Diploma Thesis

Prognostic Impact of PD-L1 Expression in Soft Tissue Sarcoma Microenvironment

submitted by

Barbara Maria Granegger

for the attainment of the academic degree

Doctor medicinae universae

(Dr. med. univ.)

at the

Medical University of Graz

accomplished at the

Department of Internal Medicine, Division of Oncology

supervised by

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

and

Dr. med. univ. Dr. scient.med. Maria Anna Smolle

Graz, 21st January 2022

Statutory Declaration

I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

Graz, 21st January 2022 *Barbara Granegger eh*

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Abbreviations

Organizations and Classification Systems:

AJCC	American Joint Committee on Cancer
ESMO	European Society for Medical Oncology
FNCLCC	Fédération Nationale des Centres de Lutte Contre le Cancer
NCCN	National Comprehensive Cancer Network
UICC	Union for International Cancer Control
WHO	World Health Organization

Diagnosis and Therapy:

CNB	Core needle biopsy
CT	Computer tomography
CTX	Chemotherapy
DM / FM	Distant metastasis / Fernmetastasierung
FISH	Fluorescence *in situ* hybridisation
FNA	Fine needle aspiration
IHC	Immunohistochemistry
LR / LR	Local recurrence / Lokalrezidiv
MDT	Multidisciplinary team
MP-IHC	Multiplex Immunohistochemistry
MRI	Magnetic resonance imaging
NGS	Next generation sequencing
OS / GÜ	Overall survival / Gesamtüberleben
PET	Positron emission tomography
RTX	Radiotherapy
SI	Signal intensity
TMA	Tissue microarray
TNM	Tumour size, tumour necrosis, mitotic count

Histological Tumour Subtypes:

AS	Angiosarcoma
CC	Cervical cancer
DDLPS	Dedifferentiated liposaroma
EBV	Epstein-Barr-virus
GIST	Gastrointestinal stromal tumour
HNSCC	Head and neck squamous cell carcinoma
hSTS	High grade soft tissue sarcoma

LMS	Leiomyosarcoma / Leiomyosarkoma
LPS	Liposarcoma / Liposarkoma
MFH	Myxofibrohistiocytoma
MFS	Myxofibrosarcoma / Myxofibrosarkoma
MLPS	Myxoid liposarcoma
MM	Malignant melanoma
MPNST	Malignant peripheral nerve sheath tumour
NOS	Not other specified
PLS	Pleomorphic liposarcoma
PNST	Peripheral nerve sheath tumours
SCS	Spindle Cell Sarcoma
SS	Synovial sarcoma / Synovial Sarkoma
STS / WTS	Soft tissue sarcoma / Weichteilsarkoma
UPS	Undifferentiated Pleomorphic Sarcoma
WDLPS	Well-differentiated liposarcoma

Immune Markers and Proteins:

APC	Antigen presenting cell
CDK4	Cycline dependent kinase 4
CTLA-4	Cytotoxic T lymphocyte antigen 4
ECM	Extracellular matrix
ICI	Immune checkpoint inhibitor
IDO	2,3-Dioxygenase
KIT	Tyrosine protein kinase KIT
LAG-3	Lymphocyte activation gene-3 receptor
MDM2	Murine double minute 2-protein
MHC	Multihistocompatibility complex
NK	Natural killer cell
p53	Tumour protein 53
PD-1	Programmed death 1
PD-L1/2	Programmed death ligand 1/2
Rb	Retinoblastoma protein
SIRPα	Signal regulatory proein α
TCR	T cell receptor
TIL	Tumour infiltrating lymphocytes
TIM	Tumour infiltrating macrophage
TIM-3	T cell immunoglobulin and mucin domain 3 receptor
TME / TMU	Tumour microenvironment / Tumormikroumgebung
Treg	Regulatory T cell

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Zusammenfassung

Einleitung: Die (De-)Aktivierung von Immun-Checkpoints wie PD-1 und PD-L1 hat einen erheblichen Einfluss auf die Immunantwort und spielt auch in der Tumormikroumgebung (TMU) eine wichtige Rolle. Die Identifizierung dieser Immun-Checkpoints als verlässliche prognostische und therapeutische Biomarker trägt zum besseren Verständnis von Weichteilsarkomen (WTS) bei und kann zu neuen, personalisierten Behandlungsstrategien führen. Ziel dieser Studie ist die Expression von PD-L1 und PD-1, sowie von tumorinfiltrierenden Lymphozyten (TILs) in der TMU mit klinischen Prognosefaktoren zu korrelieren und ihr prognostisches Potential in Bezug auf Lokalrezidiv (LR), Fernmetastasierung (FM) und Gesamtüberleben (GÜ) bei Patient*innen mit WTS zu bewerten.

Patient*innen und Methoden: In dieser retrospektiven Analyse wurden 192 Tumorproben mittels multispektraler Bildgebung analysiert. Mit Multiplex-Immunhistochemie konnten Infiltrationsraten von TILs bestimmt werden: T-Zellen (CD3+), Helfer-T-Zellen (CD3+/CD4+), zytotoxische T-Zellen (CD3+/CD8+), regulatorische T-Zellen (CD3+/CD4+/FOXP3+; Tregs) und PD-1, PD-L1 oder FOXP3 positive Zellen. Die Infiltrationsrate von TILs und Immun-Checkpoints wurde mit klinischen Parametern und dem Risiko für LR, FM und mit GÜ der Patient*innen korreliert.

Ergebnisse: Der häufigste Biomarker war FOXP3 mit 2,6% aller Zellen, gefolgt von PD-L1 (0,82%) und PD-1 (0,57%). Signifikant erhöhte Spiegel von allen PD-1+ TILs - außer für PD-1+ Tregs - konnten in ≥ 63,5 Jahre alten Patient*innen im Vergleich zu Jüngeren bestimmt werden. Im Vergleich zu Synovialsarkomen (SS), Liposarkomen (LPS) und Leiomyosarkomen (LMS) wurden in Myxofibrosarkomen (MFS) signifikant höhere Infiltrationsraten von allen TILs - ausgenommen PD-L1+ Zellen - festgestellt. Es wurde eine stark positive Korrelation zwischen hohen PD-1+ Zellspiegeln und FOXP3+ Zellen (rho=0.737) bestimmt. Hohe Infiltrationsraten von Tregs zeigten einen signifikanten Zusammenhang mit einem erhöhten Risiko für LR (p=0.016). Außerdem wurde ein Trend hinsichtlich erhöhtem Risiko für LR bei hohen Spiegeln an PD-L1+ Zellen (p=0.248) und PD-1+ Zellen (p=0.070) beobachtet, jedoch ohne statistische Signifikanz.

Schlussfolgerung: MFS zeigten die höchsten Spiegel an TILs und Immun-Checkpoint-positiven Zellen. Hohe Infiltrationsraten an Tregs standen signifikant im Zusammenhang mit einem erhöhten Risiko von LR, unabhängig vom Resektionsrand.

Abstract

Introduction: The (de)activation of immune checkpoints such as PD-1 and PD-L1 has a significant impact on the immune response and plays an important role in the tumour microenvironment (TME). The identification of these immune checkpoints as reliable prognostic and therapeutic biomarkers contributes to a better understanding of soft tissue sarcoma (STS), eventually giving rise to novel personalized treatment strategies. The aim of this study is to correlate PD-L1 and PD-1 expression as well as tumour infiltrating lymphocytes (TILs) in the TME with clinical prognostic factors and evaluate their prognostic potential towards local recurrence (LR), distant metastasis (DM) and overall survival (OS) in patients with STS.

Patients and Methods: In this retrospective analysis, 192 tumour samples were stained and analysed with multispectral imaging. Using multiplex immunohisto-chemistry infiltration rates of TIL phenotypes were assessed: T cells (CD3+), helper T cells (CD3+/CD4+), cytotoxic T cells (CD3+/CD8+), Tregs (CD3+/CD4+/ FOXP3+) and cells positive for PD-1, PD-L1, and FOXP3. The abundance of TIL phenotypes and immune checkpoints were correlated with risk of LR and DM as well as OS.

Results: The most abundant biomarker was FOXP3 (2.6% of total cell count) followed by PD-L1 (0.82%) and PD-1 (0.57%). In patients ≥ 63.5 years significantly higher levels of all PD-1+ TIL phenotypes - except for Tregs - were found compared to younger patients. According to histological subtype all TIL phenotypes - except for PD-L1+ T cells - showed significantly higher levels in myxofibrosarcoma (MFS) compared to synovial sarcoma (SS), liposarcoma (LPS), and leiomyosarcoma (LMS). A strong positive correlation between high levels of PD-1+ cells and FOXP3+ cells (rho=0.737) was found. High infiltration rates of Tregs were significantly associated with increased risk for LR (p=0.016). Furthermore, high levels of PD-L1+ cells (p=0.248) and PD-1+ cells (p=0.070) showed a trend towards increased risk for LR, however, not reaching statistical significance.

Conclusion: MFS have shown the highest infiltration rates of TILs and immune checkpoint positive cells. High levels of Tregs were significantly associated with increased risk for LR, independent of resection margins.

1. Introduction

Sarcomas describe tumours arising from the mesenchyme, which further develops into connective tissue connecting, supporting, and surrounding various structures and organs within the body. Sarcomas in general can be divided into two subgroups based on the tissue they originate from, i.e. soft-tissue and bone sarcomas (1).

Soft tissue sarcomas (STS) represent a group of tumours with heterogeneous clinical and pathological presentation. According to the World Health Organization (WHO) classification from 2020 over 100 subtypes can be differentiated by histological and molecular biological examination (2). These include malignant as well as benign tumours, originating from any soft tissue, including fat (e.g. lipoma, liposarcoma), muscles (e.g. leiomyoma, rhabdomyosarcoma), nerves (e.g. malignant peripheral nerve sheath tumour), fibrous tissue (e.g. fibromatosis) and blood and lymph vessels (e.g. haemangioma, angiosarcoma, lymphangioma) (3). STS account for less than 1% of malignant tumours in adults, whereas the prevalence in children is higher, amounting to 15% of all cancers (4). The incidence of STS ranges between 3.3 and 4.7 per 100.000 patients per year in Eastern and Northern Europe (5). The incidence of benign soft tissue tumours is estimated to be about 100-times higher but cannot be determined precisely as small benign soft tissue lesions may remain undetected.

The aetiology of STS is unknown, though several predisposing factors have been identified. The most prevalent ones are genetic predisposition syndromes, such as *Neurofibromatosis type I* and *Li-Fraumeni syndrome*, radiation-induced sarcomas following tumours of the breast or lymphangiosarcoma due to postoperative chronic lymphoedema after axillary dissection. The latter STS is known as Stewart-Treves syndrome (6, 7). Nevertheless, the aetiology of the vast majority of STS remains unclear, warranting identification of additional aetiological factors to eventually reduce risk for STS development.

1.1. Classification of Tumours

The updated WHO Classification from 2020 lists over 100 subtypes of STSs. An overview of the different types of benign and malignant soft tissue tumours defined

by the WHO is given in Table 1 (2). In general, the name of a tumour is given according to the tissue from which the tumour cells derive from. For example, tumours originating from fat cells are called lipoma or liposarcoma (LPS), tumours deriving from smooth muscle are known as leiomyosarcoma (LMS) and mesenchymal neoplasms of the gastrointestinal tract are named gastrointestinal stromal tumours (GIST). Most common histological subtypes in adults are LMS (11.7%), undifferentiated pleomorphic sarcoma (UPS, 10.8%), GIST (7.3%), dedifferentiated liposarcoma (DDLPS, 6.4%), well-differentiated liposarcoma (WDLPS, 5.9%), myxofibrosarcoma (MFS, 3.3%), and angiosarcoma (AS, 3.1%) (8). One of the most frequently encountered STSs in childhood is rhabdomyosarcoma accounting for 40% (9). STS develop anywhere in the body, but prefer to origin in extremities (59%), trunk (19%), retroperitoneum (15%), or head and neck (9%), according to a previous study by *Coindre, Terrier et al.* (10).

Beside the location and origin of lesions, it is of great importance to differentiate between benign and malignant tumours. While less aggressive treatments (or even none) are used in patients with benign tumours, the treatment of malignant STS includes resection with wide margins, and/or adjuvant or neoadjuvant radiotherapy, and/or chemotherapy. In STS, survival is highly depending on well aimed and timely diagnosis of the disease, as well as multidisciplinary treatment. Main prognostic factors for STS are grade, size, depth, age of the patient, and stage of disease (11). In general, diagnosis of progressed stage is associated with worse prognosis and decreased disease- specific survival (12). As mentioned, differentiation of benign and malignant soft tissue tumours can be difficult. Major criteria pointing towards malignancy include large size, deep location, and heterogeneous signal intensity (SI) in magnetic resonance imaging (MRI) analysis (13). Attachment to the muscular fascia is assessed by clinical examination. While tumours above the fascia are a hint for benign lesions, malignancy is more likely to be present in tumours attached to the muscular fascia (14). If a soft tissue swelling presents one of the following features it should be viewed as potentially malignant: increasing in size, size >5cm, deep to deep fascia, and painful. This should be applied until malignancy can be ruled out as stated in referral guidelines (15). In the present diploma thesis, histological STS subtypes are divided into six different groups. Therefore, the next section will discuss characteristics of **synovial sarcoma (SS), MFS, LMS,**

malignant peripheral nerve sheath tumour (MPNST), UPS and "Others" (rare diagnoses).

Table 1: An overview of the different types of benign and malignant STSs defined by the WHO 2020 (2).

