain differentiation

(1) Synovial sarcoma

The term synovial sarcoma arises from the morphological appearance and describes a distinct entity of STS. Neither does it derive from synovium, nor has there any other (epithelial) origin been described. It can grow at any site of the body, mainly in deep soft tissue of upper and lower limbs. (118) The majority of patients with synovial carcinoma are young adults between the age of 15 to 35 years and in total it represents 5-10% of all STS. (119) Malignant spindle cells alone (monophasic type) or together with epithelial components, often forming glandular structures similar to adenocarcinoma (biphasic type, cf. Figure 8), are the main characteristics. In over 90%, the chromosomal translocation t(X;18)(p11;q11) causing SS18/SSX protein fusion can be found. (6) As slowly growing tumor, calcifications can develop in the lesion and may become apparent in radiological imaging. (94)

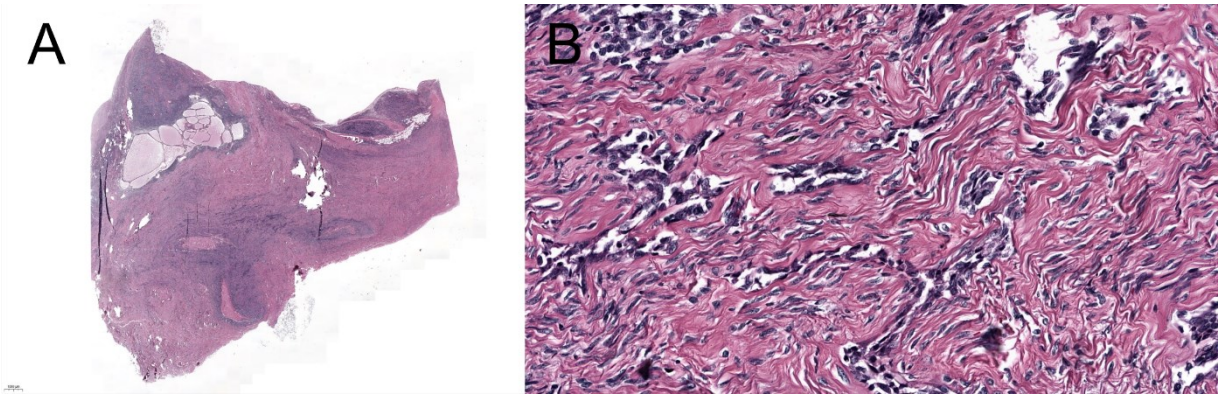

Figure 8 Morphological appearance of biphasic synovial sarcoma.
(A) The two components – eptithelial and spindle cells – are noticeable in the overview and can be confirmed in the higher magnification (B). The pictures were provided courtesy of Univ.-Prof. Priv.-Doz. Dr.med.univ. Bernadette Liegl-Atzwanger, Diagnostic and Research Institute of Pathology, Medical University of Graz, Austria.

(2) Clear cell sarcoma

Clear cell sarcoma of soft tissue is a rare entity among STS and affects mainly tendons and aponeuroses of the extremities in young adults. The tumor displays spindle and polygonal shaped cells with eosinophilic or clear cytoplasm and prominent nucleolus and is arranged in nests and fascicles, divided by fibrous septa. Melanin stains and consistent positivity for S100 protein and HMB45 reveal the melanocytic differentiation of these tumor cells. The reciprocal

translocation t(12;22)(q13;q12) causes a fusion of EWS and ATF1 gene and is specific for clear cell sarcoma. (120) Despite the slow growth, mortality is high (44-54%) and metastases have been detected in up to half of all patients, mostly in lungs and bones. (121–123)

(3) Epithelioid sarcoma

Malignant eosinophilic epithelioid and spindle cells forming a nodular lesion, defines a distinctive entity among STS with unknown origin, named epithelioid sarcoma. It affects young adults, especially men. The site of involvement is relatively specific on the surface of flexors of the fingers, hand, wrist, and forearm; knees and ankles are also commonly involved. An association with trauma is suspected. Commonly found central necrosis and chronic inflammation imitate histologically benign lesions and may be misdiagnosed as rheumatoid nodules or granuloma annulare. Cytokeratin and epithelial membrane antigen (EMA) are useful immunohistochemical markers in diagnosis. The rapid growth along tendons, fascial planes, and nerve sheaths reflects the aggressive behavior of epithelioid sarcoma with a high rate of recurrence. (94)

(4) Alveolar soft part sarcoma (ASPS)

Alveolar soft part sarcoma (ASPS) is a rare, slowly growing STS, frequently asymptomatic and undiscovered until the early metastatic spread in lung, bone and brain. (124, 125) Depending on the age, the site of involvement differs: In adults, the tumor arises mainly in the deep soft tissue of the tight, whereas tongue and orbit are most commonly affected in infants and children. Hemorrhage and necrosis in the tumor mass are frequent and large, homogenous epithelioid cells with eosinophilic cytoplasm are the histopathological characteristics of ASPS. The tumor cells are arranged in nests and divided by sinusoidal vascular channels, imitating alveolar tissue. The Robertsonian translocation der(17)t(X;18) resulting in ASPL/TFE3 fusion protein accounts for almost all cases of ASPS, but has been identified in a small subtype of renal adenocarcinomas as well, as mentioned above. (126, 58)

(5) Undifferentiated pleomorphic sarcoma (UPS)

The term undifferentiated pleomorphic sarcoma (UPS) refers to the subset of lacking a specific line of differentiaton. It is defined as pleomorphic STS with no determinable line of

differentiation by current technology. Hence, it is a diagnosis of exclusion and related to various (historic) synonyms (malignant fibrous histiocytoma [MFH], pleomorphic malignant fibrous histiocytoma, malignant fibrous xanthoma, fibroxanthosarcoma, sarcoma not otherwise specified). Typically, UPS affects patients over the age of 40 years and grows subfascial on the extremities, especially on the lower limb. By its definition, histological appearance of the tumor is heterogenous and the only common features are a storiform growth pattern and signs of inflammation, as well as general sings of malignancy, such as cytological and nuclear pleomorphism. Aberrant giant cells, spindle cells, and histiocyte-like cells with round shape and foamy cytoplasm are present in the majority of UPS (cf. Figure 9). Any major line of origin should be excluded by common immunohistochemical markers for the diagnosis of UPS. Genetical findings usually show an extremely complex, triploid or tetraploid, karyotype but also haploid chromosome numbers are common. (94)

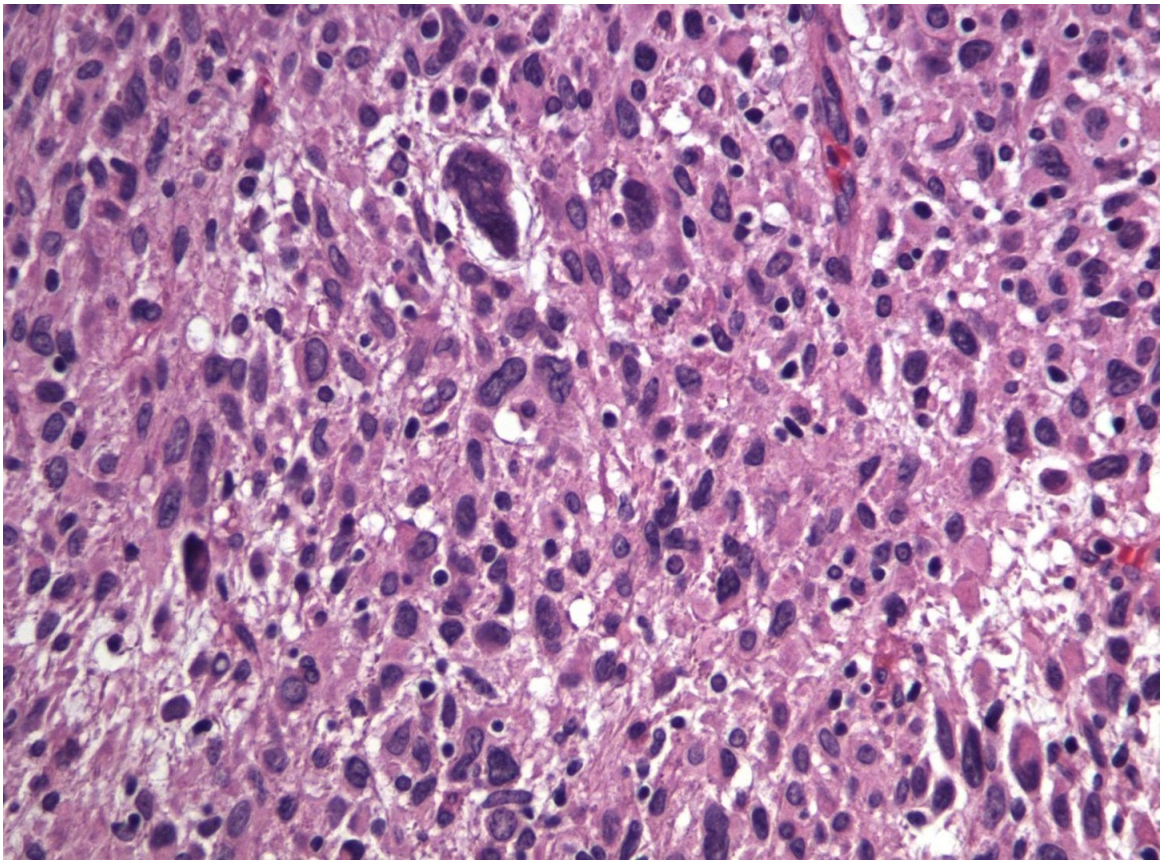

Figure 9 Undifferentiated pleomorphic sarcoma stained with hematoxilin and eosin showing atypical, heterogenous cells. The picture was provided courtesy of Univ.-Prof. Priv.-Doz. Dr.med.univ. Bernadette Liegl-Atzwanger, Diagnostic and Research Institute of Pathology, Medical University of Graz, Austria.

2 Treatment

Generally, complete surgical resection of the tumor mass is the treatment of choice in all STS. Radiation therapy (RTX) and chemotherapy (CTX), however, may help to decrease the tumor size, reduce the risk of recurrence, or control symptoms in palliative settings. In selecting the optimal treatment for the individual patient, all the options, limitations, and patient related factors are usually discussed in multidisciplinary team (MDT). (1) The three main factors in determining the therapy are (a) current stage of disease, (b) possibility of a surgery, and (c) patient's conditions and will.

a) Surgery

Owing to the continuous improvement in surgical methods and reconstruction of large soft tissue or bone defect, limb-sparing procedures with functional preservation of extremities have broadly replaced amputation over the last three decades. (127) Nevertheless, sometimes resection of functionally important structures is necessary in minimalizing the risk of recurrence, depending on the infiltration of nerves, vessels, or bones. (32) The surgical staging system for musculoskeletal sarcoma categorizes the surgical approach into the following four procedures: (128)

1. Intralesional surgery: The tumor's capsule is being opened during surgery with the intention to preserve essential structures despite the high risk of recurrence.
2. Marginal surgery: The resection margin is planned close to the tumor capsule.
3. Wide surgery: A safety margin to the tumor, mostly muscle tissue or fascia, is maintained.
4. Radical surgery: The entire anatomical compartment affected by the STS is removed en bloc.