1. Adipocytic tumours		
Benign	*Intermediate (locally aggressive)*	*Malignant*
Lipoma, lipomatosis Lipomatosis of nerve Lipoblastoma / lipoblastomatosis Angiolipoma Myolipoma of soft parts Chondroid lipoma Spindle cell / pleomorphic lipoma Atypical spindle cell / pleomorphic atypical lipomatous t. Hibernoma	Atypical lipomatous tumour	Dedifferentiated liposarcoma Well differentiated liposarcoma - adipocytic (lipoma-like) - sclerosing - inflammatory types Myxoid liposarcoma Pleomorphic liposarcoma Myxoid pleomorphic liposarcoma
2. Fibroblastic / myofibroblastic tumours		
Benign	*Intermediate (locally aggressive)*	*Malignant*
Nodular fasciitis Proliferative fasciitis Proliferative myositis Myositis ossificans Fibro-osseous pseudotumour of digits Ischemic 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 Angiomyofibroblastoma Cellular angiofibroma Nuchal-type fibroma Acral fibromyxoma	Plamar / plantar fibromatosis Desmoids-type fibromatosis Lipofibromatosis Giant cell fibroblastoma Dermatofibrosarcoma protuberans *Intermediate (rarely metastasizing)* Dermatofibrosarcoma protuberans - Fibrosarcomatous d. p. Solitary fibrous tumour Inflammatory myofibroblastic tumour Low grade myofibroblastic sarcoma Myxoinflammatory fibroblastic sarcoma Infantile fibrosarcoma Superficial CD34-positive fibroblastic t.	Solitary fibrous tumour, malignant Myxofibrosarcoma Fibrosarcoma NOS Low-grade fibromyxoid sarcoma Sclerosing epithelioid fibrosarcoma

3. So-called fibrohistiocytic tumours		
Benign	*Intermediate (rarely metastasizing)*	*Malignant*
Tenosynovial giant cell tumour - localized type - diffuse type - malignant Deep benign fibrous histiocytoma	Plexiform fibrohistiocytic tumour Giant cell tumour of soft parts NOS	Malignant tenosynovial giant cell tumour
4. Smooth-muscle tumours		
Benign	*Intermediate*	*Malignant*
Leiomyoma of deep soft tissue	Smooth muscle tumour of uncertain malignant potential EBV-associated smooth muscle tumour	Inflammatory leiomyosarcoma Leiomyosarcoma
5. Pericytic (perivascular) tumours		
Benign		*Malignant*
Glomus tumour NOS Myopericytoma, including myofibroma Angioleiomyoma		Glomus tumour, malignant
6. Skeletal-muscle tumours		
Benign		*Malignant*
Rhabdomyoma		Embryonal rhabdomyosarcoma Alveolar rhabdomyosarcoma Pleomorphic rhabdomyosarcoma Spindle cell / Sclerosing rhabdomyos. Ectomesenchymoma
7. Vascular tumours		
Benign	*Intermediate (locally aggressive)*	*Malignant*
Synovial hemangioma Venous hemangioma Arteriovenous hemangioma Intramuscular hemangioma Anastomosing hemangioma Epithelioid hemangioma Lymphangioma / lymphangiomatosis Acquired tufted hemangioma	Kaposiform hemangioendothelioma Retiform hemangioendothelioma Papillary intralymphatic angioendothelioma Composite haemangioendothelioma Pseudomyogenic (epithelioid sarcoma-like) h. Kapsoi sarcoma	Epithelioid hemangioendothelioma Angiosarcoma of soft tissue
8. Gastointestinal stromal tumours (GIST)		
Benign GIST	*GIST of uncertain malignant potential*	*Malign GIST*
MicroGIST		Gastointestinal stromal tumours

9. Peripheral nerve sheath tumours (PNST)		
Benign		*Malignant*
Schwannoma Neurofibroma Perineurioma Granuar cell tumour Nerve sheath myxoma Solitary circumscribed neuroma Meningioma Hybrid nerve sheath tumours		Malignant peripheral nerve sheath tumour (MPNST) Melanotic malignant nerve sheath tumour (MMNST) Granular cell tumour, malignant Perineurioma, malignant

10. Tumuors of uncertain differentiation		
Benign	*Intermediate (locally aggressive)*	*Malignant*
Myxoma (cellular myxoma) Deep (aggressive) angiomyxoma Pleomorphic hyalinising angiectatic tumour Phosphaturic mesenchymal tumour Perivascular epithelioid tumour, benign Angiomyolipoma	Hemosiderotic fibrolipomatous tumour Angiomyolipoma, epithelioid *Intermediate (rarely metastasizing)* Atypical fibroxanthoma Angiomatoid fibrous histiocytoma Ossifying fibromyxoid tumour Myoepithelioma	Synovial sarcoma NOS Epithelioid sarcoma Alveolar soft-part sarcoma Clear cell sarcoma Extraskeletal myxoid chondrosarcoma Desmoplastic small round cell tumour Rhabdoid tumour Perivascular epithelioid tumour, malignant Intimal sarcoma Ossifying fibromyxoid tumour, malignant Myoepthelial carcinoma Undifferentiated sarcoma Spindle cell sarcoma, undifferentiated Pleomorphic sarcoma, undifferentiated Round cell sarcoma, undifferentiated

1.1.1. Leiomyosarcoma (LMS)

Leiomyosarcomas are malignant tumours emerging from smooth muscle cells with an incidence ranging between 10% and 20% of all newly diagnosed STS (16). Smooth muscles belong to the non-voluntary controllable musculature. The tumours can origin in a wide variety of locations but are most commonly found in the retroperitoneum and proximal extremities. Histologically, they are identified by structural resemblance to smooth muscle cells but can also undergo dedifferentiation (17, 18). Smooth muscle actin and desmin-positivity is usually found upon immunohistochemical staining. This, and the absence of Tyrosine protein kinase (KIT) expression distinguishes LMS from GIST, an important differential diagnosis (19). Compared with other STSs, LMS is known to be genetically complex, with unbalanced karyotypic defects being the only shared features observed among LMS subtypes (20). Some LMSs can arise from the tunica intermedia of larger veins, for example inferior vena cava or the renal or iliac veins (21). Tumour size and the revised *American Joint Committee on Cancer* (AJCC) staging play a crucial role in prognosis of LMS patients. Tumour size $\geq$5 cm and high AJCC staging have been shown to be related to poor prognosis (22, 23).

1.1.2. Myxofibrosarcoma (MFS)

Myxofibrosarcomas represent another group of mesenchymal neoplasms. Formerly described as a myxoid variant of malignant histiocytoma in 1977, MFS has recently become a distinct subgroup thanks to immunohistochemistry and molecular biology (24). MFS is described as one of the most aggressive types of STS generally characterized by its non-pathognomonic and high heterogeneity of histological features. This heterogeneous presentation may lead to misdiagnosis in first place as case reports have demonstrated (25, 26). Cells sharing fibroblastic as well as histiocytic features observed in the examination under the light microscope are common within MFS (27). High risk for local recurrence because of the very aggressive loco-regional behaviour and a significant metastatic potential further worsens prognosis of patients with MFS. Most MFS are found in the extremities followed by the trunk as well as the head and neck region (28).

1.1.3. Synovial Sarcoma (SS)

Despite their name, synovial sarcomas do not originate from nor have morphological similarities to the synovium. They are assumed to differentiate from an undefined mesenchymal cell and are associated with a poor prognosis (29). With an incidence of 5 to 10% of all STS (30), SS constitutes a rare tumour entity. They are detected at any age, yet most patients are aged between 15 and 35 years, which is younger than the 50 to 60 years of age in whom other STS subtypes are most frequently diagnosed (31). Clinical behaviour and outcome vary in patients with SS. Most of the time, slow growth is observed, and patients complain about symptoms that have already persisted for months up to years (32). However, the lesion may also be described as a deep-seated mass with the possibility of local pain and tenderness, restricted mobility and paraesthesia (33). SS can occur at any site but nearly 90% of cases appear within the extremities, of whom 30% are located close to the knee. The origin within a joint or a bursa (34) is rather rare with less than 5%. More usual is an occurrence remote from joint or joint-near structures as reviewed by *Cyril Fisher et al.* (33). Tumours that do not occur within the extremities – for example at the head or neck – are associated with a worse prognosis (35). Especially in the lower limbs, SS may present with scattered calcifications that can be seen radiographically, being typical for this histological subtype (36). SS tend to recur and metastasize blood-borne most commonly to lungs, but also bone and other organs (37). Approximately 20% of patients already present with metastases at the first diagnosis but usually they occur two to five years after resection. From a histological point of view three different SS can be differentiated: The monophasic type with spindle cells with no or very few glandular epithelial cells, the biphasic type with spindle cell and epithelial components, and the poorly differentiated type which is identified by its distinctive immunohistological, ultrastructural and cytogenetic characteristics (33). Pathognomonic and therefore an important diagnostic genetic marker is the translocation t(X;18)(p11.2;q11.2) encoding for a number of various fusion proteins which are found in over 95% of patients (2).

1.1.4. Liposarcoma (LPS)

Liposarcomas are a group of malignant tumours arising from adipocytes. LPS account for approximately 15-20% of all STS and can be further classified into four distinct subtypes according to their status of differentiation and myxoid or pleomorphic character (2). In general, LPS are typically found in the extremities while WDLPS, DDLPS and pleomorphic LPS can also present in the retroperitoneum (38). WDLPS show low metastatic potential and if completely removed by excision are associated with favourable prospect of cure. DDLPS is a more aggressive and high-grade lesion often developing within primary WDLPS tumours (39). While good prognosis is expected for WDLPS, prognosis of DDLPS is poorer, with high rates of local recurrence and metastasis. Well-differentiated and dedifferentiated variants of LPS share morphological and molecular features. In the retrospective analysis of *Jones et al*., analysing 88 patients suffering from different types of LPS, myxoid LPS was found to be significantly more sensitive to chemotherapy (CTX) compared to other LPS subtypes, in particular WDLPS and DDLPS (40). Localized atypical lipomatous tumours (ALT) and WDLPS are known to exhibit considerably different behaviour compared to other LPS subtypes and were found to be radiosensitive to some extent when analysing the influence of adjuvant radiotherapy (RTX) on local recurrence (LR) risk of ALT/WDLPS (41). One common genetic feature found within all subtypes of LPS are amplified segments of 12q13-15 containing several cancer-related genes (42). Amplification of the *MDM2* is found in nearly 100% of patients suffering from WDLPS or DDLPS together with a co-amplification of *CDK4* in over 90% of patients. The two genes, encoding key regulators for the negative regulation of tumour protein 53 (TP53) and regulation of the G1/S cell cycle checkpoint represent important tumour-promoting genes (43).

A pathognomonic t(12;16)(q13;p11) translocation is found in over 90% of patients suffering from myxoid liposarcomas (MLPS) (44). Compared to WDLPS and DDLPS, myxoid forms of LPS show noticeable higher chemo- and radiosensitivity. Curative treatment of localized LPS consists of wide local resection with clear surgical margins. The pleomorphic variant of liposarcoma (PLS) is the rarest of the LS subtypes and exhibits aggressive behaviour (45). Although cytogenetic and molecular descriptions of these tumours are limited to a few cases, all present

complex genomic imbalances characterized by multiple chromosome duplications, complex rearrangements, and deletions (46). However, the progressive understanding of the molecular pathology – in particular in advanced stages of disease – has led to development of novel systemic, subtype-tailored therapeutic approaches for treatment of distinct LPS variants (38).

1.1.5. Undifferentiated Pleomorphic Sarcoma (UPS)

The undifferentiated pleomorphic sarcoma was considered the most common soft tissue sarcoma subtype in adults, previously termed malignant fibrous histiocytoma. The designation of these STS as malignant fibrous histiocytoma was removed according to the WHO in 2002 because it is not evident if these lesions display fibroblastic and/or histiocytic differentiation (47). Nowadays, mesenchymal stem cells are thought to be the most likely origin of UPS (48). Cytogenetic and immunohistochemical analyses of lesions previously classified as MFH have demonstrated that a number of those tumours actually relate to other types of STS (49). UPS is a high-grade aggressive STS subtype emerging within soft tissues, in the retroperitoneum, and - most commonly – the extremities (50). It is usually diagnosed in patients aged between 50 to 70 years, without gender preference. The most important factors to improve overall prognosis of patients suffering from UPS are early diagnosis and establishment of an adequate treatment according to stage, location and size of the tumour. With increasing size of the lesion, the probability for recurrence and metastases subsequently rises (51). Nowadays, distinct diagnosis of UPS is obtained by excluding other well-classified soft tissues sarcomas by histopathological, immunohistochemical, and molecular biological examination (52).

1.1.6. Spindle Cell Sarcoma (SCS)

Spindle cell sarcomas belong to the subgroup of undifferentiated/unclassified STS, characterized by the absence of a specific line of differentiation. Subsets of this group comprise pleomorphic, round cell, and spindle cell variants defined by their histomorphology (2). Only a few sporadic case reports and retrospective case series have been published about SCS in the medical literature due to their infrequent occurrence (53). For this reason, there is a deficiency of basic information on tumour incidence, specific clinical features, treatment outcomes, and disease-specific

prognostic factors. SCS are most commonly diagnosed in the respiratory system, but also occur in similar sites to other undifferentiated/unclassified sarcomas including the head and neck, extremities, or retroperitoneum (54).

1.1.7. Angiosarcoma (AS)

Angiosarcoma is a rare but aggressive endothelial cell malignancy of lymphatic or vascular onset and therefore can develop in any body region. AS accounts for <1% of all STS and is categorized as primarily cutaneous, parenchymal or visceral, deep soft tissue, lymphedema-associated, and post-irradiation AS. As few as 10% of all AS originate in deep soft tissue (55). AS can occur at any age, however, they are more common in the elderly, with the average age ranging from 60 to 70 years (56). There are noted risk factors for development of AS; the two most prominent are chronic lymphedema and previous radiation therapy. Additional factors may include familial syndromes, environmental chemical toxin exposition, and foreign bodies (55, 57). A high rate of LR and metastasis is evident (58). AS spreads primarily hematogenous, with the lung being the most common site for metastasis. Clinically, AS of the extremities and chest or abdominal wall usually appear as fast-growing, palpable masses, while deeper masses such as peritoneal, retroperitoneal, and mediastinal ones are accompanied rather by pain or discomfort related to the mass's size oppressing surrounding organs (59). Overall, AS have a poor prognosis with a 5-year survival rate of 35% (55). Treatment options include surgery with wide margins, radiation therapy, and CTX, but outcomes vary widely and depend on tumour location, size, resectability, and type (i.e., de novo or radiation induced) (60).

1.2. Grading and Staging

1.2.1. Grading

Pre-resection biopsy is normally used for pathological grading of the lesion. For unequivocal determination of grading, cytologic preparations only were found to be inadequate (61). It should be pointed out that grading is done in the context of the histologic type and subtype of a lesion. The criteria for assessing histologic features such as mitotic activity and necrosis cannot be universally applied to all STS. For example, alveolar rhabdomyosarcomas and extra skeletal Ewing sarcomas are

always classified as high-grade lesions, whereas WDLPS and dermatofibrosarcoma protuberans are classified as low grade (61).

In the grading system developed by the *French Federation of Cancer Centres* (FNCLCC), three different histological parameters are required to determine tumour grade; (1) tumour differentiation, (2) mitotic count, and (3) pattern of tumour necrosis. The occurrence of metastases and the survival rate of patients are highly correlated with these criteria (62). A score between 1 and 3 is given for features tumour differentiation as well as mitotic count, and a score between 0 and 2 for tumour necrosis.

1.2.2. Staging

Adequate and thorough histologic diagnosis and staging are crucial for accurate therapeutic decision making, improved patient outcomes, development of effective multimodality treatment programs, and comparison of treatment outcomes from different centres. A number of staging systems based on clinicopathologic classifications (63) and prognostic factors (64) have been established over the years. Nevertheless, the *AJCC/International Union Against Cancer* (UICC) (65) is the most accepted STS classification system worldwide, and recently the 8th version of their cancer staging system has been released (66). The AJCC STS staging system is a mixed clinico-pathological algorithm including excision and pathologic assessment of the primary tumour as well as regional lymph node metastases and distant metastases (DM). Tumour size and grade of the lesion are the essential parameters in the 8th AJCC staging system. The staging of STS differs from that of carcinomas by the inclusion of the histologic grade. This is of significance as the biological behaviour of STS mainly depends on this particular feature.

Tumour size (T) is categorized into T1 tumours, which are less than or equal to 5.0cm, T2 tumours, which are >5.0cm but ≤10cm, T3 tumours, including neoplasms >10cm but ≤15cm, and T4, referring to STS greater than 15cm in greatest dimension. These are determined by radiologic or physical examination. In the 8th edition (66) of the AJCC classification no further categorization according to depth is included as seen in the previous 7th edition (67). A predictive variable for its overall

outcome in patients with STS is the presence (N1) or absence (N0) of regional lymph node involvement (61).

The AJCC staging system applies a four-stage scheme for grading, starting from G1 (well-differentiated) to G4 (poorly differentiated or undifferentiated). Classification of the disease as M1 at first presentation is the most important prognostic factor for survival (66, 68–70).

Table 2: AJCC TNM Classification for STS – Adapted from *Amin, Greene et al.* 2017 - Staging STS (66).

Primary Tumour (T)	
T1	Tumour ≤ 5cm in greatest dimension
T2	Tumour > 5cm and ≤ 10cm in greates dimension
T3	Tumour > 10cm and ≤ 15cm in greates dimension
T4	Tumour > 15cm in greatest dimension
Regional Lymph Nodes (N)	
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
Distant Metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis
Histological Grade (G)	
G1	Well-differentiated
G2	Moderately differentiated
G3	Poorly differentiated
G4	Poorly differentiated or undifferentiated

Tumour Size	Lymph Node	Metastasis	Grade	Stage
T1	N0	M0	G1	IA
T2	N0	M0	G1	IB
T3	N0	M0	G1	
T4	N0	M0	G1	
T1	N0	M0	G2, G3	II
T2	N0	M0	G2, G3	IIIA
T3	N0	M0	G2, G3	IIIB
T4	N0	M0	G2, G3	
any	N1	M0	any	IV
any	any	M1	any	

1.3. Diagnostic Evaluation

The following brief description of the diagnostic approach will refer to the recommendations of the ESMO Clinical Practice guidelines for soft tissue and visceral sarcomas (71), the NCCN Clinical Practice Guidelines in Oncology Soft Tissue Sarcoma (3) and the recommendation described in a review article by *Ryan et al*. (72).

1.3.1. Clinical Presentation

For patients with any suspicion of STS, the first examination begins with a thorough clinical examination, which should precede diagnostic imaging. The typical appearance of an STS can be described as a painless and enlarging mass, whilst a history of tumour growth is not persistently described (73). High-grade tumours tend to grow rapidly over weeks or months, whereas low-grade tumours tend to grow slowly over months and years. Approximately 60% of STS occur in the extremities, but can develop anywhere in the body, particularly the extremities, trunk, retroperitoneum, or the head and neck (73).

In the medical history it should be ascertained when the mass was first noticed, whether the size has changed and if so, how quickly, and whether the patient has noted any other symptoms. Clinical examination focuses on the size and depth of the mass, fixation to adjacent structures, related oedema or signs of nerve entrapment. Furthermore, the most appropriate imaging modality should be determined. However, diagnosis of STS is often delayed as – on the one hand – patients with painless swellings do not consult a doctor immediately, and – on the other hand – delays occur by doctor's mistaken assumption of benignity (32). Within the diagnostic process, it is of great importance to first determine whether the lesion is more likely benign or malignant. In the case of soft tissue tumours, benign lesions occur about 100 times more frequently than malignant ones (2). The most sensitive clinical parameter to assess malignity is tumour location beneath the fascia, followed by tumour size >5 cm and rapid growth (74).

1.3.2. Radiological Presentation

In case a growing and suspicious soft tissue mass is present, imaging should be performed to narrow differential diagnoses and prevent an unplanned excision of the potential malignancy. A variety of imaging techniques are utilized to assess the local and regional extent of the lesion or the presence of metastases. In case of a primary tumour of the extremities, trunk, head or neck, cross-sectional imaging with MRI is preferred, as especially the soft tissue components can be depicted (75). For sarcomas occurring primarily in the retroperitoneum, abdomen, or viscera, computer tomography (CT) may first be used due to artefacts occurring upon MRI.

Furthermore, the ESMO recommends for all initially diagnosed STS, irrespective of site of occurrence, a spiral chest CT to detect potential metastases (71). Positron emission tomography (PET) and integrated PET/computed tomography (PET/CT) are imaging technologies mainly used in patients with suspected tumour recurrence. Furthermore, PET has been shown to be a suitable tool to discriminate between benign and malignant STS with best sensitivity for high-grade sarcomas (76). However, PET/CT is not routinely recommended upon diagnosis of STS (77). Apart from being the most sensitive initial diagnostic tool, MRI is also regarded the most valuable imaging technology for the detection of LR (78).

1.3.3. Biopsy

Histological examination is essential for diagnosis and further treatment planning. For tissue sampling, different methods can be used, such as core needle biopsy (CNB), fine needle aspiration (FNA) (79), and incisional biopsy (IB) considered as the gold standard (72). The standard procedure is either CNB, which is favoured by some centres, or IB. Excisional biopsy – meaning complete resection of the lesion without prior sample taking – is only performed in case of superficial lesions with the size smaller than 5 cm and in acral areas (80). In case negative margins are obtained upon excisional biopsy, the rate of LR is expected to be around 8% (81). There are several advantages of CNB over open biopsy, including short time to diagnosis, cost-effectiveness, and reduced number of patient visits required. Disadvantages of CNB arise from potential diagnostic inaccuracy or limited access to deep-seated masses, as well as limited amount of material to be used for histopathological, immunohistochemical, and molecular pathological examination. Therefore, the preferred method of biopsy taking is still controversially discussed among pathologists and clinicians (82, 83). Disadvantages of open biopsy include wound complications, tumour spread, and inappropriate incisions with regards to later surgery (84). Biopsy is ideally performed after imaging, as post-procedure oedema and radiologic artefacts complicate the interpretation of MRI. Notably, in one study, CNB correctly distinguished benign from malignant lesions with a probability of 97.5%, correctly determined histologic grading in 86.3%, and successfully achieved subtype subdivision in 88% (85).

1.4. Therapy / Treatment of STS

As seen from previous discussion concerning diagnosis and management of STS, it is highly recommended that all patients with suspected or diagnosed STS are taken under supervision by a sarcoma multidisciplinary team (MDT) at a specialist centre consisting of core members including a specialist sarcoma surgeon working in close collaboration with a reconstructive surgeon, sarcoma imaging specialists for MRI, CT and potentially PET, pathologist, clinical oncologist and palliative care specialist (86, 87).

The three main aims following biopsy are to (1) obtain enough tissue for evaluation of the soft tissue mass on histological, immunohistochemical and molecular pathological level, (2) determine the surgical approach and tissue to be sacrificed upon wide resection and (3) minimize patient morbidity (88). The standard treatment for STS includes surgical resection with wide margins, often accompanied by neoadjuvant or adjuvant RTX. In case of high-grade tumours and lesions exhibiting metastatic potential, neoadjuvant or adjuvant CTX should be considered.

1.4.1. Surgical Resection

Surgical resection is the standard treatment for patients suffering from STS. As outlined in Table 3, four different types of surgical margins are distinguished and summarized in the Enneking classification of STS tumour margins. This classification closely correlates type of margin with risk for LR (89, 90).

Due to high incidence of LR observed when only parts of the tumour are removed by intralesional excision, the so-called ‘shell-out’ procedure with margins running through the malignant tumour tissue is nowadays only used in the palliative setting (91). Marginal resection with removal of the tumour together with its pseudo-capsule and parts of the surrounding reactive zone is still associated with increased risk for LR. Radical excision equals resection of the tumour and all the affected compartments, which often results in amputation of extremities. Due to the observation that tumour clearance achieved by amputation does not significantly increase overall survival (OS), preservation of the affected limb is sought to improve the patient’s post-operative quality of life (92). Therefore, the recommended margin

for surgical resection of STS is wide local resection. This corresponds to *en bloc* excision of tumour together with the surrounding reactive zone and a safety margin (93).

Table 3: Four types of surgical margins according to the Enneking classification (adapted from *A. Misra et al.* [87]).

Resection margin	Description	Local recurrence (LR) risk
intra-lesional	The margin runs through the tumour - tumour will remain to some extent.	80 - 100%
marginal	The surgical plane runs through the pseudo-capsule (reactive zone).	40 - 60%
wide surgical	The plane of excision is through normal tissue, but within the same anatomical compartment as the tumour.	10%
radical	The tumour and affected compartments are removed together.	0.5%

The definition of the margin for surgical resection is also dependent on relative resistance of the adjacent healthy tissue against STS growth. The high resistance against expansion of the lesion into the deep fascia or periosteum acts as a natural barrier. Thus, a thinner margin may be accepted if formed by muscular fascia or periosteum than a margin formed by loose adipose tissue only. Thus, in contrast, adipose and muscle tissue are known for their low resistance to tumour growth and therefore an extended resection margin of 2 to 3 cm into healthy surrounding tissue is necessary to minimize LR risk (87). In case of more advanced STS, surgical resection may be preceded by neoadjuvant RTX and/or CTX, which eventually facilitates limb-salvage surgery.

1.4.2. Radiotherapy (RTX)

Radiotherapy as treatment prior or after wide surgical resection of STS can further improve local control rate. Adjuvant RTX may permit preservation of critical limb structures in close vicinity to the tumour by eventually destroying remnant tumour cells and thus reducing LR risk (91). Although adjuvant RTX has been shown to effectively decrease LR rate to <5% compared to rates of up to 22% in patients without adjuvant RTX (94), its use could not reduce DM rate or improve OS (94). Altogether, the use of RTX after resection of STS is recommended for high-grade (G2-3) lesions located deep to the fascia and >5 cm in size (95, 96).

The effect of preoperative (neoadjuvant) RTX on the outcome of patients suffering from STS has also been investigated. A study by *O'Sullivan B. et al.* comparing the effects of neoadjuvant and adjuvant RTX found that LR rates and progression-free survival did not significantly differ between the groups (97). On the other hand, an increased rate of radiation-associated wound complications was observed for patients treated with neo-adjuvant RTX (97). One beneficial factor observed in this study was a lowered rate of late fibrosis and fewer toxic skin effects after neoadjuvant RTX compared with adjuvant treatment and therefore improved long-term functional outcomes (94).

1.4.3. Chemotherapy (CTX)

Despite promising results with surgical resection followed by adjuvant RTX, as discussed in the previous section, poor 5-year survival rates are still encountered for patients with high-risk STS. Therefore, the administration of adjuvant or neoadjuvant chemotherapy as a potential treatment to improve patient survival has gained attention of medical scientists. Up to now, the role and impact of adjuvant CTX in STS remains controversial due to high toxicity rates (98). However, the preoperative use of CTX has been shown to achieve beneficial results in the treatment of soft tissue neoplasms with metastatic character and if STS are found to be unresectable (98).

Nowadays, the use of neo-adjuvant CTX is in focus to improve the OS of patients suffering from high-grad STS. Despite the routine application of neo-adjuvant CTX in rhabdomyosarcomas and soft tissue Ewing's sarcomas (99), most STS show only low response to CTX (100). Chemosensitivity is increased in small tumours showing a higher growth rate. With increasing size of the lesion the advance of a chemo-insensitive clone of the tumour gets more probable (101).

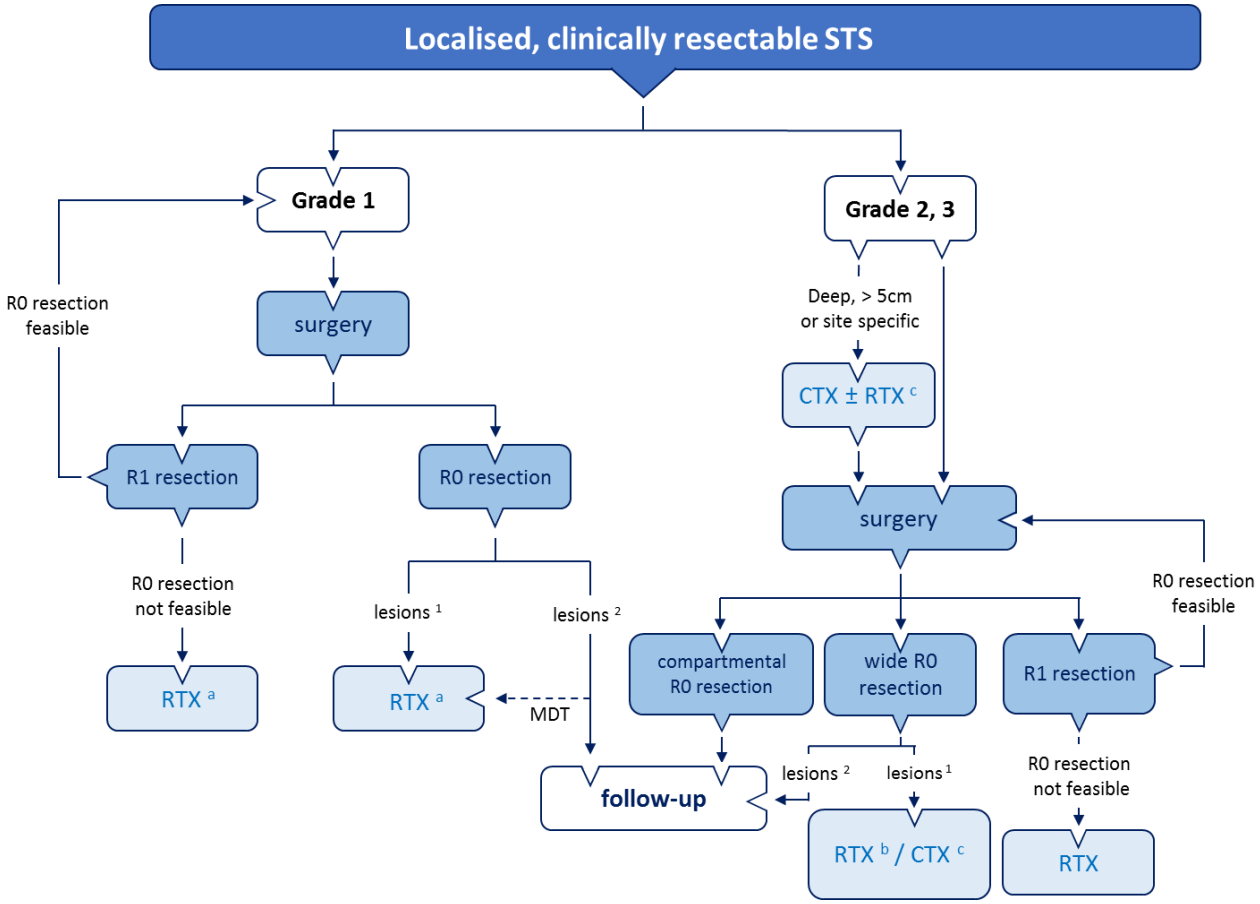

Figure 1: Management of localised, clinically resectable STS (Adapted from *Casali, Abecassis et al.* Soft tissue and visceral sarcomas [71]).

[a] RTX can be skipped in selected cases.

[b] RTX can be skipped in selected deep cases and added in selected superficial cases.

[c] Extremity and superficial trunk, G3, deep, >5cm.

[1] MDT risk assessment or selected > 5cm or deep lesions

[2] Superficial, <5cm lesions

R0, no tumour at the margin; R1, microscopic tumour at the margin; RTX, radiotherapy; CTX, chemotherapy; MDT, multidisciplinary team.

In Figures 1 and 2, the recommended management of local resectable and unresectable STSs is visualized, respectively. As mentioned before, for lesions found to be resectable, surgery with wide resection margins (no tumour at the margin, R0) is the preferred treatment option. The standard treatment of high-grade STS (G2 and G3) includes adjuvant RTX after surgical removal of the tumorous tissue.