The complete resection of the malignant tumor with a microscopic clear margin (R0) is one of the most important factors for the risk of local recurrence. (129–132) Hence, the surgical approach ought to be planned carefully regarding the optimal balance between a minimal risk of recurrence and maximally preserved functionality. However, regarding certain STS subtypes

with low risk of metastasis, such as well-differentiated liposarcoma, a marginal resection is justified, even if local recurrence is more likely. (1, 127, 133)

(1) Unplanned excisions

As a result of the low incidence, physicians are often not aware of the possibility of STS regarding a soft tissue lump and tend to excise the tumor mass like a benign lesion with sometimes disastrous consequences. An unplanned excision – also called "whoops" procedure – represents a challenging situation because of missing adequate presurgical imaging, unnecessarily contaminated healthy tissue, and inappropriate surgical interventions leading to extensive re-resection, amputation, and poorer outcome. (134, 135)

(2) Isolated hyperthermic limb perfusion (ILP)

The concept of vascular obliteration of tumor vessels has become an important neoadjuvant option in patients where limb-sparing resection is initially not feasible. After connecting the heart-lung-machine to the main blood vessels of the limb and warming it up to the level of 38-40°C for a higher effect, tumor necrosis factor-alpha (TNF-α) is injected together with melphalan to obliterate the capillaries of the STS. Ninety minutes later, the injected substances are washed out with crystalloids or colloids. (136)

ILP may be applied to both, the primary tumor as well as to the local recurrence, and the definitive surgical treatment can be arranged six to ten weeks afterwards. In 80%, ILP improves the surgical setting and enables a resection with (partially) preserved functionality of the extremity instead of amputation. (137) The effectiveness of this method depends mainly on the vascularity of the tumor and is especially potent in liposarcoma. (138)

b) Radiation therapy (RTX)

Radiation is applied at various stages of the tumor disease: In neo-adjuvant settings, it might reduce tumor size and the risk of micro-metastasis; intraoperatively and perioperatively (brachytherapy), RTX can be performed to achieve higher local control, but it is most commonly used as adjuvant therapy after surgical excision. (139, 140) Finally, in patients with palliative

treatment and severe medical condition due to the local expansion of the STS, RTX may improve the symptoms. (141)

Generally, postoperative RTX of the surgical region is indicated in STS with high risk factors for local recurrence such as high grade (G2, G3), tumor size over 5cm, and deep localization, but irradiation can be administered – depending on the MDT's decision – in other cases as well. (1, 142)

Adjuvant, external beam radiation is carried out in fractions of 1.8 to 2 Gray (Gy) up to the total amount of 50 Gy, in the majority of cases. (1) Control of local recurrence is generally equally effective in pre- and postoperative application. (143) However, neoadjuvant RTX potentially increases the risk of postoperative complications as wound healing deficits and infections, due to irradiation damage caused to skin and subcutaneous tissue.

c) Chemotherapy (CTX)

Although the benefit of general treatment with chemotherapeutics in patients with STS is doubtful, a selected subtype might profit from CTX. Due to the variety and relatively low number of STS, no standardized chemotherapeutic strategy has been established so far and an individual approach depending on staging, localization and surgical options, histological subtype, patient's conditions, and the multidisciplinary board's experience is necessary. (144) A trend away from histological type tailored CTX towards the use of CTX in patients with high-risk STS in general is noticeable in neo-adjuvant and adjuvant settings likewise. (145–147)

The major limiting effects of the current chemotherapeutical drugs are severe nausea and vomiting, cardiotoxicity (reduced in pegylated liposomal doxorubicin), mucositis, myelotoxicity, as well as skin toxicity, and fatigue. (148) Thus, the decision to perform a CTX – whether with or without RTX – should always consider the patient's individual physical condition including renal function.

(1) Neoadjuvant chemotherapy

Neoadjuvant CTX is primarily used to decrease risk of recurrence and tumor size aiming for a less radical resection and maintained functionality, and thus, to improve overall survival (OS).

(149) Studies with neoadjuvant CTX with anthracyclines (e.g. epirubicin) plus ifosfamide (AI-scheme) for minimum three cycles have shown an advantage regarding recurrence free survival and OS in patients with high-risk STS, i.e. high grade, deep localization, and tumor size exceeding 5cm – independently of histological subtype. (146, 150) Thus, neoadjuvant CTX should be discussed individually with high-risk patients in the process of shared decision making. (1)

There is evidence for the effectivity of neo-adjuvant simultaneous or sequential combination of regional CTX and RTX, especially with doxorubicin, resulting in a higher rate of limb-sparing resection and OS, and lowering the risk of local recurrence. (151, 152) However, this combination comes along with a higher rate of toxicity and different efforts were made to reduce severe side effects of the therapy, but the method has never prevailed broadly. (153, 154)

(2) Adjuvant chemotherapy

Since two meta-analyses of adjuvant CTX with doxorubicin in STS yield controversial results, (155, 156) there is no general recommendation for adjuvant CTX, but the AI-scheme should be proposed to high-risk patients. (1)

(3) Palliative systemic treatment

Patients with advanced, metastatic, progressive, or unresectable STS might benefit from CTX, even if they have been treated already with anthracyclines first line. The approved drugs in Austria for palliative settings are ifosfamid, trabectedin, doxorubicin, eribulin, and pazopanib. In particular, trabectedin showed favorable effects in patients with advanced, previously treated liposarcoma and leiomyosarcoma. (157, 158) Eribulin, too, is approved for pre-treated liposarcoma based on a randomized phase III trial with prolonged OS compared to dacarbazine. (1, 159) Regarding non-lipomatous STS, pazopanib has been reported to be beneficial in palliative patients. (1, 160)

Targeted therapies with less adverse effects are slowly gaining importance in treating STS, such as pazopanib (multitargeted tyrosine kinase inhibitor) in patients with metastasized non-lipomatous sarcoma. (160, 161) However, we know little about the possibilities and efficacy of targeted therapies until now, and further investigations are needed.

Response evaluation remains a challenging question regarding both, standard CTX and even more in targeted therapeutics. The Response Evaluation Criteria In Solid Tumors (RECIST) are probably the most widely used guideline for tumor change assessment and are mainly based on the measurement of tumor size and lymph node diameter, applicable in both, in CT scan as well as in MRI. (162) However, targeted therapy might lead to tumor infiltration with immune cells resulting in a progression of tumor size, although there is a therapeutic response. Hence, tumor size alone can be misleading and the use of the Choi criteria with additional focus on tumor density measured in Hounsfield Units (HU) has been established in response evaluation after targeted therapies in different tumor entities. (163) Both assessment tools can be applied in neoadjuvant and palliative settings. Recent research has focused on detecting tumor specific protein transcripts or genomic particles in patient's blood in order to estimate treatment response or to identify recurrent disease at an early stage in follow-up. (164–167)

d) Management algorithms according to the ESMO guidelines

(1) Localized, clinically resectable STS

Grade 1 STS show the lowest risk of metastasis and if curative resection (R0 – no cancer cells can be microscopically detected at the tumor site) is possible, only follow-up and no further treatment is required in case of superficial lesions smaller than 5cm and low risk assessment by the multidisciplinary team (MDT). On the contrary, if tumor cells reach the resection margin (R1) and no further resection is feasible, RTX is recommended. In patients with STS grade 2 or 3 with deep localization, tumor size over 5cm or at specific sites, a neo-adjuvant CTX or RTX should be discussed in the MDT. Alternatively, in case of grade 2 or 3 STS, an adjuvant chemotherapy and radiotherapy should be considered in high-risk patients and site-specific situations. If after an R1 resection of a grade 2/3 tumor a curative resection (R0) is not feasible, an adjuvant RTX is recommended (cf. Figure 10). (1)

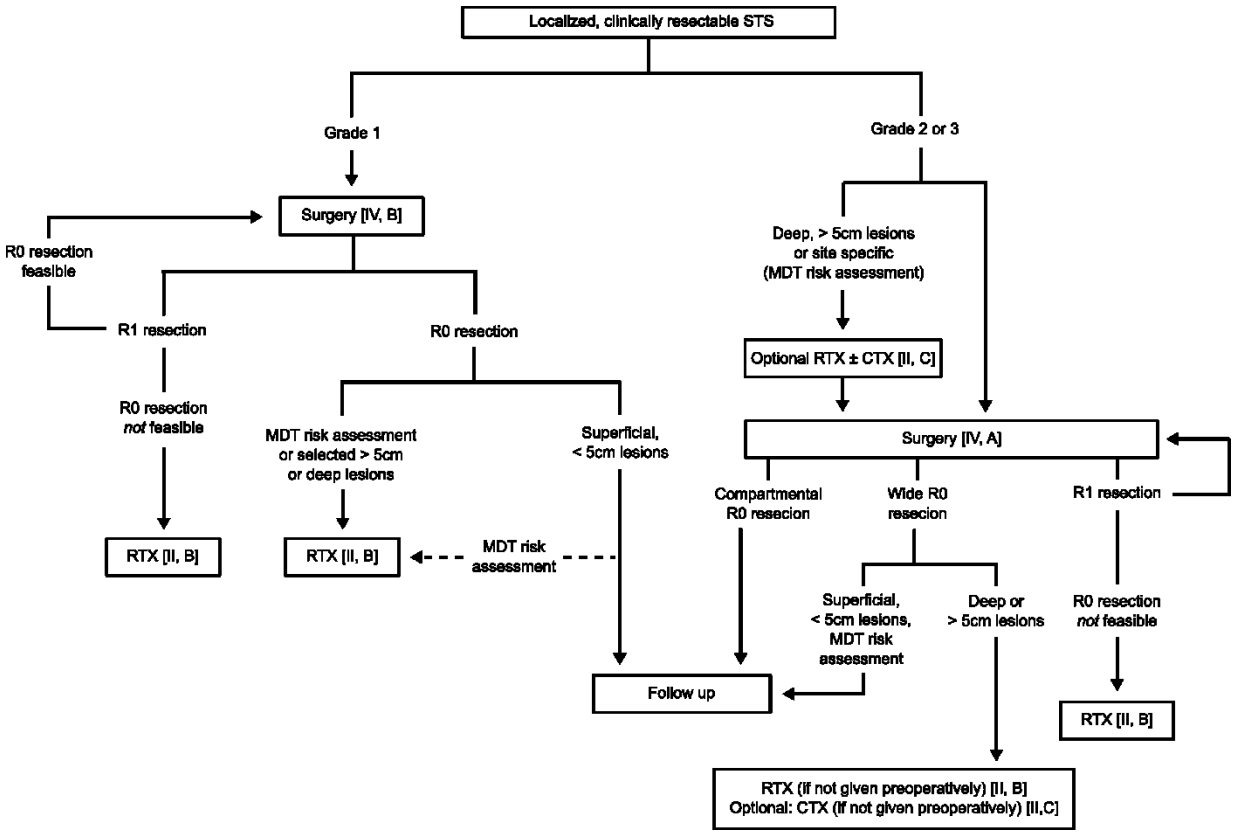

Figure 10 Management of localized, clinically resectable soft tissue sarcoma.
Adapted from Casali PG et al. (1) CTX, chemotherapy. MDT, multidisciplinary team. R0, negative margins. R1, marginal margins. RTX, radiation therapy. Level of evidence in squared brackets.