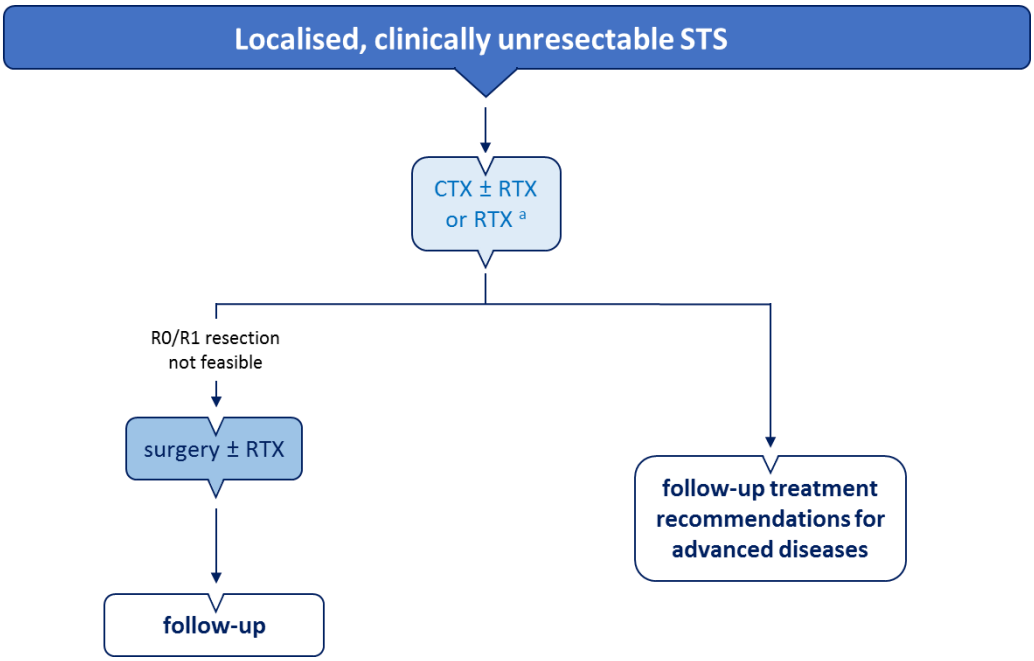

Figure 2: Management of localised, clinically unresectable STS. (Taken from *Casali, Abecassis et al.* Soft tissue and visceral sarcomas [71]).

[a] Optional: isolated limb perfusion in selected cases.
R0, no tumour at the margin; R1, microscopic tumour at the margin; RTX, radiotherapy; CTX, chemotherapy.

1.5. Biomarkers

Although progress has been made in respect of radiology, pathology, and surgery, STS still presents with high rates of LR and/or DM. Thus, STS are associated with high mortality. For instance, the overall 5-year survival rate for STS patients with primarily localised disease is 50-60% (102). Due to the wide variety of histologic subtypes and great diversity in presentation, soft tissue tumours are regarded as a diagnostic – and even more therapeutic – challenge. In this respect, molecular profiling is an important research field for the identification of STS subtypes, eventually improving the understanding of the biology of different malignancies and therefore allowing for more accurate molecular characterization of sarcoma subtypes (103).

The lack of useful and reliable therapeutic and prognostic biomarkers impairs treatment decision making. As mentioned before, detection and consequently response to tumour cells is mediated by different types of T lymphocytes infiltrating

the TME (104). The expression of immune checkpoint markers by tumour cells can establish tumour evasion from the hosts immune system and have a tremendous effect on the efficacy of cancer treatments (105).

Therefore, identification of e.g. immune checkpoint markers is warranted to improve prognosis and potentially invent novel therapeutic strategies for personalized treatment, especially for patients at high LR- and metastasis-risk. Furthermore, biomarkers may play a vital role in the investigation of underlying mechanisms that would explain why some patients are responding to therapies whereas others are not. Biomarkers exhibit high potential to be used as therapeutic markers, and eventually provide a basis for assessment of certain drugs' potential efficacy and toxicity (106).

Considerable efforts have been made in recent years to elucidate histotype-targeted therapies. Trabecedin used for myxoid or round cell liposarcomas and LMSs is one of the rare representative examples with promising results thus far. Regarding discovery of novel prognostic and predictive biomarkers in STS, two different aspects have to be considered: (1) pharmacogenetic properties exerted by biomarkers and (2) characteristics of the tumour microenvironment.

Discussing the cytogenetic presentation of tumour cells, STS can be classified into malignancies with simple or complex karyotypes, as shown in Table 4. The oncogenic mechanisms in tumours with simple karyotypes mainly reflect recurrent gene mutations and genomic rearrangements resulting in transcriptional deregulation or deregulated signalling (107). Although this group of STSs are defined by a low mutational burden, they present in a wide range of clinical behaviour. Furthermore, there are no novel therapeutic approaches successfully targeting chimeric transcription factors or alterations of the epigenetic transcription programme, such as DNA methylation, histone modification and chromatin remodelling (107, 108). STS with mutations of specific oncogenes also constitute simple karyotype malignancies. One of the most prominent representatives is an STS with deregulated kinase signalling. Tremendous progress has been achieved by the generation of various kinase inhibitors providing substantial clinical benefits in patients suffering from this type of tumours (107).

Some sarcomas exhibit a more complex karyotype and are defined by the presence of non-specific and nonrecurring alterations. The main characteristics of this class of neoplasms are high chromosomal instability, limited number of specific molecular markers and prevalent loss of distinct tumour suppressor genes, such as *TP53* and retinoblastoma protein (*Rb)* (107). However, from an immunogenomic perspective, the class of STS presenting a more complex karyotype shows high rates of immune cell infiltration within the tumour microenvironment. This is related to a potential of these tumours to respond to new immunotherapeutic agents, such as immune checkpoint inhibitors (ICIs) (109).

Table 4: STS Categorization into two groups according to their genomic complexity (110).

STS with Simple Karyotype	STS with Complex Karyotype
Clear cell sarcoma	Pleomorphic leiomyosarcoma
Synovial sarcoma	Undifferentiated pleomorphic sarcoma
Gastrointestinal stromal tumour	Myxoid fibrosarcoma
Angiosarcoma	Embryonal rhabdomyosarcoma
Myxoid liposarcoma	Dedifferentiated liposarcoma
Dermatofibrosarcoma protuberans	Peripheral nerve sheath tumour
Solitary fibrous tumour	
Alveolar rhabdomyosarcoma	

1.5.1. Characteristics of the Tumour Microenvironment (TME)

In the past, STSs have been considered to remain widely unrecognized by the host immune system. Employing next generation sequencing (NGS) technologies, such as transcriptomic sequencing together with proteomic-based approaches has connected tumour genetics with the tumour immune profile. Investigation of the TME by determination of the expression profile of immune-related genes, immune cell abundance, and even identification of the immune cell repertoire present within the tumorous tissue itself has highly contributed to the knowledge and understanding of the oncological mechanisms of STS (111). Besides genetics and proteomics, antibody-based staining of specific proteins expressed by tumour-infiltrating cells using immunohistochemistry (IHC) remains one of the most often employed techniques for the investigation of the TME (112, 113). Invention of multiplex-IHC

enabling analysis of a number of different markers on a single slide at the same time has added to the value of this technology for future research (114).

The main components of the TME can be divided with regards to their physical and biological nature with close interaction to each other. Tissue types together with structures of the extracellular matrix (ECM) define the architecture of the tumour (115). Tumour cells can modify ECM protein signalling to their favour by recruiting endothelial cells for angiogenesis within tumour tissue, providing nutrient supplies. This secures energy needed for tumour growth and proliferation (116). Additionally, signals released by cells in their immediate vicinity are crucial to the character of the TME. Among these cells are stromal cells including fibroblasts potentially contributing to development of therapeutic resistance (117). The TME is further characterized by infiltration of immune cells such as lymphocytes and macrophages. Presence and quantity of certain immune cells are used as prognostic and predictive biomarkers.

1.5.1.1. B cells

B cells that are responsible for the production of antigen-specific immunoglobulins, play an elementary role in the TME. Antibody-dependent cell cytotoxicity and phagocytosis against neoplastic cells are the main mechanisms induced by B cells (118). As a specific marker, expression of the *MS4A1* gene encoding for CD20+ can be determined using transcriptomics analysis. Identification of B cells can also be carried out by IHC analysis via antibodies specific for CD20+. Numerous studies consistently show that high levels of B cell infiltration is associated with improved prognosis in STS (119, 120). However, high levels of B cell involvement is only observed in about 18% of sarcomas (121). In general, STS show low abundance of CD20+ expressing cells or they are only found in the tumour's periphery, as in rhabdomyosarcoma (122).

1.5.1.2. T cells

A crucial role in the immune response against cancer cells is carried out by so-called tumour-infiltrating lymphocytes (TILs). T lymphocytes are major components of the adaptive immune system and are involved in direct killing of infected and tumorous cells, activation of other immune cells, and the production and regulation of immune response (123, 124). Due to their crucial role in various mechanisms for detection, defence, and clearance of cancer cells, different types of T cells are infiltrating the tumorous tissue (104). The biomarker presented on the cell surface of any T cell is CD3+. Using multiplex IHC, targeting various T cell biomarkers at the same time enables for deconvolution of different types of T cells found in the tumour's microenvironment including helper T cells (CD3+, CD4+), cytotoxic T cells (CD3+, CD8+), helper memory T cells (CD3+, CD4+, CD45RO+), and cytotoxic memory T cells (CD3+, CD8+, CD45RO+) (125). Additionally, the presence of regulatory T cells (Tregs, CD4+, CD25+, FOXP3+) and natural killer cells (NK, CD57+) can be obtained by multiplex ICH analysis (126).

Helper T cells (CD4+) and cytotoxic T cells (CD8+) represent the two main subpopulations of T lymphocytes found in the TME (127). Activation of these T cells by T cell receptors (TCR) binding to the Multihistocompatibility complex (MHC) II complex of antigen presenting cells (APCs) triggers the adaptive immune response (124). As with CD20+, the presence of CD8+ and CD4+ T cells is likewise correlated with better outcome and treatment response in STS patients (128), while the occurrence of FOXP3+ Tregs is correlated with an immunosuppressive response (129). Many studies have found a greater number of infiltrating CD8+ than FOXP3+ cells, which are particularly prevalent in newly diagnosed STS (130).

Tregs (positive for CD4, CD25, FOXP3) have been identified to restrain and regulate other immune cells by producing cytokines and by expressing inhibitory molecules. This suppression of the overall anti-tumour immune response ultimately results in promotion of tumour growth.

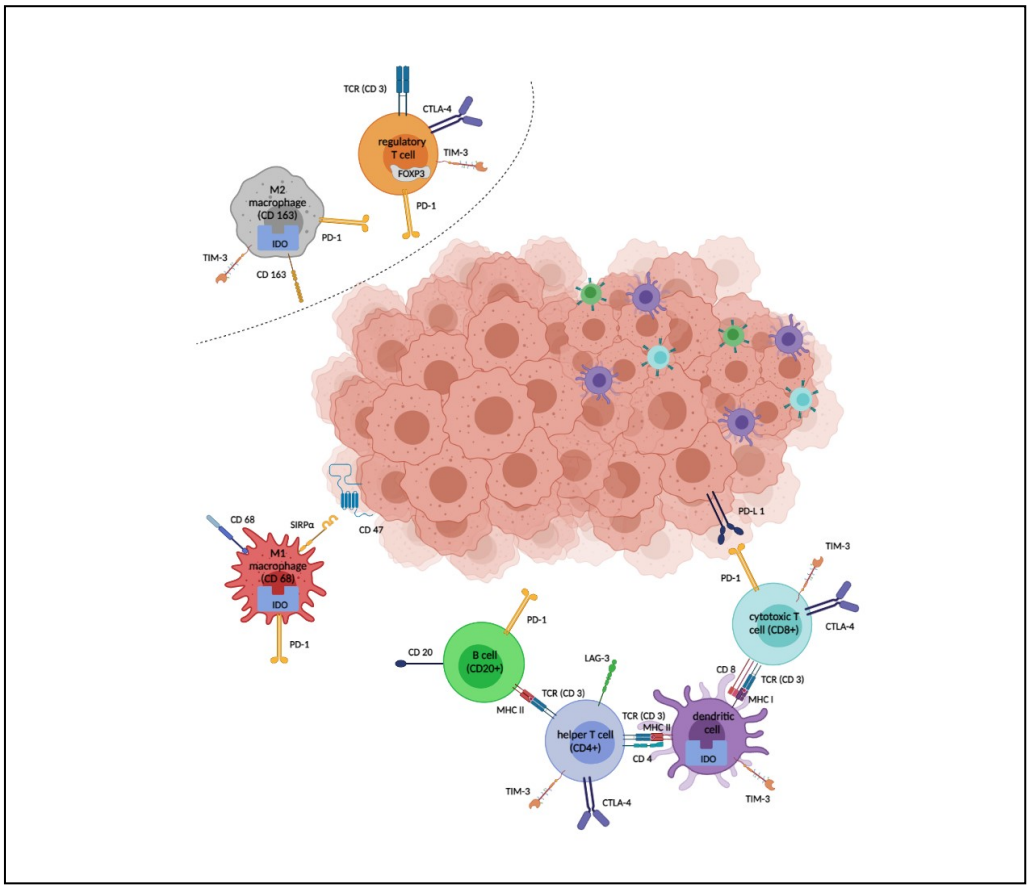

Figure 3: Schematic illustration of infiltrating immune cells and immune checkpoint markers identified in the tumour microenvironment of STSs (created with BioRender.com; adapted from *Zhu et al.* [131]) Infiltrating immune cells, macrophages, dendritic cells, T cells, and B cells exhibit anti-tumour responses, but potentially can be reprogrammed by the cancer cells showing immune-suppressive activities: PD-1/PD-L1, CTLA-4, TIM-3, LAG-3 and IDO. Two types of macrophages are present CD68+ (M1) with pro-inflammatory and anti-tumorous activity and CD163+ (M2) showing anti-inflammatory and pro-tumour effects. Dendritic cells activate CD8+ cytotoxic T cells through CD8/MHC I/TCR complexes and CD4+ helper T cells through CD4/MHCII/TCR complex. FOXP3+ regulatory T cells (Tregs) are immunosuppressive.

1.5.1.3. Macrophages

Another important type of cells found in the TME are so-called tumour-infiltrating macrophages (TIMs) often outnumbering TILs (132). The main function of TIMs are phagocytosis of altered host cells and regulation of tissue maintenance (133). Two subtypes of TIMs can be distinguished by the presence of different biomarkers, namely CD68+ and CD163+, specific for M1 and M2, respectively (120). Even though the M1 type (CD68+) exhibits proinflammatory and anti-tumour

characteristics, the far more common M2 type (CD163+) shows immunosuppression by recruiting Tregs. Therefore, the presence of macrophages at higher levels is considered a marker for poor prognosis and reduced OS (134).

Certain proteins expressed by activated lymphocytes, macrophages, monocytes, and dendritic cells are responsible for modulation of immune response and are collectively termed immune checkpoints. Immune checkpoints identified to exhibit anti-inflammatory and immune suppressive effects in STS include the programmed cell death-1 (PD-1/PD-L1) axis, co-receptor cytotoxic T-lymphocyte antigen 4 (CTLA-4), the enzyme 2,3-dioxygenase (IDO), the T cell immunoglobulin and mucin domain 3 receptor (TIM) signalling, and the lymphocyte activation gene-3 receptor (LAG3) interaction as shown in Figure 3. Lately, another immune checkpoint under investigation is the receptor signal regulatory protein α (SIRPα)/CD47 pathway (135). The identification of these immune checkpoints has opened the avenue for the development of novel immunotherapeutics following a personalized treatment approach (136).

1.5.2. Immune Checkpoint PD-1/PD-L1

In recent years, immunotherapy has been considered a promising treatment option, targeting immune checkpoint molecules such as PD-1 and its ligand (PD-L1) or cytotoxic T-lymphocyte antigen-4 (CTLA-4). With immunotherapy, significant results have been achieved in late-stage melanoma, lung, kidney, and bladder cancer (137). Following these promising results, immune checkpoint molecules as PD-1/PD-L1 and CTLA-4 have gained interest of researchers as potential prognostic and predictive biomarkers in high grade STS (hSTS). It is well established that the prognosis of a malignant tumour is strongly associated with the immune response of the host (138). An indicator of a productive anti-tumour response of hosts' immune system is the immigration and activation of TILs as well as a balance of positive and negative signalling pathways (139).