(2) Advanced or metastatic, clinically resectable STS

Isolated pulmonary metastases are seen to be the only metastatic setting where surgical resection should be considered, if R0 excision is possible. Both, a preoperative or postoperative chemotherapy might be recommended in high-risk STS (Figure 11). Extrapulmonary metastasis and not feasible R0 resections should be treated like clinically unresectable advanced or metastatic disease (cf. Figure 12). (1)

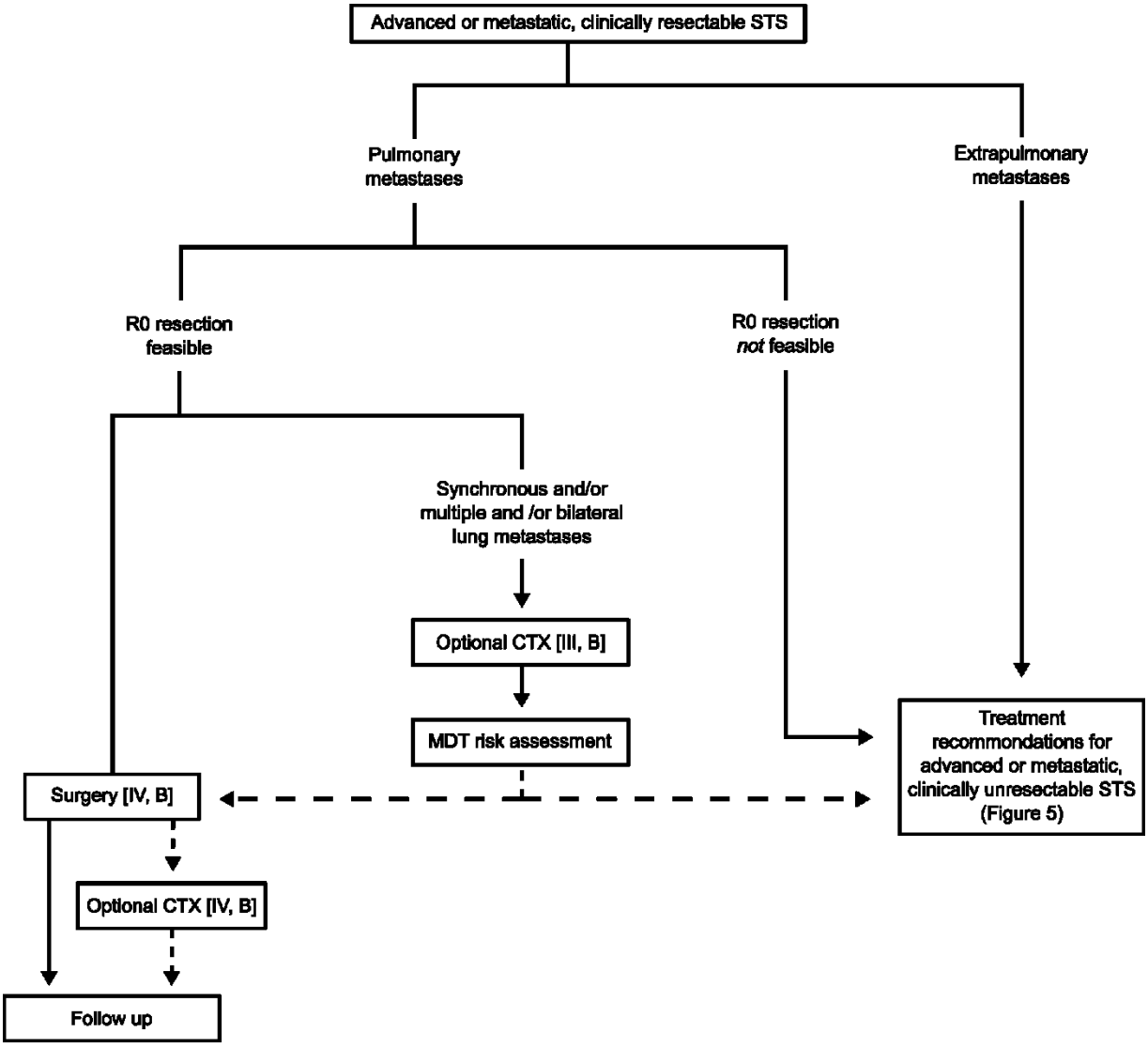

Figure 11 Management of advanced or metastatic, clinically resectable soft tissue sarcoma.
Adapted from Casali PG et al. (1) CTX, chemotherapy. MTD, multidisciplinary team. R0, negative margins. R1, marginal margins. Level of evidence in squared brackets.

(3) Clinically unresectable STS

If the patient's conditions or the anatomical localization of the STS does not allow a surgical resection, an attempt of tumor size reduction via radiotherapy and chemotherapy should be performed. Neo-adjuvant CTX consists of doxorubicin plus ifosfamid. In case of progressive disease despite neo-adjuvant therapy, a second-line histology-driven therapy should be considered in palliative settings. (1)

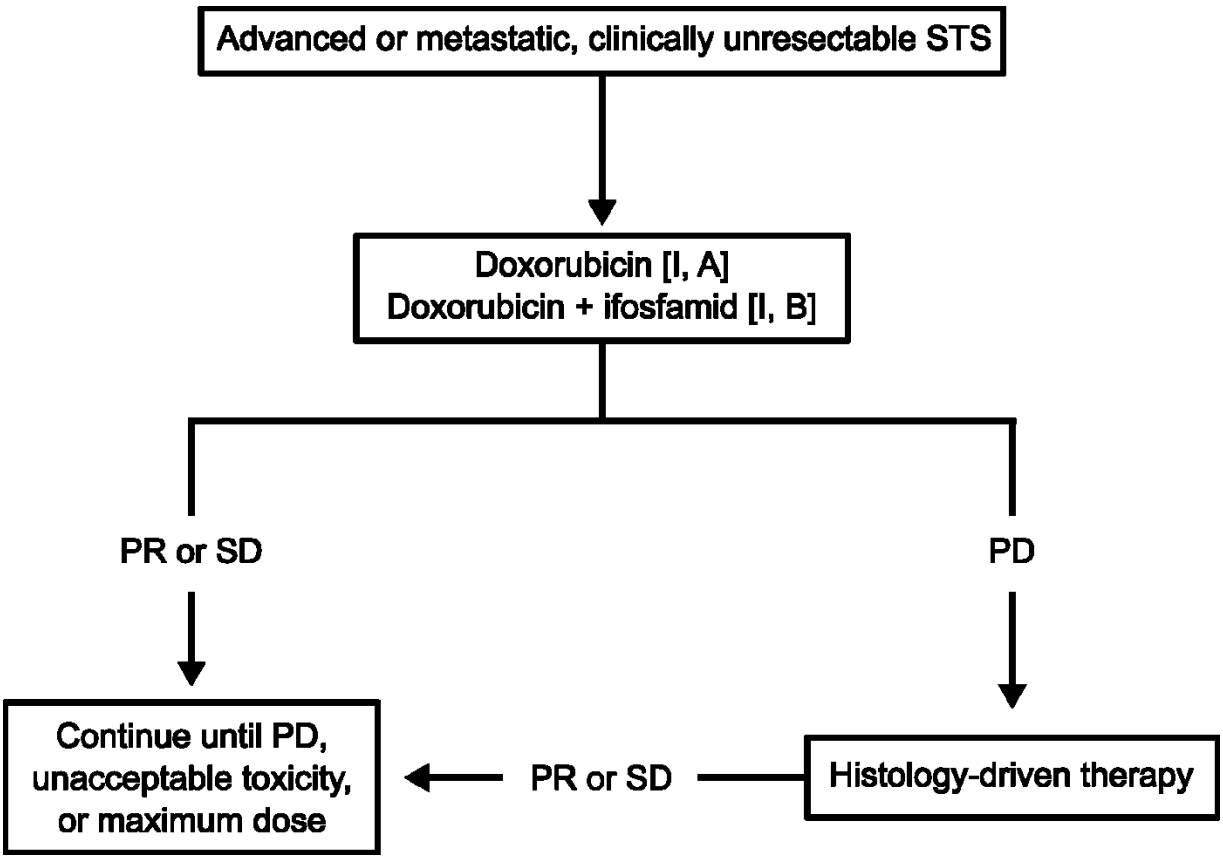

Figure 12 Management of advanced or metastatic, clinically unresectable soft tissue sarcoma.
Adapted from Casali PG et al. (1) The therapeutic option with doxorubicin plus olaratumab has been ruled out due to the negative phase III trial. (170) PD, progressive disease. PR, partial remission. SD, stable disease. Level of evidence in squared brackets.

In case of advanced disease without chances for curative resection (Figure 12), doxorubicin as single-agent is the first choice in metastatic setting, particularly for the intention of pulmonary disease control and life prolongation, but may be combined with ifosfamide, if the rapid tumor growth induces severe symptoms. (1, 148) A double-blind, randomized phase III trial could not confirm a significant benefit of doxorubicin plus olaratumab compared to doxorubicin plus placebo in patients with advanced or metastasized STS. (168) Hence, olaratumab was withdrawn as therapeutic agent in advanced STS.

3 Prognosis and follow-up

The most important determinants for prognostic assessment are tumor size, grade, and stage, but also the histological subtype, anatomic site, surgical margins, and patient's age are

independent factors for estimating the patient outcome. (95, 88) Since prognosis is not only relevant for patients themselves, but also regarding treatment modalities, validated prognostic nomograms like the “Sarcoma-Specific Death After Surgery” nomogram from the Memorial Sloan Kettering Cancer Center (MSKCC) and digital applications such as Sarculator and Personalised Sarcoma Care (PERSARC) support the optimal patient management and outcome evaluation. (169–171) Even more, there are first attempts to objectify biological tumor behavior for prognosis via molecular findings, like detecting microRNA as product of fusion protein PAX3-FOXO1 in alveolar rhabdomyosarcoma. (172)

Generally, distant metastases arise in up to 60% of patients with high grade STS, whereas G1 STS metastasizes in 5-10% only. Local recurrences develop in 12 to 26% within five years and require aggressive treatment approaches with re-resection and additional RTX. (173) Atypical lipomatous tumor has one of the highest five-year OS (95%), as opposed to alveolar rhabdomyosarcoma (26%) and desmoplastic round cell tumor (11%) with the poorest prognosis among the STS subtypes. (5) The average two-year survival rate for patients with metastatic disease amount about 39%. (174)

The most common site of metastases is the lung and isolated pulmonary metastases are usually asymptomatic but can be treated. Therefore, a frequent follow-up with pulmonary screening is reasonable, especially within the first two years, where most of the recurrences arise. (175) In patients with stage I disease, the National Comprehensive Cancer Network (NCCN) suggests a history and clinical examination every three to six months for the first three years (then every year once) together with chest imaging every six to twelve months. Patients in stage II and III should be seen for clinical evaluation and chest imaging every three to six months in the first two to three years after treatment, then twice a year for the next two years, and then annually. In both groups, a regular MRI or CT of the primary site, depending on the risk for local recurrence, is recommended. (83)

The clinical data for an ideal follow-up regiment in general is poor, but newest research is aiming for personalized follow-up according to different risk assessments, such as the flexible parametric competing risk regression models (FPCRRMs) by estimating the risk for local and distant recurrence. (176)

3) The importance of tumor microenvironment

The development of a malignant neoplasm happens through specific pathophysiological steps like the evasion of apoptosis, the resistance to anti-growth signals and the self-sufficiency in growth-signals resulting in an unlimited potential of replication, the invasion of surrounding tissue, or the induction of angiogenesis. In large part, these hallmarks of malignancy are closely dependent on the tumor microenvironment (TME). (177) Thus, tumor research has been focusing on different cells, chemical factors and tissue surrounding the tumor already for a long time, but since the great success of immunotherapy in certain tumor entities like malignant melanoma or renal cell carcinoma, particularly tumor-infiltrating immune cells (TIIC) have gained major significance.

In general, tumor-infiltrating regulatory T-cells, T_{H17}-cells, granulocytes, mast cells, immature dendritic cells, myeloid-derived suppressor cells, and M2-type macrophages are associated with unfavorable patient outcome as opposed to cytotoxic T-cells, B-cells, NK-cells, mature dendritic cells as well as M1-type macrophages with positive correlation regarding prognosis. (178) Nevertheless, not the single count of certain immune cell types is distinctive alone; in fact, the combination of different cell lineages is essential regarding tumor behavior and prognosis. For example, in lung adenocarcinoma a mutual abundance of T-cells and B-cells was found to be a favorable prognostic marker, presumably by representing a tertiary lymphoid structure within the tumor. (179, 180) Among the immune cells, the most relevant ones regarding tumor evolution seem to be lymphocytes and macrophages.

Although there are different methodologies to analyze cellular components within the TME on histopathological samples, two different methods are largely used nowadays:
(a) Immunohistochemistry and immunofluorescence, on the one hand, mark cells on the basis of defined markers with the advantage of incorporating localization and tissue structure into the assessment. In automatic evaluation, tissue microarrays have prevailed as adequate method for TIIC recognition (181). (b) On the other hand, transcriptomic analysis can detect a large number of surface molecules, cytokines, or other markers at once by genetic sequencing, mainly of RNA. (178)

1 T-cells

T-cells represent a major part of the adaptive immune system and originate in the bone marrow. However, maturation to naïve T-cells takes place in the thymus. Once T-cell receptors are created by random genetic recombination and are proven to be both, specific against a certain antigen and tolerant to the body's own cells, the lymphocytes emigrate through the blood to secondary lymphoid organs such as the spleen, lymph nodes, or the mucosa of the gastrointestinal tract and start proliferating as soon as a T-lymphocyte binds to its specific antigen. T-cells are categorized according to their cluster of differentiation (CD) surface molecules. T-cells that developed CD3 and CD4 surface proteins belong to the subgroup of helper T- cells (T_H-cells) and support B-cells and immune cells of the innate immune system in the defense against intracellular pathogens (T_{H1}) and extracellular parasites (T_{H2}). CD3 and CD8 are markers for cytotoxic T-cells, responsible for the apoptosis of the body's own cells that are either infected by a virus or have been mutated to a tumor. Consequently, a high density of cytotoxic T-cells or helper T-cells in the TME is associated with better prognosis in different tumor entities. (182) In high-grade STS, infiltration of TME with CD3+ and CD8+ cells have been associated with improved disease-free survival (DFS), as well as disease-specific survival (DSS) and OS (additionally, it also correlated positively with expression of programmed death ligand 1 [PD-L1]). (183) Another study has described a significant link between CD4+ tumor infiltrating lymphocytes and prolonged DSS in STS patients with wide resection margins. (184) A shift in the amount of CD8+ cytotoxic T-cells in TME between the first and the second resection in recurrent STS was positively associated with OS and a decrease turned out to be an independent negative prognostic parameter. (185)

The abundance of T-cells in STS might depend on the histopathological subtype and seems to be higher in subtypes with increased mutational burden like UPS, as the study of Pollack et al. suggests. (186) However, tumor growth may arise despite a high rate of tumor-infiltrating T-lymphocytes and has been explained by a T-cell exhaustion phenomenon, characterized by T-cells losing their effector function with a change in the transcriptional program including the expression of self-inhibitory receptors like PD-L1. (187, 188)

Tumor cells with abundance of inhibitory PD-1 receptors are able to escape apoptosis mediated by T-cells by inhibiting the immune response, once receptors are activated by PD-L1 on the

surface of T-cells. (189) Thus, high levels of PD-1 on tumor cells, or PD-L1 on lymphocytes are known to be pro-tumorigenic, whilst pharmacological inhibitors of the PD-1/PD-L1 system have turned out to be efficient agents against certain tumor entities like small-lung cancer, melanoma, or renal cell carcinoma. (190) In STS, however, PD-1 expression is rather low and treatment with immune checkpoint inhibitors has been disappointing so far. (191–193) Interestingly enough, the first successful treatment based on "immunotherapy" was performed on sarcomas by Coley in 1891 by using bacterial toxins, thus inducing an inflammatory reaction also targeting malignant cells. (194) Generally, better respond rates to PD-1 inhibitors are observed in tumors with high mutational burden, like colorectal carcinoma with microsatellite instability (MSI). (178) Among STS subtypes, UPS is known to have high mutation rates, as well as increased expression of PD-1 and PD-L1, indicating that a promising respond to checkpoint inhibitors could be expected, analogously to colorectal cancer. (186)

In contrast to the majority of T-lymphocytes, regulatory T-cells – characterized by the expression of forkhead box protein 3 (FOXP3) in addition to positive CD3 and CD4 markers – suppress the immune response and represent an unfavorable prognosis marker, described in clear cell renal cell carcinoma type 4. (195)

2 B-cells

B-cells derive from lymphoid stem-cells in the bone marrow and are released through the blood system to secondary lymphoid organs. As soon as they get in contact with their specific antigen, naïve B-cells adsorb it and present the antigen to T-cells with the fitting antigen-receptor and start proliferating in the germinal center of lymph nodes. Subsequently, activated B-cells turn into plasma cells and produce immunoglobulins against the presented antigen. In case of neoplasms, B-cells are capable of fighting tumor cells through antibody production leading to complement mediated cell lysis, to opsonization for phagocytosis, or to immunoglobulin-dependent cytotoxicity. In addition, B-cells promote CD4+ helper T-cells and CD8+ cytotoxic T-cells by antigen presentation in the secondary lymphoid organs. (196, 197) CD20, the most common marker, is present on B-cells at every single stage of development, except for plasma cells and at the very early stage of Pro-B-cells.

Multiple clinical studies have already demonstrated an antitumoral effect of B-cells, not only in lung cancer and ovarian cancer, but also in STS. (198, 199) The infiltration of TME with CD20+ B-cells in patients with STS and clear resection margins (R0) is positively correlated with DSS, for instance. (200, 201) Additionally, high counts of TIIC and tertiary lymphoid structures indicate a better patient outcome and increased response rate to PD-1 checkpoint inhibitors in patients with STS, especially in tissues rich in B-cells. (202)

However, further investigations on the role of B-cells in STS in regard to prognosis and therapy is required.

3 Macrophages

Macrophages account for the majority of phagocytes in TME and are present in most STS types. (193, 203) In particular, high-grade leiomyosarcoma was shown to be rich in CD168+ macrophages. (204) Generally, tumor-associated macrophages (TAM) impair the prognosis and are suspected to reduce the therapeutic efficacy of chemotherapy, angiogenesis inhibitors, and irradiation. (205–207)

However, TAM's biological behavior is heterogeneous, not all macrophages support tumor growth. Traditionally, TAM are divided into two main subgroups (203):

M1-macrophages are classically activated by interferon-gamma (INFγ) or bacterial lipopolysaccharide and considered being *pro-inflammatory* and *anti-tumorigenic* by expressing MHC class II molecules, tumor necrosis factor (TNF), or inducible nitric oxide synthase (iNOS).

M2-macrophages or alternatively activated macrophages (by IL-4 or IL-10) are characterized by producing high levels of IL-10, CD163, CD204, CD206, or arginase 1 and represent the *anti-inflammatory* and *pro-tumorigenic* type of TAMs. A common marker in research for detecting M2-type macrophages is CD163.

The three major factors deciding between pro-tumorigenic or anti-tumorigenic behavior are (A) the origin of the macrophage (embryonic precursors versus hematopoietic stem cells or both), (B) the tissue of residence, and (C) present microenvironmental cues such as fibrosis, nutrition supply, hypoxia, or lymphocyte-derived factors. (203) The functional differentiation of TAMS is, therefore, closely related to the tumor itself. Table 3 provides an overview of influential

parameters on the differentiation of macrophages between the two classical types M1 and M2. (203)

Promoters of anti-tumorigenic M1-type	Promoters of pro-tumorigenic M2-type
Normoxia	Hypoxia, Cellular Stress, ROS
Functional vascular supply	Dysfunctional vascular supply
T_{H1}, Cytotoxic T-cells, and NK cells	T_{H2} and regulatory T-cells, CAFs, B-cells, Immunoglobulins
IFNγ, TNF, Lipopolysaccharides	IL-4, IL-6, IL-10, IL-13, IL-17
HMGB1 docking on TLR4	GM-CSF, CSF1, CCL2, TGFβ
CD40 ligand	ECM, Fibrosis, FAK, DAMPs, MERK-R

Table 3 Factors influencing the differentiation to M1- or M2-like macrophages.
CAF, cancer-associated fibroblast. CCL2, colony-stimulating factor 1 (also known as Macrophage colony-stimulating factor [M-CSF]). CD, cluster of differentiation. DAMPs, Damage-associated molecular patterns. ECM, extracellular matrix. FAK, focal adhesion kinases. GM-CSF, granulocyte–macrophage colony-stimulating factor (also known as colony-stimulating factor 2 [CSF2]). HMGB1, high mobility group protein B1. IFNγ, interferon-gamma. IL, interleukin. MERK-R, mer tyrosine protein kinase receptor. NK cells, natural killer cells. ROS, reactive oxygen species. TGFβ, transforming growth factor-beta. TH1, helper T-cell type 1. TH2, helper T-cell type 2. TLR4, Toll-like receptor 4. TNF, tumor necrosis factor.