Priming and consequently the activation of T cells are critical processes playing a vital role in the initiation of an immune response by the adaptive immune system. The resulting amplitude of this response to foreign structures found in infected or tumorous cells is regulated by the expression of checkpoint molecules responsible

for a balanced immune reaction. Positive signals ensure that altered host cells are properly identified, targeted, and cleared by the immune system while negative signalling pathways prevent attack of healthy host cells and therefore establish self-tolerance (140, 141).

An upregulation of negative signals that are initiated by ligand interactions with cell-surface molecules such as PD-1 and CTLA-4 is dependent on tumour-specific antigen recognition (142, 143). However, expression of PD-1/PD-L1, CTLA-4, and markers of the associated TME have rarely been investigated in STS, (144) and identification of novel therapeutic targets for personalized treatment is still at an early stage.

PD-1 belongs to the CD28 receptor family and is bound by its two ligands PD-L1 or PD-L2, both members of the B7 family. PD-1 is expressed by activated B cells, T cells, and natural killer cells and presented on their surface. The ligands PD-L1 and PD-L2 are found on peripheral tissue cells, APCs, and tumour cells as membrane bound proteins (145). The main function of the PD-1/PD-L1 receptor-ligand interaction in the human body is protection against excessive immune response. By limiting sustained T cell activation during chronic inflammation, infection or cancer, the PD-1/PD-L1 pathway has been identified as a key player in limitation of autoimmunity (146). In cooperation with anti-tumour immune response by cytotoxic TILs (CD8+) that are able to kill tumour cells directly, and other subpopulations of CD3+ T cells, such as helper T cells (CD4+), negative signalling of checkpoint markers balance the overall amplitude of adaptive immunity allowing suppression of tumour growth and preventing autoimmunity at the same time (142, 141).

Tumour cells can use the PD-1 signalling pathway to evade immune control expressing PD-L1 and PD-L2 on their surface. The PD-1/PD-L1 interaction between cancer cells and activated TILs inhibits proliferation, survival, and cytokine release of lymphocytes (147). Further immune inhibitory effects mediated by receptor-ligand interaction include induction of tumour-specific T cell apoptosis (148) and resistance of tumour cells to CD8+ T cell attack (149). Consequently, PD-L1 expression by tumour cells and interaction with PD-1 of T cells in the TME results in inactivation of T cells and escape from the host's adaptive immune system (144).

The level of PD-L1 expression is associated with poor prognosis in a variety of different tumours (148). Furthermore, the clinical response correlates with PD-L1 expression by tumour cells (TCs) and tumour infiltrating lymphocytes (TILs). Another important predictive factor is the presence of high levels of tumour-specific T cells in the TME. Therefore, these immune checkpoint markers are potential targets for therapeutic intervention. In accordance with recent findings, the absence of T cell immigration implies weak to no response to the use of so-called immune checkpoint inhibitors (ICIs) (142).

PD-L1 expression has been shown to be of prognostic significance in several tumour entities. However, the role beyond this prognostic value remains uncertain (148). Due to the heterogeneous patient sample in various studies, the found correlation between clinical features, OS, and PD-L1 expression detected in the TME is limited (150). High levels of PD-1/PD-L1 expression have been associated with improved clinical event in head and neck squamous cell carcinoma (HNSCC). In line with this finding, the lack of significant PD-1/PD-L1 expression indicates worse clinical outcome in gastric cancer, whereas for malignant melanoma (MM) altered PD-1/PD-L1 expression has been correlated with both positive as well as negative clinical outcomes. In cervical cancer (CC), no association between PD-1/PD-L1 abundance with clinical outcome could be found (151).

Additionally, CTLA-4 (cytotoxic T lymphocyte antigen 4) has also been identified as potential target for immune checkpoint inhibitors. CTLA-4 is an inhibitory co-receptor expressed on the surface of T cells. Interaction with ligands found on the cell membrane of APCs leads to downregulation of T cell activity (152). The inhibitory signalling results in prevention of the priming phase of the immune response within the lymph nodes (138). As seen for PD-L1 and PD-L2, the ligand of CTLA-4 is also a member of the B7 family (152).

2. Aim of the Study

STS represent a rare and heterogeneous tumour entity that include diverse histologic subtypes of mesenchymal origin. Depending on size, histology, grading, and location, the prognosis of patients with STSs varies. Oncological treatment outcome focusses on rate of LR, DM, disease-specific survival (DSS) and OS. Well-known prognostic factors for LR and DM in STS are tumour size, grading, histology, and localization (10, 63).

In recent years, the importance of the immune system in tumour defence, development and maintenance of cancer has been discovered. Thus, the presence of tumour-infiltrating immune cells plays a crucial role in the defence or maintenance of the microenvironment of various malignancies of epithelial origin, such as non-small cell bronchus carcinoma or renal cell carcinoma. In mesenchymal neoplasms as STS, the importance of the immune system is less certain, though.

In this study, IHC and multispectral imaging are used to investigate a correlation between presence and amount of immune checkpoint markers, as well as tumour-infiltrating lymphocytes, and outcome of patients with STS. Immune checkpoints are special molecular switch points in the host's immune system, whose (de)activation have a significant influence on various processes in the immune response. Among others, there are PD-1, PD-L1, and CTLA-4, which all act as immune checkpoints (140).

The aim of this study is to correlate PD-L1 and PD-1 expression within the TME with already known clinical prognostic factors and outcome of patients with STS. T cell subpopulations will likewise be differentiated using markers CD3, CD4, CD8, and FOXP3. Both the incidence of recurrence (local or DM) and OS will be investigated. Immune checkpoint markers are expected to correlate with outcome in patients with STS or with other known clinico-pathogenetic risk factors as seen for other cancers, recently. Immunotherapy has achieved substantial success in the treatment of patients with tumours, but its effectiveness in sarcomas remains to be explored.

3. Materials and Methods

For this retrospective analysis exploring the potential of immune checkpoint markers to be used as prognostic factors for LR and DM-risk, as well as OS, all patients having undergone surgical resection of localized STS with curative intent at one single institution were potentially eligible. Over 400 patients were treated with curative intent between 1998 and 2016 at the *Department of Orthopaedics and Trauma, Medical University of Graz, Austria* and the *Division of Clinical Oncology, Internal Medicine, Medical University of Graz, Austria*. Due to expected alterations in the immune phenotype abundance induced by preoperative treatments, 10 patients having received neoadjuvant CTX, and 4 patients having been treated with neoadjuvant RTX were excluded. The resulting 192 patients found eligible for this study had a median age of 63.5 years (interquartile range [IQR]: 49.5-76.0 years) and included 103 males (53.6%). The median timespan of follow-up after surgical treatment was 45.5 months (IQR: 19.0-99.0 months).

3.1 Clinical Data

Preoperative MRI scans and histopathological reports were used to determine maximal tumour size. Using the *Union International Contre le Cancer* (UICC) guidelines (153), margins of resection were defined, discriminating between R0, R1, and R2. Negative margins containing at least 1mm of healthy tissue between the surface and tumour tissue are defined as R0. R1 and R2 margins include marginal margins with healthy tissue of <1mm thickness separating surface and tumour and contaminated margins where tumour cells are found directly at the surface, respectively. In the statistical analysis, R0 margins were compared with R1 and R2 margins as one single group. Definition of histological subtypes was done following the World Health Organisation Classification of Tumours of Soft Tissue and Bone 2013 (154). Based on the frequency of present histologies, six different categories were differentiated, being MFS, SS, UPS, LPS and “Others”, where rare histological subtypes were grouped together. A complete list of all different sarcoma subtypes included in this study is found in Table 5 (155).

Grading of tumour entities was done according to the *French Federation of Cancer Centres Sarcoma Group* (FNCLCC) grading system, differentiating low grade (G1), intermediate grade (G2), and high grade (G3) STS (63).

Table 5: Complete list of frequency of histological STS subtypes included in this study (155).

Histological STS Subtype	Frequency	Percent (%)
Myxofibrosarcoma (MFS)	78	40.6
Undifferentiated pleomorphic sarcoma (UPS)	32	16.7
Liposarcoma (LPS)	22	11.5
Leiomyosarcoma (LMS)	22	11.5
Synovial sarcoma (SS)	12	6.2
"others"	26	13.5
Frequency of Rare Histological Subtypes: "Others"	**Frequency**	**Percent (%)**
Spindle cell sarcoma	5	19.2
Angiosarcoma	3	11.5
Fibrosarcoma, NOS	3	11.5
Low-grade Fibromyxosarcoma, NOS	3	11.5
MPNST	2	7.7
Clear cell sarcoma	2	7.7
Alveolar soft part sarcoma	2	7.7
Soft tissue sarcoma, NOS	2	7.7
Dermatofibrosarcoma, NOS	1	3.8
Pleomorphic rhabdomyosarcoma	1	3.8
Psammomatous melanotic schwannoma, malignant	1	3.8
Extraskeletal myxoid chondrosarcoma	1	3.8

Postoperative follow-up examinations including clinical inspection, local MRI scans, as well as alternating computed tomography (CT) scans of the thorax and chest x-rays were conducted on a regular basis. In the first three years after surgical resection patients underwent follow-up every 3 months. In the subsequent years 4 and 5, follow-up examinations were scheduled twice a year, and once a year thereafter. Recurrence observed on MRI scans or by histopathological analysis after repeated excision at the original tumour site was defined as LR, whereas tumour seeding far from the original tumour site was defined as DM. The time interval from surgery to diagnosis of LR or DM, or last follow-up was defined as time to LR and DM. Timespan from surgery to last follow-up or ultimately death was defined as time

to last follow-up. Before initiation of the current study, approval by the local institutional review board (IRB-approval number: 29-205 ex 16/17) was obtained.

3.2 Construction of Tissue Microarrays for IHC Analyses

Prior to construction of tissue microarrays (TMAs) for following IHC analyses, all initially included paraffin-embedded tumour samples (n=206) were revalued independently by two specialized soft tissue tumour pathologists. The next step was to punch paraffin blocks at regions indicated as tumour areas to be representative of the histological subtype into cores of 4µm thickness. The obtained 1621 cores were transferred to five recipient paraffin blocks. From this TMA-paraffin blocks, sections of 3-5µm were cut out, which were used for the analysis of immune checkpoint markers and the abundance of TILs in the TME using multiplex IHC. Throughout the pre-procession and cutting, 541 cores were lost due to damage resulting in 1080 cores finally analysed. These amounted to 7 cores per tumour sample on average to be analysed and evaluated.

3.3 Multiplex Immunohistochemistry Analysis (MP-IHC)

DAPI (4',6-diamidino-2-phenylindole) was used as fluorescent dye for the labelling of cell nuclei and therefore identification of separate cells withing the TMA-cores. Six antibodies conjugated to fluorescent dyes of different colours were used for MP-IHC. To increase sensitivity, the tyramide signal amplification (TSA) kit (Akoya Biosciences, Marlborough, USA) was used for MP-IHC. Reagents used for the identification and detection of immune checkpoint markers and TILs are listed in Table 6 (155).

Table 6: Reagents used for multiplex immunohistochemistry (MP-IHC, [155]).

Target / Marker	Antibody	Additional Information
PD-1	clone NAT105	Abcam plc, Cambridge, UK, ab234444
PD-L1	clone 22C3	Dako, M3653
FOXP3	clone D6O8R	part of Opal 7 TIL Kit from Akoya Biosciences, OP7TL3001KT)
CD3	clone LN10	Leica Biosystems Inc., Vienna, Austria, NCL-L-CD3-565
CD4	clone EP R6855	Abcam plc, Cambridge, UK, ab133616
CD8	C8/144B	Abcam plx, Cambridge, UK, ab75129

3.3.1. Positive and Negative Controls

According to the recommendations of the manufacturer, all monoclonal-specific primary antibodies (listed in Table 6) were tested individually following monoplex staining on control tissue as positive control setup. Furthermore, testing on non specific binding to avoid false-positive results, staining for the secondary antibodies was performed on human tissue as a negative control. This is of great importance to minimize false-positive results in the actual MP-IHC analysis. The complete multiplex panel including all six fluorescence-coupled antibodies was checked on formalin-fixed paraffin-embedded sarcoma tissue sections prior to application and staining of the constructed STS TMA sections.

3.3.2. Staining and Scanning of TMA Slides

TMA slides were stained using the autostainer system *Bond RX* (*Leica Boisystems Inc., Vienna, Austria*) and subsequent scanning was performed on the *Vectra*® 3 (*Akoya Biosystems, Marlborough, USA; software version 3.0.7*) microscope. In the first step, whole-slide scans were recorded for identification and localization of individual TMA cores withing the TMA slides at 4-fold magnification. Increasing the spectral resolution by zooming in up to 20-fold magnification multispectral images of selected TMA cores were obtained. This results in one image of the same magnification for each individual antibody-coupled fluorescent dye (in total 6 different images per TMA section).

3.3.3. Image Analysis

Obtaining one overall image covering the entire TMA slide was achieved by stitching Individual images recorded at 20-fold magnification together. Image processing, including spectral deconvolution and removal of autofluorescence was done by employing the *inform* software (*Akoya Biosystems, Marlborough, USA; software version 2.4.8*.). For further analysis and evaluation of multispectral images, the *HALO® Image Analysis Platform* (*Indica Labs, Albuquerque, NM, USA; version 3.1.1076.342*) was applied.

As mentioned before, identification of individual cells was done using DAPI for staining of cell nuclei setting a threshold for the following parameters: nucleus size, roundness, and signal intensity. Based on the signal intensity obtained for the entire cell thresholds for the six fluorescence-labelled markers were defined.

Based on the combination of the three immune cell markers CD3, CD4, and CD8, four different TIL phenotypes were identified to be present in all TMA cores as listed in Table 7. Furthermore, cells expressing the immune checkpoint markers PD-1, PD-L1, or marker FOXP3, were also identified in TMAs. Moreover, three additional phenotypes could be determined by assessment of the co-expression of PD-1 and PD-L1, also shown in Table 7. Furthermore, differentiation of TILs expressing either PD-1 or PD-L1 was performed (see Table 7). Additionally, cells negative for CD3, but found positive for expression of PD-1 or PD-L1 were distinguished.

Table 7: List of cell phenotypes differentiated by multiplex immunohistochemistry (155). Three different CD3+ T cell phenotypes (PD-1+ or PD-L1+ or none); two different CD3+/CD4+ helper T cells, CD3+/CD8+ cytotoxic T cells, and CD3+/CD4+/FOXP3+ Tregs (PD-1+ or none); two cell phenotypes CD3- (PD-1+ or PD-L1+) and four additional cells positive for different immune checkpoint markers (PD-1+/PD-L1+ or PD-1+/PD-L1- or PD-1-/PD-L1+ or FOXP3+).

TIL marker	T cell subtype	Immune checkpoint markers / Phenotypes		
CD3+	T cell	PD-1+	PD-L1+	
CD3+ / CD4+	helper T cell	PD-1+		
CD3+ / CD8+	cytotoxic T cell	PD-1+		
CD3+ / CD4+ / FOXP3+	regulatory T cell	PD-1+		
CD3-	non T cell	PD-1+	PD-L1+	
-----	all cells	PD-1+	PD-L1+	FOXP3+
-----	all cells	PD-1+ / PD-L1+	PD-1+ / PD-L1-	PD-1- / PD-L1+

All 17 different cell phenotypes were counted automatically. Initially, cell counting was performed individually for each TMA core and results were combined for cores originating from the same tumour tissue sample, consequently corresponding to one patient. Presence of TIL phenotypes together with cell levels expressing immune checkpoint markers in percent of total cell count were calculated from the combined results for the same person to minimize the bias of local fluctuations when looking at only one single core.