However, the differentiation of macrophages is not irreversible. Rather, it is changeable by different stimuli, in particular IFNγ and IL-10. (208, 209) In general, macrophages show a great diversity in biological function and recent preclinical studies question the binary division, suggesting a model of continuous phenotypes. (210, 211) The categorization of macrophages by chemokines and other markers is probably not a differentiation between two subtypes, but rather between the macrophages' functional state. (210)

Via direct and indirect mechanisms, macrophages have a great impact on tumor development and behavior. In particular, the effects of TAMs on T-cells play a decisive role. To name a few examples, macrophages produce iNOS that directly harms T-cells. (212) Indirectly, T-cell proliferation and recruitment is suppressed by the production of peroxynitrites that leads to a nitrition of CC-chemokine ligand 2 (CCL2) and CC-chemokine ligand 5 (CCL5), subsequently impeding the interaction of MHC with T-cell receptors. (213–217) On the other side, iNOS seem to have an anti-tumorigenic effect, as well. The presence of iNOS-producing macrophages

promotes the expression of vascular cell adhesion molecule 1 (VACM1) on endothelium cells of tumor tissue, resulting in the recruitment of CD8+ cytotoxic T-cells, at least during low-dose radiation therapy. (218, 219)

In general, one must be aware of the caveat that same molecular mechanisms might result in contrary biological effects on tumors. The fact that iNOS might lead to both, depletion and promotion of T-cells depending on the circumstances, highlights the importance of context-dependent investigations of tumor-influential factors. Therefore, the simple division into pro-tumorigenic and anti-tumorigenic macrophages remains questionable.

Another indirect mechanism of macrophages promoting tumor growth might be the expression of epidermal growth factor (EGF). In mammary adenocarcinoma, a positive correlation between macrophages counts in TME and pulmonary metastasis was observed. (220) M2-considered macrophages show a preference to perivascular localization and might contribute to tumor vascularization and recurrence, particularly by expressing vascular endothelial growth factor A (VEGF-A). (221) Moreover, the depletion of macrophages in mice with mammary carcinoma was significantly associated with delayed tumor recurrence after irradiation. (222) Regarding the influence of other immune cells, autoantibodies from B-cells docking to M2-like macrophages' Fcγ receptor, lead to macrophage mediated angiogenesis in tumor tissue. (223)

Thus, evidence suggests that targeting macrophages may help fighting tumors in general. The two main approaches of macrophage-affecting agents are (a) the depletion of tumor-favoring TAMs on the one side, (b) and the inducement of anti-tumoral mechanisms of macrophages on the other side. Depletion can mainly be mediated by either blocking the receptors of colony-stimulating factor 1 (CSF1, also known as macrophage colony-stimulating factor [M-CSF]) or by impeding the CCL2-CCR-2 signal axis. Potential macrophage-stimulating agents against tumor growth are for example agonistic CD40 antibodies or inhibition of phosphoinositide 3-kinase gamma (PI3Kγ), but also epigenetic shifting by the inhibition of histone deacetylases might be a therapeutic option in reprogramming macrophages. (224–230) In addition, the depletion of macrophages by antagonizing the myeloid growth receptor CSF1R enhances also the efficacy of checkpoint inhibitors, suggesting a augmented effect by the conjoint use of both. (205, 231)

4 Aim of the study

The impact of TIIC in STS regarding prognosis and treatment has not been investigated sufficiently. The aim of this study is to investigate the correlation between the counts of tumor-infiltrating immune cells in STS and patient outcome regarding local recurrence (LR), distant metastasis (DM), and overall survival (OS). The goal in the long term is to provide clinical data for individual patient prognosis based on histopathological features and to contribute to further pharmaceutical investigations and to the selection of STS patients regarding immunotherapeutic treatment.

II Material and Methods

1 Inclusion and exclusion criteria

Patients with STS other than GIST and with definitive surgery in curative intention and with evaluable histopathological tissue were considered for our retrospective single center study. Since therapies prior to the biopsy could possibly change the tumor microenvironment and influence the infiltrating immune cells, patients who received either neo-adjuvant chemotherapy or neo-adjuvant radiation were excluded from the analysis (cf. Table 4).

Inclusion criteria	Exclusion criteria
• Curative intent with definitive surgery • Viable histopathological tissue	• Neoadjuvant chemotherapy • Neoadjuvant radiotherapy • No follow up

Table 4 Inclusion and exclusion criteria.

Of patients with STS treated with definite surgery with curative intent at the Department of Orthopedics and Trauma and Division of Clinical Oncology, Internal Medicine, Medical University of Graz, Austria, between 1998 and 2016, 202 patients met the inclusion criteria for

the study. An ethical approval was affirmed by the Institutional Review Board of the Medical University of Graz (protocol number 29-205 ex 16/17). Tissues were provided by Biobank Graz. Fourteen patients were excluded due to neo-adjuvant therapeutical interventions (10 patients with neo-adjuvant CTX, 4 patients with neo-adjuvant RTX). Hence, we analyzed 188 patients in this retrospective analysis.

2 Clinical data

In our study, we categorized the histological subtypes of STS into six groups according to the World Health Organization Classification of "Soft Tissue and Bone Tumours" (65):

1. Synovial sarcoma
2. Myxofibrosarcoma
3. Leiomyosarcoma
4. Malignant peripheral nerve sheath tumor (MPNST)
5. Undifferentiated pleomorphic sarcoma (UPS)
6. Others (rare diagnoses)

For the grading, we relied on the criteria of the Fédération Nationale des Centres de Lutte Contre le Cancer (FNCLCC) and performed histopathological evaluation according to its grading system into grades G1, G2, and G3. Tumor size was defined as the maximum diameter of the tumor in the resected tissue. The classification of tumor margins was performed according to the guidelines of the Union Internationale Contre le Cancer (UICC) into the following three categories:

- R0 – negative margins: ≥1mm healthy tissue between tumor and surface
- R1 – marginal margins: <1mm healthy tissue between tumor and surface
- R2 – microscopically positive margins: no healthy tissue between tumor and surface

Upon statistical evaluation, R1 and R2 were grouped together in contrast to R0 margins.

Regular follow-up after definite surgery consisted of clinical examinations, local imaging with MRI, and thorax scans for metastasis (alternating CT and x-ray) every three months for the first

three postoperative years. From the third until the end of the fifth postoperative year, the same checkup was done every six months, and finally once a year thereafter (cf. Figure 13). Recurrent tumor tissue growing in the tissue bed at the original site of affection and diagnosed via local imaging or upon re-resection, was regarded as local recurrence. In contrast, distant metastases were determined as STS arising from the primary lesion but different in location and detected by imaging with CT-scans, chest x-rays, or MRI, either in the course of regular follow-up or based upon clinical suspicion. The period of time from definite surgery to diagnosis of either local recurrence or distant metastasis was defined as time to LR or time to DM, respectively.

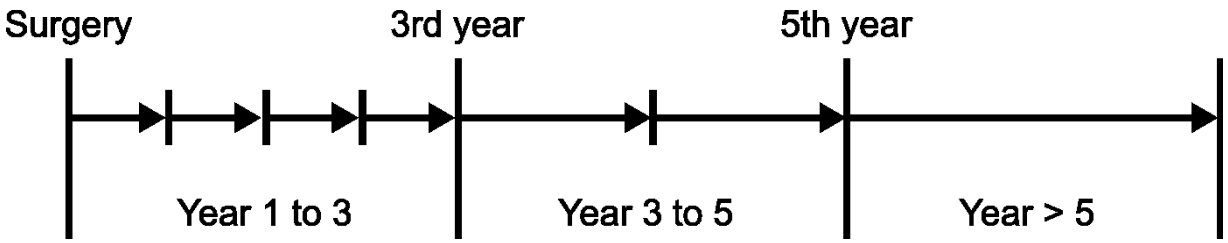

Figure 13 Regular follow-up regime in our study.

3 Analysis of tissue microarrays

Formalin-fixed histopathological tissue of resected STS of 202 patients were embedded in paraffin blocks, sliced, and stained with hematoxylin and eosin (HE). Two independent soft-tissue pathologists confirmed diagnosis and grading of STS and marked the regions of representative tumor tissue on the HE slides. Afterwards, seven cores of the selected areas were punched from the original paraffine blocks with 4µm thickness per core on average. After embedding the cores into another paraffin block, tissue microarrays (TMA) of 3-5µm in diameter were constructed and put on five TMA slides in total.

Multiplex immunohistochemistry with the following six antibodies was applied to all TMAs by using the technique of the Tyramide-signal-amplification (TSA) kit by Akoya Biosciences (Marlborough, USA):

a) CD3: LN10, Leica Biosystems Inc. (Vienna, Austria)

b) CD4: EPR6855, Abcam plc (Cambridge, UK)

c) CD8: C8/144B, Abcam plc (Cambridge, UK)

d) CD20: L26, Abcam plc (Cambridge, UK)

e) CD45RO: UCHL1, Cell Signaling Technology Europe, B.V. (Leiden, Netherlands)

f) CD68: PG-M1, Dako Agilent Pathology Solutions, Agilent (Vienna, Austria)

In addition, TMAs were stained with fluorescent immunomarker 4′,6-diamidino-2-phenylindole (DAPI) that binds to adenine-thymine and thus, highlighting the nucleus as area of DNA accumulation.

The specific combination of the listed cluster of differentiation (CD) enables the differentiation between seven immune cell phenotypes:

1) T-cells: CD3+

2) Helper T-cells: CD3+, CD4+

3) Cytotoxic T-cells: CD3+, CD8+

4) Helper memory T-cells: CD3+, CD4+. CD45RO+

5) B-cells: CD20+

6) Macrophages: CD68+

All TMAs were stained with the autostainer system Bond RX (Leica Biosystems Inc., Vienna, Austria). Vectra® 3 microscope (Akoya Biosystems, Marlborough, USA; software version 3.0.7) was used to scan TMA slides. The detection of individual TMA cores on the TMA slide was performed at 4x magnification, whereas the multispectral image analysis with one color channel per immunomarker was realized at 20x magnification.

Digital image evaluation along with autofluorescence removal and spectral unmixing was obtained by inForm software (Akoya Biosystems, Marlborough, USA; software version 2.4.8). For the multispectral image analysis with HALO® Image Analysis Platform (indica labs, Albuquerque, NM, US; Version 3.1.1076.342), separately recorded images at 20x magnification were united to a complete scan of the whole TMA slide. Subsequently, individual cells were distinguished from one another on the basis of DAPI stains by defining a threshold for (i) signal intensity, (ii) nucleus size, and (iii) roundness in advance. Regarding the other six

immunohistochemical markers, different intensity cut-offs were predetermined to assess cell marker's positivity. The number of immune cells was counted in every single TMA core separately and, as final step, added up for the same patient. The percentage of the recorded immune cells in relation to the total number of cells in the entire TMA core, represents the phenotype abundance for the statistical evaluation.

4 Statistical analysis

In the statistical analysis, distribution of continuous variables were evaluated using Shapiro-Wilk test. In case of normal distribution, the data was described by means and standard deviations (SD), whilst medians and interquartile ranges (IQR) between the 25^{th} and 75^{th} percentile were applied to non-normally distributed variables. Abundance of immune cell phenotypes was assessed in percentage (%) in relation to total cell count on TMA. For the analysis of differences in immune cell counts between patient groups (binominal or categorical variables), Wilcoxon rank-sum test and Kuskal Wallis test with post-hoc Dunn test, adjusted to multiple comparisons according to Benjamini and Hochberg, were used. (232) Correlations between the various immune cell markers were calculated based on Spearman's rank correlation coefficients. The impact of specific variables on time to LR or DM were determined by uni- and multivariate Fine & Gray competing risk-regression models, whereas the influence of variables on OS was assessed by uni- and multivariate Cox regression models. A p-value of ≤ 0.1 was the cut-off for applying multivariate computation. Statistical significance was defined with a p-value of < 0.05.

III Results

1 General clinical and histopathological features

In our retrospective study with 188 patients, 101 patients were male (53.7%) and 87 female (46.3%) with a median age of 62.5 years (IQR 49.5 - 75 years). Regarding patient outcome, the median follow-up was 46.5 months with an IQR of 19 to 99 months (Table 5).

Age	Median 62.5 years	IQR 49.5 – 75.0 years
Gender	Male	101 (53.7%)
	Female	87 (46.3%)
Follow-Up	Median 46.5 months	IQR 19 – 99 months

Table 5 Age, gender, and time of follow-up of patient cohort. IQR, interquartile range.

STS were predominantly localized in the lower limb (65.8%), whereas the upper limb (25.1%) and the trunk (9.1%) were less often affected. Over two third of STS were partly or entirely located in deep tissue (70.2%). Moreover, 72.9% were classified as grade 3. In 143 (76.1%) STS patients, R0 and R1 resection had been feasible, whilst four patients (2.1%) had a margin status of R2 at the initial definitive surgery.

Regarding the histological subtypes in our collective, myxofibrosarcoma was the most common, found in 76 patients (40.4%). The other subtypes were distributed as follows: undifferentiated pleomorphic sarcoma (UPS) 17.0%, liposarcoma 11.7%, leiomyosarcoma 11.2%, synovial sarcoma 6.4%, and other subtypes in 13.3% (Table 6).

Localization	Upper limb	47 (25.1%)
	Lower limb	123 (65.8%)
	Trunk	17 (9.1%)
Depth	Superficial	56 (29.8%)
	Deep	105 (55.8%)
	Superficial and deep	27 (14.4%)

Tumor size	Mean 8.5cm	Standard deviation ± 5.3cm
Histology	Myxofibrosarcoma	76 (40.4%)
	Synovial sarcoma	12 (6.4%)
	UPS	32 (17.0%)
	Liposarcoma	22 (11.7%)
	Leiomyosarcoma	21 (11.2%)
	Other	25 (13.3%)
Grade	G1	17 (9.6%)
	G2	31 (17.5%)
	G3	129 (72.9%)
Margins	R0	143 (76.1%)
	R1	41 (21.8%)
	R2	4 (2.1%)
Status	Alive	120 (63.8%)
	Died	68 (36.2%)

Table 6 General clinical and histopathological features.
R0, negative margins. R1/2, marginal or positive margins. UPS, undifferentiated pleomorphic sarcoma.

The majority of patients was alive at time of last follow-up (63.8% vs. 36.2% dead). The relative risk of local recurrence was significantly higher in cases with R1 or R2 margins at resection compared to R0 surgical margins (hazard ratio [HR] 2.47, p-value 0.037), whilst grading showed no significant influence on rate of local recurrence (grading G3 HR 0.789 compared to G1/2, p-value 0.619). Large tumors over the median size of 8.5cm were found to have a significantly higher risk for distant metastasis (p-value 0.001), as well as a worse overall survival (p-value 0.019), independent of grading in the multivariate model (Table 8).

LOCAL RECURRENCE				
		Univariate Model		
		HR	**95%CI**	**p-value**
Gender	Male	1		0.632
	Female	0.813	0.348 – 1.900	
Age at surgery (in years)		1.010	0.989 – 1.032	0.332

Margins	R0	1		**0.037**
	R1/2	2.470	1.058 – 5.767	
Tumor Location	Upper limb	1		
	Lower limb	0.882	0.346 – 2.253	0.794
	Trunk	0.476	0.058 – 3.891	0.489
Grading	G1/2	1		0.619
	G3	0.789	0.329 – 1.940	
Tumour Size (in cm)		0.959	0.875 – 1.051	0.371
Histology	Myxofibrosarcoma	1		
	Synovial sarcoma	NA	NA	NA
	UPS	1.043	0.277 – 3.928	0.950
	Liposarcoma	0.738	0.168 – 3.235	0.687
	Leiomyosarcoma	2.140	0.627 – 7.296	0.224
	Others	2.078	0.694 – 6.220	0.191
	Multivariate Model			
Margins	R0	1		0.089
	R1/2	2.230	0.885 – 5.621	
B-cells		1.311	0.812 – 2.117	0.268
Macrophages		1.049	1.011 – 1.088	**0.012**

Table 7 Hazard ratio (HR) for the risk of local recurrence.

CI, confidence interval. HR, hazard ratio. NA, not applicable for too few variables. R0, negative margins. R1/2, marginal or positive margins. UPS, undifferentiated pleomorphic sarcoma. Significant values in bold.

Independent of grading, too, UPS was associated with a risk of HR 2.188 for distant metastasis in comparison to myxofibrosarcoma (p-value 0.028) in the multivariate analysis (Table 8). In addition, among the histopathological subtypes, overall survival was significantly worse in patients with synovial sarcoma compared to myxofibrosarcoma in the multivariate Cox regression analysis (HR 5.482, p-value 000.1). However, the univariate calculation of risk regression displayed that 61.3% more patients survived with liposarcoma than with myxofibrosarcoma, though not significantly (HR 0.387, p-value 0.076). Finally, OS was negatively associated with seldom histopathological subtypes summarized under the label "other" (HR 1.411, p-value 0.010), independently of patient age or tumor size in the multivariate regression model (Table 9).

DISTANT METASTASIS				
		Univariate Model		
		HR	95%CI	p-value
Gender	Male	1		0.848
	Female	1.050	0.636 – 1.736	
Age at Surgery (in years)		1.007	0.992 – 1.023	0.344
Margins	R0	1		0.347
	R1/2	0.784	0.428 – 1.438	
Tumor Location	Upper Limb	1		
	Lower Limb	1.347	0.724 – 2.509	0.347
	Trunk	1.283	0.463 – 3.557	0.631
Grading	G1/2	1		0.084
	G3	1.673	0.933 – 3.001	
Tumour Size (in cm)		1.056	1.017 – 1.097	**0.005**
Histology	Myxofibrosarcoma	1		
	Synovial sarcoma	1.835	0.717 – 4.499	0.206
	UPS	2.188	1.090 – 4.393	**0.028**
	Liposarcoma	0.517	0.183 – 1.460	0.213
	Leiomyosarcoma	1.865	0.846 – 4.111	0.122
	Others	1.088	0.479 – 2.469	0.841
		Multivariate Model		
Grading	G1/2	1		0.667
	G3	1.142	0.623 – 2.093	
Tumour Size (in cm)		1.070	1.027 – 1.114	**0.001**
Histology	Myxofibrosarcoma	1		
	Synovial sarcoma	1.701	0.574 – 5.035	0.338
	UPS	2.572	1.240– 5.338	**0.011**
	Liposarcoma	0.400	0.138 – 1.156	0.090
	Leiomyosarcoma	1.878	0.912 – 3.867	0.087
	Others	1.191	0.486 – 2.919	0.702

Table 8 Hazard ratio (HR) for the risk of distant metastasis.

CI, confidence interval. HR, hazard ratio. R0, negative margins. R1/2, marginal or positive margins. UPS, undifferentiated pleomorphic sarcoma. Significant values in bold. Significant values in bold.

OVERALL SURVIVAL				
		Univariate Model		
		HR	**95%CI**	**p-value**
Gender	Male	1		0.547
	Female	0.862	0.531 – 1.398	
Age at Surgery (in years)		1.036	1.019 – 1.054	**<0.001**
Margins	R0	1		0.173
	R1/2	0.648	0.347 – 1.209	
Tumor Location	Upper Limb	1		
	Lower Limb	0.957	0.541 – 1.695	0.881
	Trunk	1.375	0.563 – 3.355	0.881
Grading	G1/2	1		0.067
	G3	1.738	0.963 – 3.137	
Tumour Size (in cm)		1.040	0.999 – 1.083	0.054
Histology	Myxofibrosarcoma	1		
	Synovial sarcoma	1.683	0.694 – 4.081	0.249
	UPS	1.691	0.866 – 3.301	0.124
	Liposarcoma	0.387	0.135 – 1.106	0.076
	Leiomyosarcoma	1.244	0.563 – 2.747	0.590
	Others	1.411	0.681 – 2.926	0.354
		Multivariate Model		
Grading	G1/2	1		0.694
	G3	1.134	0.606 – 2.121	
Tumour Size (in cm)		1.053	1.008 – 1.100	**0.019**
Histology	Myxofibrosarcoma	1		
	Synovial sarcoma	5.482	2.076 – 14.474	**0.001**
	UPS	1.919	0.976 – 3.776	0.059
	Liposarcoma	0.589	0.191 – 1.821	0.358
	Leiomyosarcoma	1.571	0.704 – 3.504	0.270
	Others	2.889	1.296 – 6.442	**0.010**

Table 9 Hazard ratio (HR) for overall survival.

CI, confidence interval. HR, hazard ratio. NA, not applicable for too few variables. R0, negative margins. R1/2, marginal or positive margins. UPS, undifferentiated pleomorphic sarcoma. Significant values in bold. Significant values in bold.

2 Immune cell abundance

a) Overall frequency and clinical correlations

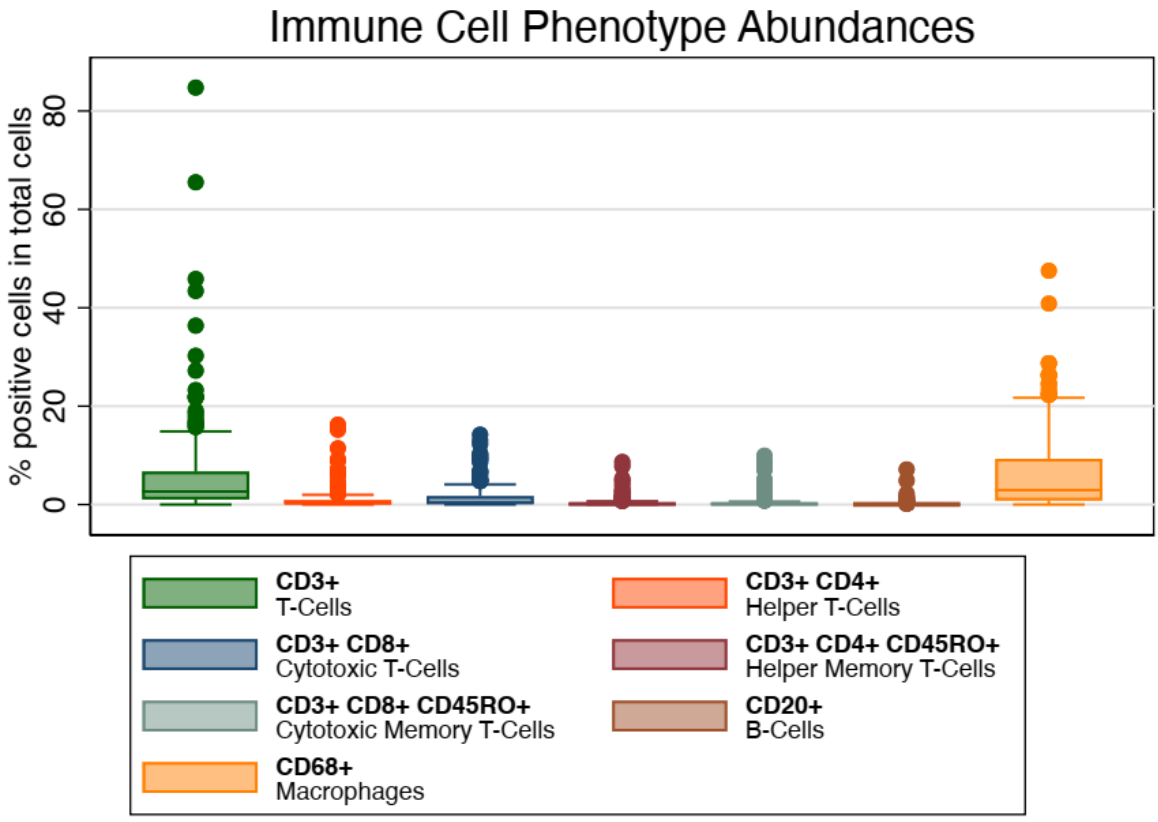

Figure 14 Overall phenotype abundance of tumor-infiltrating immune cells in percentage of total cell counts.