The cut-off values for tumour samples showing “low” or “high” density of distinct TIL phenotypes and cells expressing immune checkpoint markers were defined as <1% or >1% of total cell count within the descriptive analysis (see Table 8-10 in the results section). Notably, statistical analyses were done based on median values rather than means, due to non-normal distribution of the presence of cell phenotype levels across the whole patient cohort.

3.4 Statistical Analysis

All statistical analyses were carried out with *Stata Version 16.1* for Mac (*StataCorp, College Station, Texas, US*). Descriptive analysis used means with corresponding standard deviations (SDs) for normally distributed continuous variables, and medians with corresponding interquartile ranges (IQRs) for non-normally distributed continuous variables. Quantitative differences in immune phenotype abundances between patient subpopulations and binominal variables and were assessed using Wilcoxon’s rank sum test. Differences between patient subpopulations and categorical variables were assessed with Kruskal Wallis test. For analysis of multiple comparators, post-hoc Dunn-tests (with Benjamini-Hochberg adjustment for multiple comparisons) were carried out (156). Associations between TILs and immune checkpoint markers were assessed with Spearman’s rank correlation coefficient.

Univariate and multivariate Fine&Gray models for LR and DM were constructed to assess the potential impact of prognostic variables, using time to LR/last follow-up, or time to DM/last follow-up. Death was entered as the competing event for both models. In order to investigate a potential impact of variables on OS, uni- and multivariate Cox-regression analyses taking time from surgery to last follow-up or death, were carried out. Any factor with a *p*-value <0.01 in the univariate time-to-event models was entered in the multivariate models, as long as the one-in-10 rule was respected (157). A *p*-value of <0.05 was considered as being of statistical significance.

4. Results

The results of this diploma thesis, that will be subsequently presented, have been published in the *British Journal of Medicine* by *Smolle et al.* (155).

4.1 Patient Characteristics

As postoperative treatment, 117 (63.9%) patients were administered adjuvant RTX, and 20 (10.4%) patients received adjuvant CTX. By the last follow-up appointment, 123 (74.1%) patients were alive, while 69 (35.9%) patients had died. More detailed information on patient characteristics and follow-up are listed in Table 8 (155).

Table 8: Summary of general patient characteristics (155).

Age at Diagnosis		
Years [median ± IQR]	63.5	49.5 - 76.0
Gender		
Male	103	53.6 %
Female	89	46.4 %
Adjuvant Therapy		
Radiotherapy	117	63.9 %
Chemotherapy	20	10.4 %
Follow-up		
Months [median ± IQR]	45.5	19.0 - 99.0)
Status		
Alive	123	74.1 %
Died	69	35.9 %

As mentioned in the methods section (Table 5) and visualized in Figure 4, STS were classified into six different groups according to the frequency of histological subtype. The most frequent histological presentation was MFS (n=78; 40.6%) followed by UPS (n=32; 16.7%) as well as LPS (n=22; 11.5%) and LMS (n=22; 11.5%). In total, 26 STS (16.7%) of rare histological subtype were summarized in the subgroup “Others” including SCS, AS, fibrosarcoma (NOS) and low-grade fibromyxosarcoma (NOS) being the most frequent rare STS counting for 5 (19.2%), 3 (11.5%), 3 (11.5%), and 3 (11.5%), respectively.

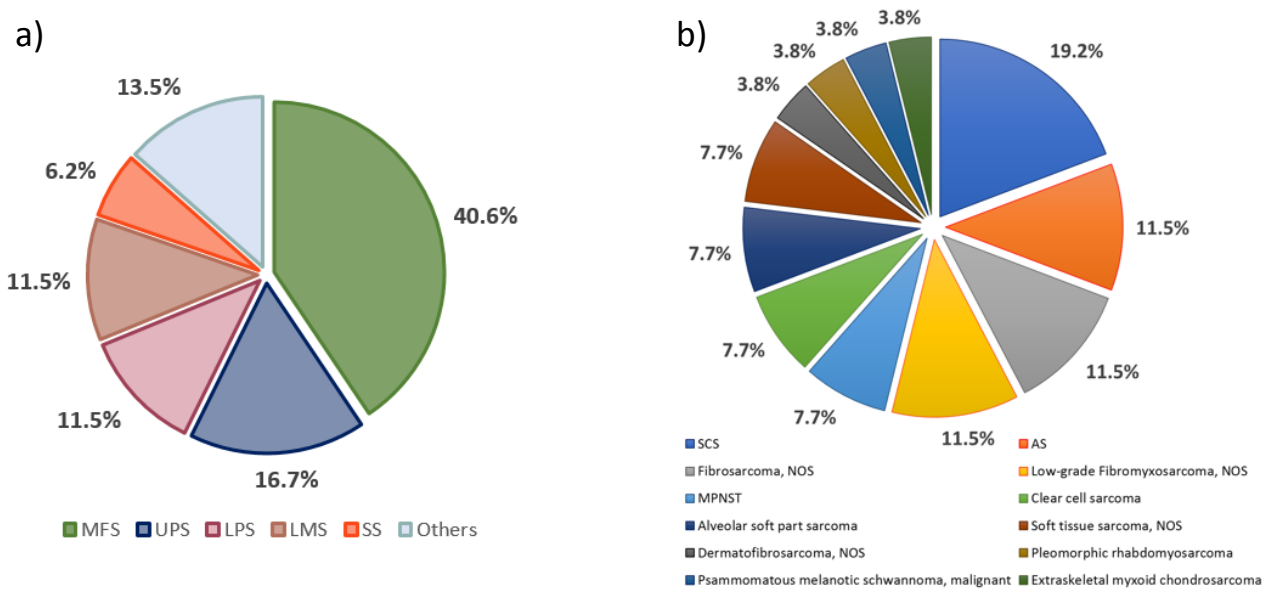

Figure 4: Distribution of histological subtypes (155). a) Classification of tumour samples include 6 different histological subtypes which are used for comparison. b) Detailed information on histological subtype distribution of rare STS encountered in this study and summarized as "Others". NOS = not other specified; MFS = myxofibrosarcoma; UPS = undifferentiated pleomorphic sarcoma; LPS = liposarcoma; LMS = leiomyosarcoma; SS = synovial sarcoma; SCS = spindle cell sarcoma; MPNST = malignant pleomorphic nerve sheath tumour.

4.2 Tumour Characteristics

According to preoperative MRI scans and histopathology reports, mean tumour size was 8.5±5.2cm. Regarding tumour grade, the majority of STS 132 (72.5%) were classified as G3 compared to 17 (9.4%) grouped together as G1 and 33 (18.1%) as G2. Irrespective of subtype all STSs were located in the upper or lower limb as well as in the trunk, with a distribution of 48 (25.1%), 125 (65.5%), and 18 (9.4%), respectively.

More than half of the tumours (n=111; 57.8%) were located deep to the fascia. According to the definition of surgical margins by the UICC, most patients (n=146; 76.0%) had undergone resection with R0 margins. Further information on tumour characteristics is shown in Table 9 and visualized in Figure 5.

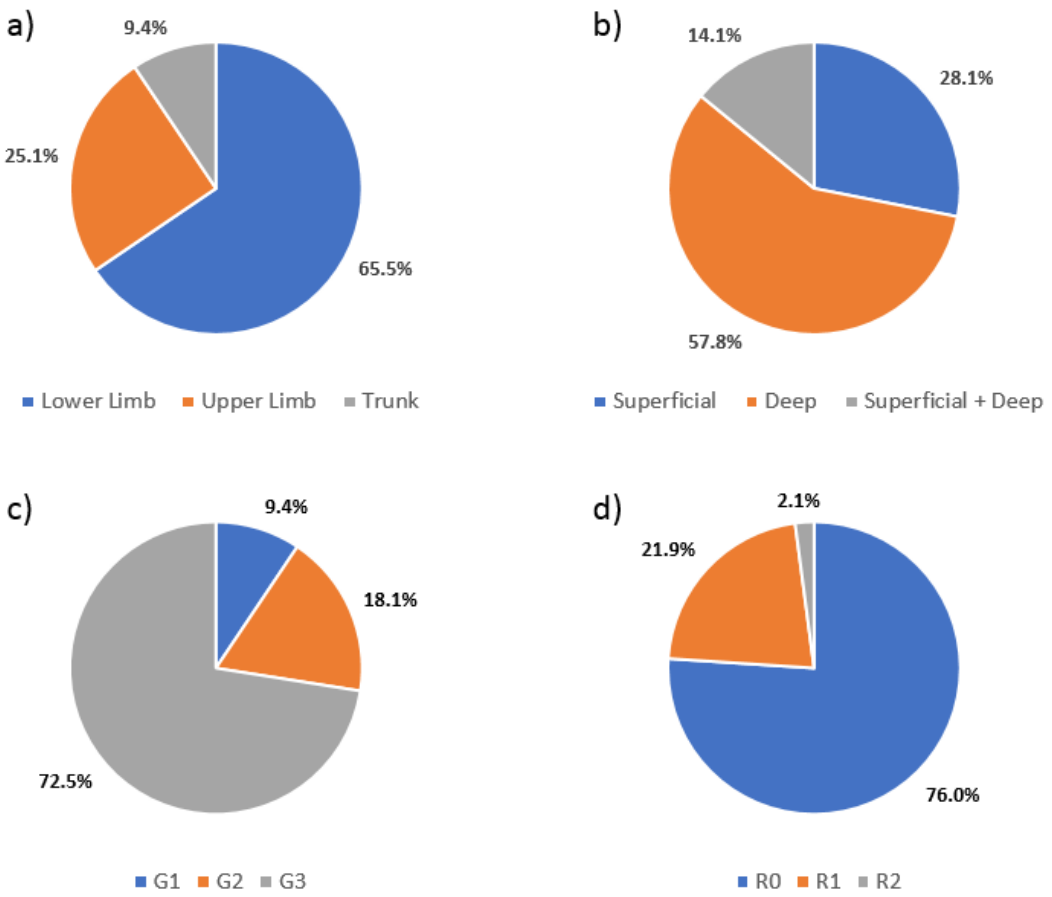

Figure 5: Representation of the distribution of clinical data and tumour characteristics included in this thesis: a) Location of the neoplasm; b) depth of tumorous tissue; c) grade of the STS; d) surgical margins achieved in the resection (155).

Table 9: Summary of clinical data and tumour characteristics (155).

Size in cm [mean ± SD]		8.5 ± 5.2	
Location	Upper Limb	48	25.1 %
	Lower Limb	125	65.5 %
	Trunk	18	9.4 %
Depth	Superficial	54	28.1 %
	Deep	111	57.8 %
	Superficial + Deep	27	14.1 %
Grade	G1	17	9.4 %
	G2	33	18.1 %
	G3	132	72.5 %
Margins	R0	146	76.0 %
	R1	42	21.9 %
	R2	4	2.1 %

4.3 Tumour Microenvironment Characteristics

4.3.1. Immune Checkpoint Markers

In the automated count of multiplex IHC analyses, the most abundant marker was FOXP3, accounting for a median of 2.6% (IQR: 0.38-16.85%) positive cells of total cell count, followed by PD-L1 and PD-1 with 0.82% (IQR: 0.06-5.27%) and 0.57% (IQR: 0.13-1.70%). Concerning density, in 122 (63.5%) of STS samples, FOXP3+ abundancy exceeding >1% of total cell count was present. This density was termed as “high”, in comparison to <1% termed as “low” density. For PD-L1, in approximately half of the tumour samples (93; 48.4%) a high abundance of PD-L1+ cells (>1%) was observed, whereas only one third of STS (n=63; 32.8%) had PD-1 expression in >1% of cells. Moreover, in the assessment of co-expression patterns of PD-1 and PD-L1, the most prevalent subtypes were PD-1-/PD-L1+ (0.59%; IQR: 0.05-5.54%) and PD-1+/PD-L1- (0.47%; IQR: 0.01-1.51%). Co- expression of PD-1 and its ligand PD-L1 was observed in 0.01% (IQR: 0.00-0.17%) of total cell count.

Table 10: Quantitative and qualitative immune checkpoint marker and TIL phenotype abundances (155). Results of phenotype percentages are presented as median and interquartile range (IQR) against all cells.

All Cells							
Checkpoint Markers	cell type	[% of all cells]	(IQR)	Density (n, %)			
				low < 1%		high > 1%	
PD-1+	cells	0.57	(0.13-1.70)	129	67.2	63	32.8
PD-L1+	cells	0.82	(0.06-5.27)	99	51.6	93	48.4
PD-1+ / PD-L1-	cells	0.47	(0.01-1.51)	176	91.7	16	8.3
PD-1- / PD-L1+	cells	0.59	(0.05-5.54)	144	75.0	48	25.0
PD-1+ / PD-L1+	cells	0.01	(0.00-0.17)	189	98.4	3	1.6
FOXP3+	cells	2.60	(0.38-16.85)	70	36.5	122	63.5
Tumour-Infiltrating Lymphocytes (TILs)							
TIL Markers	cell type	[% of all cells]	(IQR)	Density (n, %)			
				low < 1%		high > 1%	
CD3+	T cells	1.02	(0.30-3.15)	95	49.5	97	50.5
CD3+ / CD4+	helper T cells	0.11	(0.01-0.47)	167	87.0	25	13.0
CD3+ / CD8+	cytotoxic T cells	0.20	(0.03-0.82)	150	78.1	42	21.9
CD3+ / CD4+ / FOXP3+	regulatory T cells	0.05	(0.00-0.30)	177	92.2	15	7.8

4.3.2. Tumour-Infiltrating Lymphocyte Subpopulations

Of all T cell subpopulations, CD3+ T cells represented the most abundant subset with a median abundance of 1.02% (IQR: 0.30-3.15%) of total cell count. In other words, in half of the tumour samples (n=97; 50.5%) more than 1% of CD3+ T cells in comparison to the entire cell count were present. Regarding the other cell types, cytotoxic T cells, helper T cells, and regulatory T cells, MP-IHC analyses determined a median abundance of 0.20% (IQR: 0.03-0.82%), 0.11% (IQR: 0.01-0.47%), and 0.05% (IQR: 0.00-0.30%), respectively. A detailed representation of the quantitative abundance of immune checkpoint markers and TIL phenotype distribution can be found in Table 10. Representative images of different MFS obtained from the MP-IHC analyses and examined with multispectral imaging analysis are shown in Figure 4.