Regardless of the histopathological subtype, the most common immune cells were CD68+ macrophages (median percentage [MP] 2.93%) followed by CD3+ T-cells (MP 2.65%). The remaining immune cell types constituted in the median percentage less than 0.35% of all cells in a stain, respectively (Figure 14, Table 10).

Tumor-infiltrating immune cells (TIIC)	MP	IQR
T-cells (CD3+)	2.65 %	1.02 – 6.70 %
Helper T-cells (CD3+ CD4+)	0.25 %	0.06 – 0.89 %
Cytotoxic T-cells (CD3+ CD8+)	0.34 %	0.08 – 1.71 %
Helper memory T-cells (CD3+ CD4+ CD45RO+)	0.06 %	0.00 – 0.28 %
Cytotoxic memory T-cells (CD3+ CD8+ CD45RO+)	0.05 %	0.00 – 0.30 %
B-cells (CD20+)	0.01 %	0.00 – 0.07 %
Macrophages (CD68+)	2.93 %	0.85 – 9.30 %

Table 10 Median percentage of immune cells in STS.

IQR, interquartile range. MP, median percentage.

Spearman's correlation analysis revealed a significant positive correlation between the counts of every single immune cell subtype with one another, apart from CD68+ macrophages and CD3+ T-cells, as well as CD68+ macrophages and CD20+ B-cells (Table 11).

	1.	2.	3.	4.	5.	6.
1. Helper T-Cells	0.645*					
2. Cytotoxic T-cells	0.668*	0.746*				
3. Helper memory T-cells	0.371*	0.768*	0.526*			
4. Cytotoxic memory T-cells	0.505*	0.736*	0.704*	0.842*		
5. B-cells	0.316*	0.438*	0.308*	0.561*	0.483*	
6. Macrophages	0.198†	0.545*	0.449*	0.598*	0.569*	0.202‡

Table 11 Spearman's correlation analysis for immune cell subtype count.

**p-value < 0.0001; † p-value = 0.140; ‡ p-value = 0.118*

The patient age at the time of STS resection showed a significant association for two immune cell subgroups: In patients older than the median age of 62.5 years, the count of CD68+ macrophages was significantly higher compared to the subpopulations under the median (MP 5.16% [IQR 0.00-0.03%] vs. 1.9% [IQR 0.64-4.83%], p-value 0.002), as opposed to the number of CD20+ B-cells that decreased with increasing age (MP 0.00% in patients under the median age of 62.5 years [IQR 0.00-0.03%], vs. MP 0.02% in patients over the median age [IQR 0.00-0.08%], p-value 0.013). In contrast, gender was not associated with a statistically significant difference in immune cell infiltration.

In STS larger than median diameter of 8.5cm, CD3+ T-cells were more frequent than in tumors smaller than the median size (MP 1.74% in patients under the median size [IQR 1.04-5.61%], vs. MP 3.62% [IQR 1.05-8.65%], p-value 0.029). The rest of the analyzed immune cells did not differ compared to the tumor size. Neither tumor depth, nor location showed a significant effect on immune cell counts.

b) Comparison to histopathological features

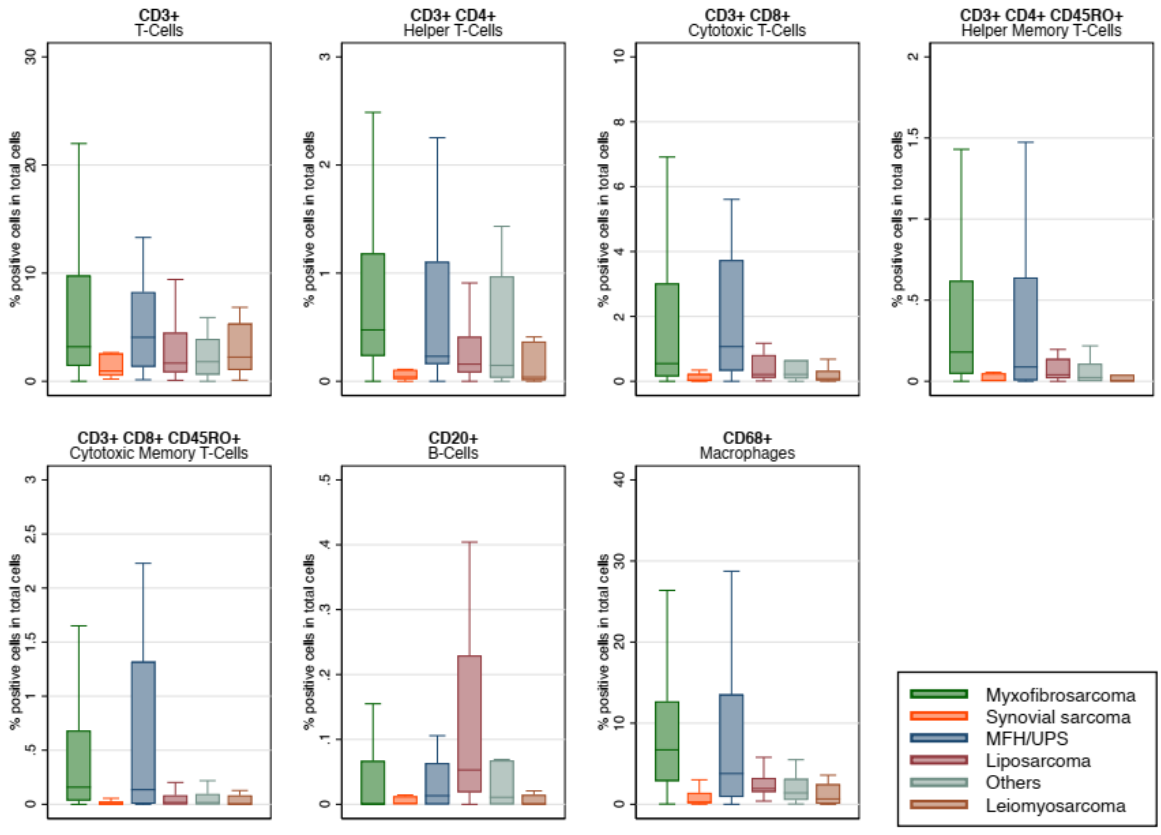

Figure 15 Immune cell counts in different STS subtypes.
MFH, malignant fibrous histiocytoma. UPS, undifferentiated pleomorphic sarcoma.

Spearman's correlation analysis revealed a significant difference between histopathological subtypes and every single of the six analyzed immune cells subgroups (Figure 15). Moreover, the MP of all immune cell subgroups showed a significant or marginal positive association (p-value < 0.065) with high-grade G3 STS, except for CD20+ B-cells (Table 12).

	G1/2		G3		p-value
	MP	95%CI	MP	95%CI	
T-cells	1.87%	0.62 – 5.15%	2.94%	1.20 – 7.20%	0.063
Helper T-cells	0.14%	0.03 – 0.48%	0.33%	0.11 – 1.00%	**0.021**
Cytotoxic T-cells	0.18%	0.03 – 1.87%	0.46%	0.12 – 1.72%	**0.050**
Helper memory T-cells	0.04%	0.00 – 0.18%	0.11%	0.01 – 0.40%	**0.036**
Cytotoxic memory T-cells	0.01%	0.00 – 0.15%	0.08%	0.01 – 0.39%	**0.002**
B-cells	0.01%	0.00 – 0.08%	0.01%	0.00 – 0.07%	0.666
Macrophages	1.94%	0.65 – 5.16%	3.56%	0.98 – 10.54%	0.054

Table 12 Differences in immune cell subtype abundance compared to grading.
MP, median percentage. CI, confidence interval. Significant values in bold.

c) Correlation with prognostic parameters

A significantly increased risk of local recurrence was found in STS with elevated levels of CD68+ macrophages (p-value 0.003) and CD20+ B-cell (p-value 0.035) counts. The positive association of CD68+ macrophages stayed significant (p-value 0.012), independent from R1/2 margins (p-value 0.089) and CD20+ B-cell infiltration (p-value 0.268) in the multivariate analysis. No other significant correlation of immune cell phenotypes to the prognostic parameters – risk of LR, DM, or OS – could be found (Table 13, Table 14, Table 15, Figure 16).

LOCAL RECURRENCE			
	Univariate Model		
	HR	**95%CI**	**p-value**
T-cells (CD3+)	1.004	0.977 – 1.032	0.772
Helper T-cells (CD3+ CD4+)	1.123	0.949 – 1.329	0.178
Cytotoxic T-cells (CD3+ CD8+)	1.056	0.923 – 1.209	0.426
Helper memory T-cells (CD3+ CD4+ CD45RO+)	1.293	0.924 – 1.809	0.133
Cytotoxic memory T-cells (CD3+ CD8+ CD45RO+)	0.965	0.762 – 1.222	0.766
B-cells (CD20+)	1.547	1.032 – 2.319	**0.035**
Macrophages (CD68+)	1.046	1.016 – 1.078	**0.003**
	Multivariate Model		
B-cells (CD20+)	1.311	0.812 – 2.117	0.268
Macrophages (CD68+)	1.049	1.011 – 1.088	**0.012**

Table 13 Correlation between immune cell abundance and risk of local recurrence.

CI, confidence interval. HR, hazard ratio. Significant values in bold.

DISTANT METASTASIS			
	Univariate Model		
	HR	**95%CI**	**p-value**
T-cells (CD3+)	1.008	0.993 – 1.024	0.293
Helper T-cells (CD3+ CD4+)	1.013	0.936 – 1.096	0.752
Cytotoxic T-cells (CD3+ CD8+)	1.065	0.982 – 1.155	0.127
Helper memory T-cells (CD3+ CD4+ CD45RO+)	0.987	0.808 – 1.206	0.899
Cytotoxic memory T-cells (CD3+ CD8+ CD45RO+)	1.028	0.895 – 1.182	0.694
B-cells (CD20+)	1.031	0.824 – 1.292	0.787
Macrophages (CD68+)	1.021	0.995 – 1.047	0.114

Table 14 Correlation between immune cell abundance and risk of distant metastasis.

CI, confidence interval. HR, hazard ratio.

OVERALL SURVIVAL			
	Univariate Model		
	HR	95%CI	p-value
T-cells (CD3+)	1.005	0.987 – 1.024	0.578
Helper T-cells (CD3+ CD4+)	0.999	0.903 – 1.104	0.980
Cytotoxic T-cells (CD3+ CD8+)	1.050	0.967 – 1.141	0.247
Helper memory T-cells (CD3+ CD4+ CD45RO+)	0.934	0.734 – 1.187	0.575
Cytotoxic memory T-cells (CD3+ CD8+ CD45RO+)	1.057	0.928 – 1.205	0.403
B-cells (CD20+)	0.916	0.637 – 1.319	0.638
Macrophages (CD68+)	1.005	0.976 – 1.034	0.762

Table 15 Correlation between immune cell abundance and overall survival.
CI, confidence interval. HR, hazard ratio.

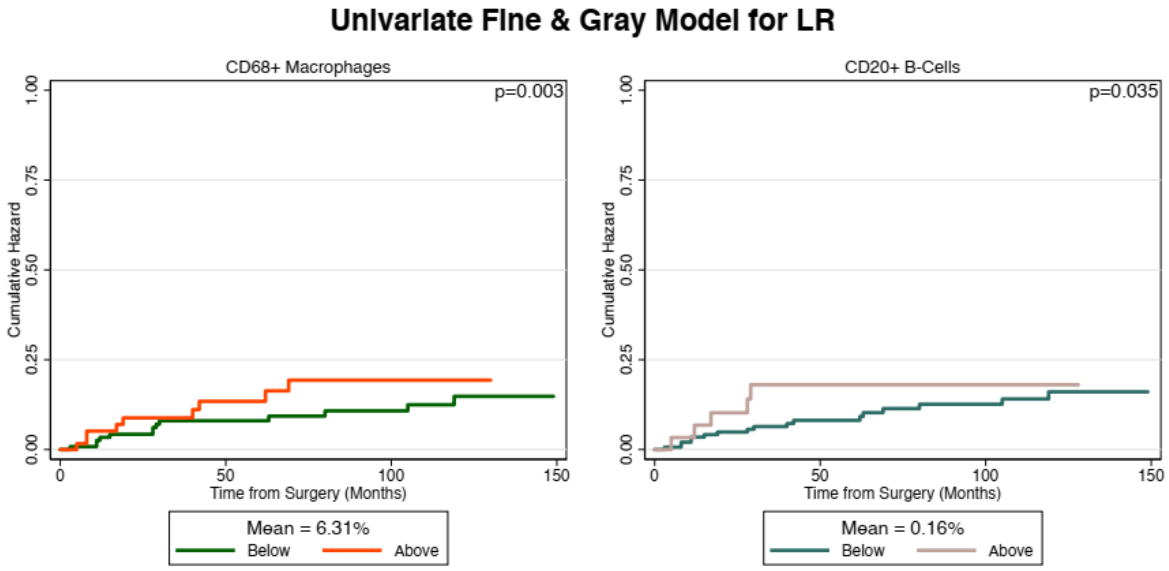

Figure 16 Univariate Fine & Gray model for CD68+ macrophages and CD20+ B-cells with cumulative Hazard ratio for local recurrence (LR), death as competing event.

IV Discussion

1 Relevance

For the first time, an association between an increase in CD68+ macrophages in TME of STS and a higher risk for LR independently of resection margins and CD20+ B-cell infiltration was found. This result is in line with one study focusing on the correlation between CD68+ or CD163+ (staining M2-type) macrophages and the DSS in nongynecological leiomyosarcoma only. (233) In contrast, Komohara et al. did not observe a correlation between macrophages infiltration and clinical outcome in patients with UPS, whereby the only analyzed markers were CD163, CD204 and CD8. (234) However, previous findings in other tumor entities support our results, too: In gastric carcinoma, for instance, high infiltration with CD68+ macrophages was correlated with more progressed TNM stage and poorer outcome regarding OS. (235, 236) A similar conclusion was drawn by Atanasov et al. from the comparison of clinical outcome and infiltration with CD68+ TAMs in hiliar cholangiocarcinoma. (237) Moreover, the presence of CD68+ and iNOS-producing (M1-type) macrophages in colorectal carcinoma was associated with a significantly reduced DFS and OS. (238) In contrast, a meta-analysis of twelve different studies investigating TIIC in early-stage non-small cell lung cancer displayed contradictory results about CD68+ TAMs and patient outcome. (198)

Secondly, in our study, the significant factors influencing tumor infiltration by immune cells were (A) histopathological subtype, (B) patient age, and (C) tumor grade.

(A) Among the analyzed STS subtypes, UPS and myxofibrosarcoma showed overall high levels of TIIC and, in particular, the highest counts of CD68+ macrophages, whilst synovial sarcoma was associated with low abundance of CD68+ macrophage. STS genetically driven by complex karyotypes (such as UPS and myxofibrosarcoma) are generally more likely to attract and activate phagocytic immune cells than STS caused by genetic translocations (like synovial sarcoma), as previous investigations suggested. (239–242)

(B) A patient age dependent immune profile with an increase of CD68+ macrophages and decrease of CD20+ B-cells in advanced age was observed regardless the histopathological subtype. This might contribute to the poorer prognosis regarding OS in elderly STS patients but is presumably of minor relevance. Nevertheless, in advanced age, the efficacy of the immune system is decreasing. This "immunosenescense" happens mainly because of the depletion and exhaustion of T-cells, and also because of higher amounts of suppressive immune cells. (243, 244)

(C) High grade STS (G3) correlated with marginally or significantly increased levels of all analyzed immune cell types except for CD20+ B-cells compared to G1/2. Aneuploidy, recombination defects, and general heterogeneity within the tumor are features of high grade tumors and indicate DNA damage that might be the cause of an upregulation of damage-associated molecular patterns attracting immune cells – in particular macrophages – to the tumor, as Dancsok et al. hypothesized. (241)

The levels of the different immune cell subtypes correlated with each other, beside CD3+ T-cells and CD68+ macrophages, and CD20+ B-cells and CD68+ macrophages, which is consistent with other studies. (234, 245) In general, CD68+ cells showed the highest abundance and outnumbered lymphocytes in STS, which is conformable with previous findings. (241, 245) In respect of chemotherapeutic agents, macrophages and monocytes are regarded quiescent compared to tumor cells and, therefore, hardly responsive to conventional DNA-damaging chemotherapeutics. Nevertheless, certain agents, such as trabectedin, trigger the extrinsic apoptotic pathway in monocytes and macrophages by activating caspase and, by that, cause the death of unfavorable macrophages in TME. (246–249) Hence, there is evidence for using chemotherapeutic agents targeting macrophages rather than lymphocytes in STS. Although UPS, for example, showed a higher risk for distant metastasis in our patient cohort, it also contained the highest level of CD68+ macrophages and TIIC in general. Consequently, UPS might be more sensitive to trabectedin.

Contrary to previous observations, we did not find a significant influence of CD20+ B-cell or CD4+ T-cell infiltration on prognostic parameters. (200, 202) This might be explained by diverging definitions of immune cell counts – Sorbye et al. rated tumor cell infiltration in three qualitative categories (missing, low, high) only and Petitprez et al. created five clusters based

on immune profiles, as opposed to our approach of quantifying the percentage of the immune cells in relation to the total number of cells within the TME.

2 Limitations

The interpretation of the results should consider the following limitations:

1. The immunohistochemical staining applied to the tissues might not detect all relevant immune cells. Accurately validated, standardized, and uniformly available IHC staining methods for the identification of immune cells have not been established so far. (250, 251) Therefore, the study focused on commonly used and affirmed markers for macrophages, B- and T-cells.
2. In our study, no distinction between M1- and M2-type macrophages was made, but CD68+ was applied as general marker for both types of macrophages. The two types are not definitive states but rather seem to represent the two extremes of a spectrum of various mechanisms and functions of macrophages, as said above. (203, 210) Moreover, all macrophages, regardless their typing, are affected by macrophage-targeted therapeutics such as trabectedin, which is already approved for certain STS subtypes. (249, 1) Hence, the clinical relevance of the distinction into M1- and M2-like macrophages is questionable.
3. In Addition, depending on the fixation method and the antibody clones, CD68 may also be present on the surface of dendritic cells and non-myeloid cells like fibroblasts or endothelial cells. (252) However, by using PG-M1 by Dako Agilent Pathology Solutions, Agilent (Vienna, Austria) for the cell counts of CD68+ macrophages, which specifically stains monocytes and macrophages, this limitation is not applicable for our study.
4. Furthermore, we have not distinguished between immune cells within the tumor nest compared to the surrounding tumor-cell free capsule, as it was performed in comparable studies. (184, 253) The reason behind the general count within the region of representative tumor tissue was the fact that all immune cells in the area

of the malignancy might affect the tumor and be susceptible for therapeutical interventions, regardless their exact position in relation to tumor cells.

5. Apart from CD68+ macrophages and CD3+ T-cells, the overall abundance of TIIC was rather low in our study but indicates the general observation of poor immunogenicity in STS compared to the TME of other tumor entities.

3 Further investigations

Although our study might contribute to a greater understanding of macrophages' role in STS and their importance regarding prognosis and treatment, the significance of different types or functional states of TAMs remains unclear. The clinical relevance of differences found in preclinical research needs to be evaluated in more specific investigations. Moreover, the effect of B-cells and T-cells in STS on patient outcome showed inconsistent results, demanding further exploration.

So far, the majority of studies focused on the correlation between clinical outcome and the presence of certain immune cell types in the TME. A more comprehensive analysis was performed by Petitprez et al. by grouping distinctive immune profiles together to specific patterns rather than counting single cells and could be considered as a more holistic approach for the future. (202)

Furthermore, the optimal therapeutical approach targeting macrophages in STS is still unexplored. The two major options are the depletion of unfavorable macrophages (e.g. inhibition of CSF1-CSF1R axis, CCR2 inhibition) on the one hand, and the reprogramming to an anti-tumor behavior (e.g. agonist CD20 antibodies, inhibition of histone deacetylases, PI3Kγ inhibition) on the other hand. (203) To answer the question, which STS subtypes benefit most of which therapeutic agents or combinations, more data on immune profiles and patient outcome, as well as pharmaceutical trials are required. Moreover, problems with pharmaceutical resistance and immune-escape mechanisms in macrophages may arise and need to be addressed, subsequently. (241)

However, the low incidence and the high variety of STS impede large-scaled studies and tempt to group different STS types or immune profiles together to enable higher accuracy at the expense of more differentiated, type-specific research.

4 Conclusion

To our knowledge, this is the first study confirming high counts of CD68+ macrophages in STS as negative prognostic factor regarding local recurrence. Moreover, the data revealed histopathological subtype, patient age, and tumor grade as three significant factors determining the level of TIIC. The findings suggest that selected STS patient groups might benefit from macrophage-targeted pharmaceutical agents. Nevertheless, further investigations are needed to gain more detailed insights regarding prognosis and therapy.

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