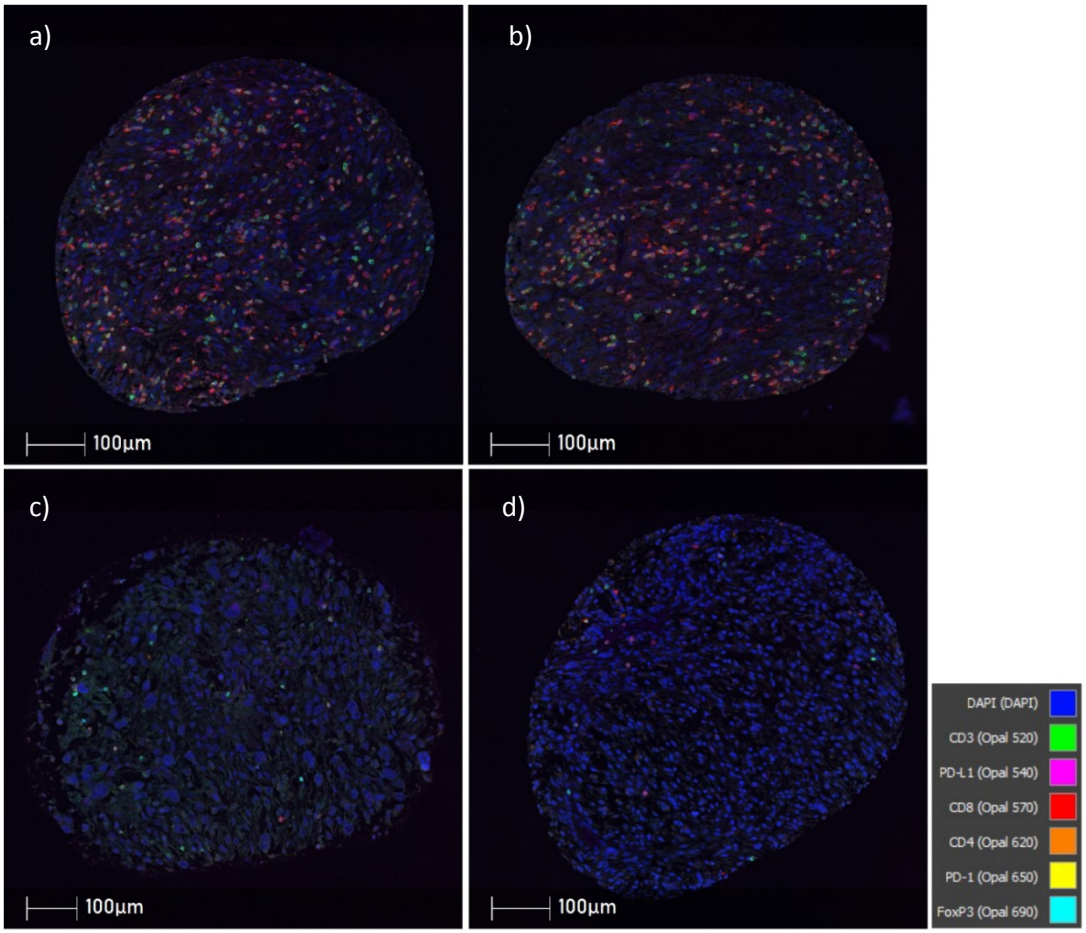

Figure 6: Comparison of multispectral images of four myxofibrosarcoma TMA slides (155). a) / b) high immune cell infiltration rate and high levels of immune checkpoint marker expressing cells; c) / d) low abundance of TILs and low levels of PD-1+ and/or PD-L1+ cells.

4.3.3. PD-1 and PD-L1 Expression by Various TIL Subtypes

Upon analysis of immune checkpoint marker (PD-1 and PD-L1) expression in different TIL subtypes, in 20 (10.4%) tumour samples high abundance (>1% of all counted cells) of PD-1+/CD3+ T cells was observed, whereas in 11 (5.7%) samples only, high levels of PD-L1+/CD3+ T cells were found. For CD3+/CD4+ helper T cells and CD3+/CD8+ cytotoxic T cells, only one tumour sample was found to have more than 1% of cytotoxic T cells also positive for PD-1. Moreover, high levels (i.e. >1%) of CD3-/PD-1+ and CD3-/PD-L1+ cells were observed in 47 (24.5%) and 36 (18.8%) tumour samples, respectively. Further details on the combined analyses of TIL marker and immune checkpoint marker expression are shown in Table 11.

Table 11: Immune checkpoint marker expressing TIL subtype abundances (155). Results of phenotype percentages are presented as median and interquartile range (IQR) against all cells.

Tumour-Infiltrating Lymphocytes (TILs) expressing Checkpoint Markers							
Checkpoint markers	cell type	[% of total cells]	(IQR)	Density (n, %)			
				low < 1%		high > 1%	
PD-1+	T cells	0.03	(0.00-0.38)	172	89.6	20	10.4
PD-L1+		0.15	(0.02-0.71)	181	94.3	11	5.7
PD-1+	helper T cells	0.00	(0.00-0.03)	191	99.5	1	0.5
PD-L1+		----	-----	-----	-----	-----	-----
PD-1+	cytotoxic T cells	0.00	(0.00-0.12)	191	99.5	1	0.5
PD-L1+		----	-----	-----	-----	-----	-----
PD-1+	regulatory T cells	0.15	(0.02-0.71)	192	100.0	0	0.0
PD-L1+		----	-----	-----	-----	-----	-----
Checkpoint Marker-Positive CD3-Negative Cells							
Type of cells		[% of total cells]	(IQR)	Density (n, %)			
				low < 1%		high > 1%	
PD-1+	CD3-	0.53	(0.04-4.69)	145	75.5	47	24.5
PD-L1+	CD3-	0.21	(0.05-0.76)	156	81.3	36	18.8

4.4 Comparison of TIL Abundance in Patient Subpopulations

4.4.1. Difference in TIL Abundance According to Patient Characteristics

Based on median age all patients included in this study were divided into two groups (≥63.5 vs. <63.5 years). Comparing the abundance of TIL phenotypes regarding age a significant increase in helper T cell (0.14 vs. 0.07%; *p*=0.030) and Treg (0.06 vs. 0.02%; *p*=0.010) count was observed in the older patient group versus younger patients. Moreover, in older patients (compared to younger patients), significantly higher levels of PD-1 expressing T cells (0.06 vs. 0.01%; *p*=0.013), helper T cells (0.01 vs. 0.00; *p*=0.010), and cytotoxic T cells (0.03 vs. 0.00%; *p*=0.019) were detected. The only PD-1+ TIL subtype level found to be not significantly different depending on patient age was the one of Tregs (*p*=0.116). A detailed view of the correlation between patient age and TIL abundance is highlighted in Table 12.

Table 12: Differences in TIL phenotypes according to patient age (155). Statistical significance is highlighted as bold *p*-values.

TIL Phenotype		≥ 63.5 years [% of total cells]	< 63.5 years [% of total cells]	*p*-value
CD3+	T cells			0.072
CD3+ / CD4+	helper T cells	0.14	0.07	**0.030**
CD3+ / CD8+	cytotoxic T cells			0.106
CD3+ / CD4+ / FOXP3+	regulatory T cells	0.06	0.02	**0.010**
PD-1+ / CD3+	T cells	0.06	0.01	**0.013**
PD-1+ / CD3+ / CD4+	helper T cells	0.01	0.00	**0.010**
PD-1+ / CD3+ / CD8+	cytotoxic T cells	0.03	0.00	**0.019**
PD-1+ / CD3+ / CD4+ / FOXP3+	regulatory T cells			0.116
PD-L1+ / CD3+	T cells			0.195

No significant difference in TIL subtype levels was observed regarding gender.

4.4.2. Difference in TIL Abundance According to Tumour Characteristics

Significantly higher levels of CD3+ T cells were found in the TME of G3 STS compared to G1 or G2 STS (G3 vs. G1/2: 1.31 vs. 0.78%; *p*=0.047). Depending on grading, no significant differences in other TIL phenotypes were observed. For other tumour characteristics such as location, depth, or tumour size, no significant alteration in TIL abundance was present.

4.4.3. Difference in TIL Abundance According to Histological Subtype

Levels of TIL phenotypes were compared between six histological subgroups, i.e. MFS, SS, UPS, LPS, LMS, and "Others" as outlined in Table 5. Significant differences (p<0.05) between all histological subtypes and TIL-abundance were found. All TIL phenotypes – except for PD-L1+/CD3+ T cells – showed significantly higher levels in MFS compared to SS, LPS, and LMS. In addition, PD-L1+/CD3+ T cells were more frequently found in the TME of MFS in comparison to SS and LMS. Regarding UPS, only levels of cytotoxic T cells and PD-1+ Tregs were significantly lower compared to MFS.

Furthermore, the UPS TME showed significantly increased infiltration rates of CD3+ T cells and CD3+/CD8+ cytotoxic T cells as well as PD-1+/CD3+ T cells and PD-1+/CD3+/CD8+ cytotoxic T cells compared to SS. In addition, also in LMS significantly higher amounts of CD3+ T cells were found compared to SS. Also, in UPS, increased levels of CD3+/CD8+ cytotoxic T cells were detected in comparison to LPS. All differences in TIL abundance according to histological subtype are visualized in Figure 5.

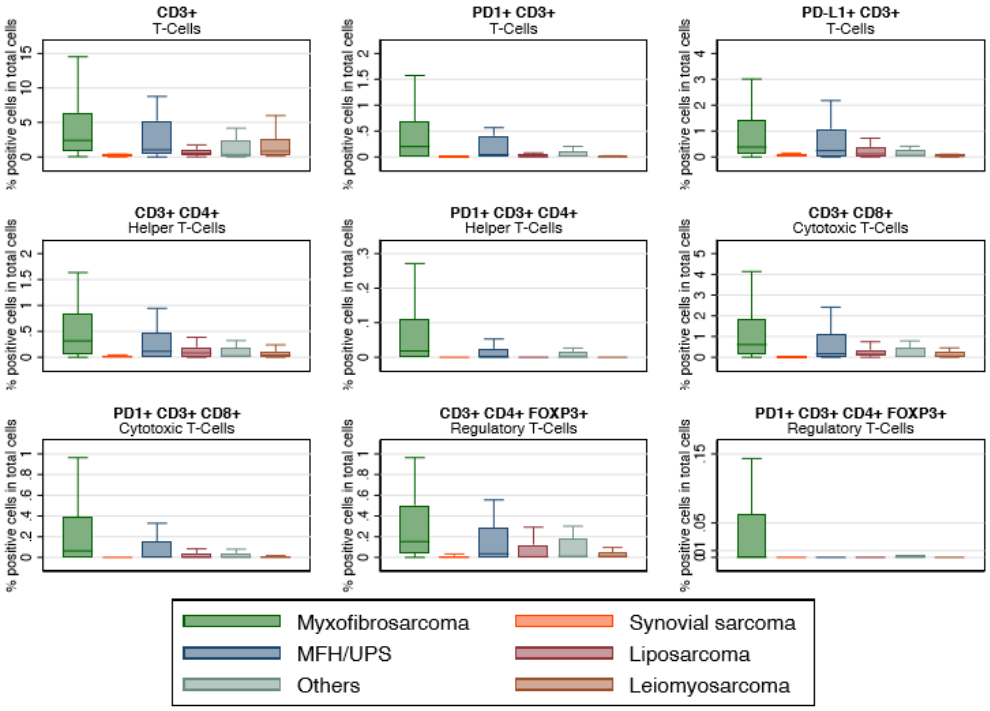

Figure 7: Difference in TIL phenotype infiltration rates depending on histological STS subtype. (Adapted from *Smolle et al.* [155]).

4.5 Comparison of Immune Checkpoints in Patient Subpopulations

4.5.1. Immune Checkpoint Markers and Patient Characteristics

Overall, significantly higher levels of PD-L1 expressing cells were present in older patients, including PD-L1+ cells (0.71 vs. 0.46%; p=0.047), PD-L1+/PD-1+ cells (0.03 vs. 0.00%; p=0.038) as well as CD3-/PD-L1+ cells (0.28 vs. 0.16%; p=0.014). Notably, no significant difference in infiltration rate was found for PD-L1+/PD-1- cells (p=0.074) depending on patient age.

Considering PD-1 expression, no significant differences between older and younger patients were found for PD-1+ cells (p=0.215), CD3-/PD-1+ cells (p=0.233), and PD-1+/PD-L1- cells (p=0.211). Furthermore, infiltration rates of FOXP3+ cells (p=0.860) were not significantly different depending on patient age (Table 13, [155]).

Table 13: Differences in immune checkpoint marker expressing cell levels in respect of patient age (155). Statistical significance is highlighted as bold *p*-values.

Immune Checkpoint Marker	≥ 63.5 years [%]	< 63.5 years [%]	*p*-value
PD-1+			0.215
PD-L1+	0.71	0.46	**0.047**
PD-1+/PD-L1-			0.211
PD-1-/PD-L1+			0.074
PD-1+/PD-L1+	0.03	0.00	**0.038**
CD3-/PD-1+			0.233
CD3-/PD-L1+	0.28	0.16	**0.014**
FOXP3+			0.860

Regarding gender, no significant difference in abundance of immune checkpoint marker positive cells was observed (all p>0.05). Moreover, according to tumour characteristics, including location, depth, tumour size, and grading, no significant alteration in infiltration rates of immune checkpoint marker positive cells was present.

4.5.2. Immune Checkpoint Marker Abundance Depending on Histology

Significant differences (p<0.05) between all histological subtypes, except for FOXP3+ and CD3-/PD-L1+ cells, were found regarding abundance of immune checkpoint marker positive cells. As highlighted in Figure 6, significantly higher levels of PD-1+, PD-L1+, PD-1-/PD-L1+, PD-1+/PD-L1-, and PD-1+/PD-L1+ cells were observed in MFS compared to LMS. Moreover, in MFS, significantly higher amounts of PD-1-/PD-L1+ and PD-1+/PD-L1+ cells were detected in comparison to SS.

Furthermore, significant higher levels of all different PD-1 expressing cells were found in the subgroup of rare STS termed “Others” compared to LMS. These include PD-1+ cells, PD-L1-/PD-1+ cells, and PD-L1+/PD-1+ cells. From the boxplot representation of immune checkpoint marker expression in different histological subtypes (Figure 6) a wide range of different expression levels of PD-1, PD-L1 and FOXP3 can be seen in the subgroup of “Others”. This may be explained by the inclusion of various rare STS subtypes with considerably different biological behaviours in one group.

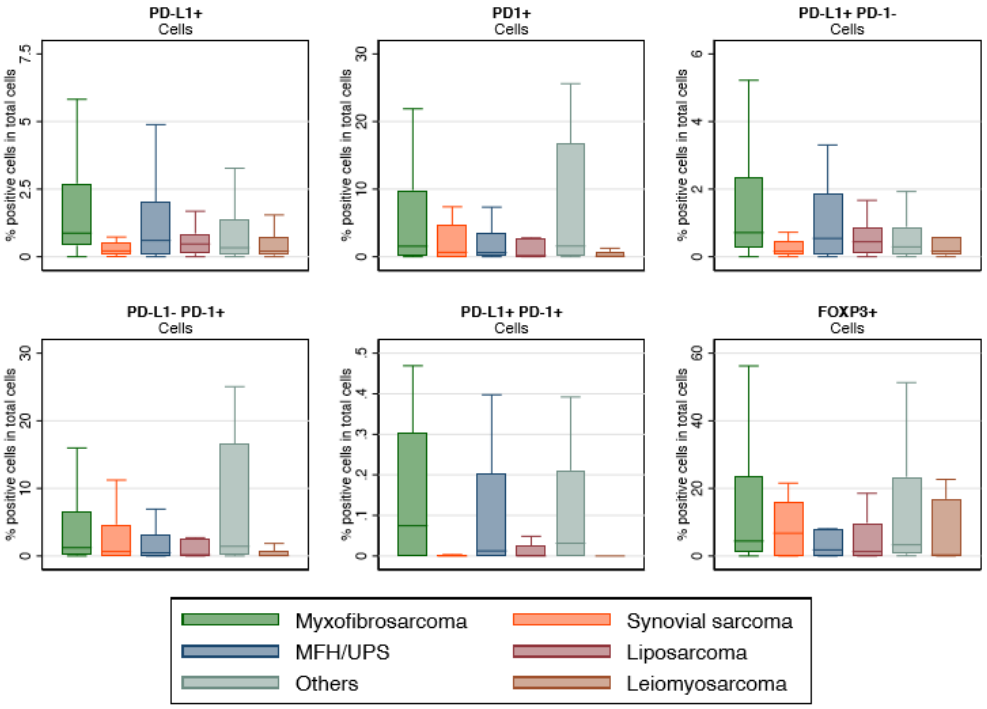

Figure 8: Difference in immune checkpoint marker positive cell infiltration rates depending on histological STS subtype. (Adapted from *Smolle et al.* [155]).

4.6 Correlation of Immune Checkpoint Markers and TIL Phenotypes

In the correlation analysis of TIL phenotype abundances, strong positive correlations between the presence of CD3+ T cells and CD3+/CD4+ helper T cells (rho=0.761), CD3+/CD8+ cytotoxic T cells (rho = 0.880), and CD3+/CD4+/FOXP3+ Tregs were found, as depicted in Figure 7 (155).

Correlation analyses of different immune checkpoint marker-expressing cells with TIL phenotypes showed a strong positive correlation between the abundance of PD-1+ cells and FOXP3+ cells (rho=0.737). Further specification of FOXP3 expressing cells led to observation of a weak positive correlation between the presence of PD-1+ cells and CD3+/CD4+/FOXP3+ Tregs (rho = 0.311).

Moreover, there was a moderate positive correlation between the presence of PD-L1+ cells and T cells, helper T cells, cytotoxic T cells, and regulatory T cells.

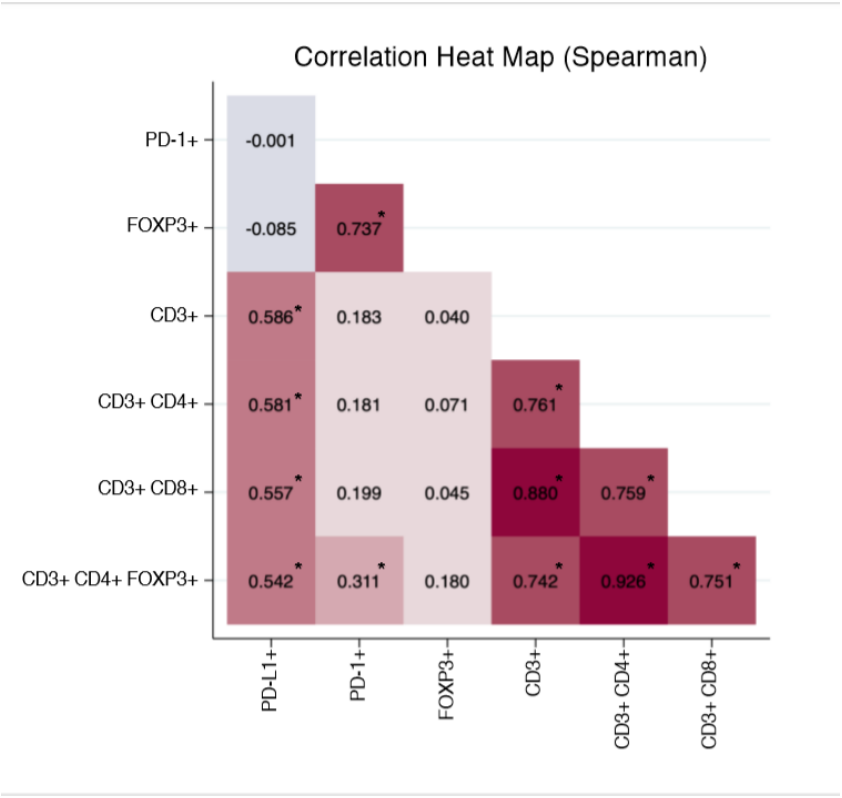

Figure 9: Correlation heat map presenting Spearman's rank correlation coefficients (rho) between immune checkpoint marker abundance and levels of TIL phenotypes (155). Significant correlations (p<0.001) highlighted with asterisk; greyish blue = very weak negative correlation; light rosa = very weak positive correlation; rosa= weak positive correlation; lila = medium positive correlation; ruby = strong positive correlation; dark ruby= very strong positive correlation.

4.7 Prognostic Value of Immune Checkpoints and TIL Phenotypes

4.7.1. Prognostic Impact on Risk of Local Recurrence (LR)

In the univariate analysis of the prognostic potential of clinical variables, immune checkpoint markers and the abundance of TIL phenotypes on LR risk, none of the patient characteristics (age, gender, receiving adjuvant treatment) were statistically significant. Tumour characteristics found to show a trend towards increased and decreased risk for LR are depth (HR: 0.40; 95% CI: 0.16-1.04; *p*=0.059 vs. superficial as ref.) and the surgical margin status (R1/R2: HR: 2.21; 95%CI: 0.96-5.10; *p*=0.063 vs. R0 as ref.;). However, no statistical significance was obtained.

Table 14: Multivariate competing risk regression analysis for LR including resection margins and Tregs (155). Statistical significance is highlighted as bold *p*-value.

Multivariate Analysis	**Local recurrence**		
	HR	**95% CI**	***p*-value**
R1/2 margins (vs. R0 as ref.)	2.14	0.94-4.89	0.072
CD3+/CD4+/FOXP3+ Tregs	1.41	1.08-1.59	**0.006**

HR (hazard ratio); 95% CI (95% confidence interval); ref. (reference).

In case of TIL phenotypes, high infiltration rates of Tregs were significantly associated with increased risk for LR (HR: 1.33; 95% CI: 1.05-1.68; *p*=0.016) in the univariate analysis and remained statistically significant in the multivariate analysis (HR: 1.41; 95% CI: 1.08-1.59; *p*=0.006), irrespective of margin status (HR: 2.14; 95%CI: 0.94-4.89; *p*=0.072). Another potential positive prognostic marker was high levels of CD3+/CD4+ helper T cells (HR: 1.24; 95% CI: 0.99-1.55; *p*=0.062), showing a trend towards decreased LR risk in the univariate setting. Furthermore, a trend towards increased risk for LR was observed for high infiltration rates of three different PD-1 expressing cell phenotypes including PD-1+/CD3+/CD4+/FOXP3+ Tregs (HR: 8.91; 95% CI: 0.90-87.81; *p*=0.061), PD-1+/PD-L1- cells (HR: 1.02; 95% CI: 1.00-1.03; *p*=0.070), and CD3-/PD-1+ cells (HR: 1.02; 95% CI: 0.99-1.03; *p*=0.078).

Table 15: Summarized results of the univariate analysis of prognostic value of patient characteristics, tumour factors, immune checkpoint markers and TIL phenotype abundances for LR (155). Statistical significance is highlighted as bold *p*-values.

Patient Characteristics	**Local recurrence**		
	HR	**95% CI**	***p*-value**
Female (vs. male, ref.)	0.91	0.40-2.07	0.817
Age at surgery (years)	1.01	0.99-1.03	0.251
Adjuvant RTX (vs. no adjuvant RTX, ref.)	1.23	0.51-2.98	0.653
Adjuvant CTX (vs. no adjuvant CTX, ref.)	0.31	0.03-2.29	0.249

Tumour Characteristics	**Local recurrence**		
	HR	**95% CI**	***p*-value**
Tumour size	0.96	0.88-1.05	0.343
Lower limb (vs. upper limb, ref.)	0.87	0.35-2.22	0.772
Trunk (vs. upper limb, ref.)	0.94	0.19-4.65	0.935
Deep (vs. superficial, ref.)	0.40	0.16-1.04	0.059
Superficial + deep (vs. superficial, ref.)	1.35	0.48-3.81	0.566
G2 (vs. G1, ref.)	3.12	0.39-24.86	0.282
G3 (vs. G1, ref.)	2.10	0.28-15.53	0.468
R1/2 margins (vs. R0, ref.)	2.21	0.96-5.10	0.063
SS (vs. MFS, ref.)	N/A	N/A	N/A
UPS (vs. MFS, ref.)	0.93	0.25-3.41	0.907
LPS (vs. MFS, ref.)	0.66	0.15-2.85	0.580
Other (vs. MFS, ref.)	1.82	0.62-5.33	0.273
LMS (vs. MFS, ref.)	1.76	0.53-5.86	0.358

Immune Checkpoint Markers	**Local recurrence**		
	HR	**95% CI**	***p*-value**
PD-L1+ cells	1.07	0.96-1.19	0.248
PD-1+ cells	1.02	1.00-1.03	0.070
PD-L1+ / PD-1- cells	1.10	0.96-1.26	0.178
PD-L1- / PD-1+ cells	1.02	1.00-1.03	0.070
PD-L1+ / PD-1+ cells	1.03	0.86-1.24	0.728
FOX3P+ cells	1.00	0.98-1.02	0.823

TIL Phenotypes	**Local recurrence**		
	HR	**95% CI**	***p*-value**
CD3+ T cells	1.02	0.96-1.10	0.503
CD3+ / CD4+ helper T cells	1.24	0.99-1.55	0.062
CD3+ / CD8+ cytotoxic T cells	0.91	0.75-1.09	0.300
CD3+ / CD4+ / FOXP3+ Tregs	1.33	1.05-1.68	**0.016**
PD-1+ / CD3+ T cells	1.07	0.89-1.28	0.473
PD-1+ / CD3+ / CD4+ helper T cells	1.28	0.79-2.05	0.319
PD-1+ / CD3+ / CD8+ cytotoxic T cells	0.77	0.42-1.53	0.493
PD-1+ / CD3+ / CD4+ / FOXP3+ Tregs	8.91	0.90-87.81	0.061
PD-L1+ / CD3+ T cells	1.09	0.93-1.28	0.293
PD-1+ / CD3- cells	1.02	0.99-1.03	0.078
PD-L1+ / CD3- cells	1.09	0.93-1.28	0.312

HR (hazard ratio); 95% CI (95% confidence interval); ref. (reference); N/A (not applicable as not enough variables in group) MFS (myxofibrosarcoma); SS (synovial sarcoma); UPS (undifferentiated pleomorphic sarcoma); LPS (liposarcoma); LMS (leiomyosarcoma).

4.7.2. Prognostic Impact on Risk of Distant Metastasis (DM)

Univariate Fine and Gray model analysis for DM showed a significant association between larger tumour size (HR: 1.05; 95% CI: 1.01-1.09; *p*=0.010) and higher grading (G3 vs. G1; HR: 4.08; 95% CI: 1.05-15.77; *p*=0.042) with regards to increased risk for metastatic spread. In the comparison to MFS, UPS was associated with a higher risk for DM (HR: 2.32; 95% CI: 1.15-4.71; *p*=0.020). Other factors such as patient age, gender or adjuvant treatment did not show any prognostic value on DM risk. High levels of immune checkpoint markers as well as high infiltration rates of TIL phenotypes could not be associated with an alteration in risk of metastatic spread either.

Table 16: Multivariate competing risk regression analysis for DM of tumour factors including grading, size and histological subtype (155). Statistical significance is highlighted as bold *p*-value.

Multivariate Analysis	**Distant metastasis**		
	HR	**95% CI**	***p*-value**
G2 (vs. G1 as ref.)	2.69	0.66-11.03	0.169
G3 (vs. G1 as ref.)	2.68	0.69-10.39	0.153
Tumour size (cm)	1.06	1.02-1.11	**0.003**
SS (vs. MFS as ref.)	1.72	0.59-5.01	0.321
UPS (vs. MFS as ref.)	2.69	1.29-5.62	**0.008**
LPS (vs. MFS as ref.)	0.48	0.17-1.35	0.163
Other (vs. MFS as ref.)	1.57	0.66-3.75	0.307
LMS (vs. MFS as ref.)	2.16	1.07-4.33	**0.031**

HR (hazard ratio); 95% CI (95% confidence interval); ref. (reference); MFS (myxofibrosarcoma); SS (synovial sarcoma); UPS (undifferentiated pleomorphic sarcoma); LPS (liposarcoma); LMS (leiomyosarcoma).

In the multivariate model, large tumour size (HR: 1.06; 95% CI: 1.02-1.11; *p*=0.003) was significantly associated with increased risk for DM. Moreover, in the multivariate model, UPS (HR: 2.69; 95% CI: 1.29-5.62; *p*=0.008) and LMS (HR: 2.16; 95% CI: 1.07-4.33; *p*=0.031) were significantly associated with increased risk for metastatic spread compared to MFS, irrespective of grading. Of note, as none of the TIL phenotypes nor immune checkpoint markers were significantly associated with altered DM-risk in the univariate analysis, they were not entered in the multivariate model.

Table 17: Summarized results of the univariate analysis of prognostic value of patient characteristics, tumour factors, immune checkpoint markers and TIL phenotype abundances for DM (155). Statistical significance is highlighted as bold *p*-values.

Patient Characteristics	**Distant metastasis**		
	HR	**95% CI**	***p*-value**
Female (vs. male, ref.)	1.01	0.62-1.67	0.962
Age at surgery (years)	1.01	0.91-1.02	0.403
Adjuvant RTX (vs. no adjuvant RTX, ref.)	0.95	0.56-1.64	0.894
Adjuvant CTX (vs. no adjuvant CTX, ref.)	1.58	0.78-3.23	0.204
Tumour Characteristics	**Distant metastasis**		
	HR	**95% CI**	***p*-value**
Tumour size	1.05	1.01-1.09	**0.010**
Lower limb (vs. upper limb, ref.)	1.36	0.73-2.53	0.332
Trunk (vs. upper limb, ref.)	1.23	0.44-3.40	0.695
Deep (vs. superficial, ref.)	1.23	0.68-2.22	0.497
Superficial + deep (vs. superficial, ref.)	1.41	0.63-3.17	0.405
G2 (vs. G1, ref.)	3.08	0.73-12.92	0.124
G3 (vs. G1, ref.)	4.08	1.05-15.77	**0.042**
R1/2 margins (vs. R0, ref.)	0.75	0.41-1.37	0.347
SS (vs. MFS, ref.)	1.93	0.75-4.96	0.173
UPS (vs. MFS, ref.)	2.32	1.15-4.71	**0.020**
LPS (vs. MFS, ref.)	0.55	0.19-1.55	0.256
Other (vs. MFS, ref.)	1.31	0.60-2.86	0.499
LMS (vs. MFS, ref.)	2.08	0.98-4.44	0.058

Immune Checkpoint Markers	**Distant metastasis**		
	HR	**95% CI**	***p*-value**
PD-L1+ cells	1.02	0.98-1.07	0.323
PD-1+ cells	1.01	0.99-1.02	0.449
PD-L1+ / PD-1- cells	1.04	0.98-1.10	0.194
PD-L1- / PD-1+ cells	1.01	0.99-1.02	0.431
PD-L1+ / PD-1+ cells	0.10	0.88-1.13	0.974
FOX3P+ cells	0.99	0.98-1.01	0.417
TIL Phenotypes	**Distant metastasis**		
	HR	**95% CI**	***p*-value**
CD3+ T cells	1.02	0.99-1.05	0.161
CD3+ / CD4+ helper T cells	0.99	0.86-1.14	0.911
CD3+ / CD8+ cytotoxic T cells	0.98	0.89-1.08	0.659
CD3+ / CD4+ / FOXP3+ Tregs	0.99	0.84-1.18	0.966
PD-1+ / CD3+ T cells	1.01	0.86-1.14	0.877
PD-1+ / CD3+ / CD4+ helper T cells	0.98	0.69-1.38	0.904
PD-1+ / CD3+ / CD8+ cytotoxic T cells	0.94	0.78-1.14	0.541
PD-1+ / CD3+ / CD4+ / FOXP3+ Tregs	0.78	0.09-6.62	0.820
PD-L1+ / CD3+ T cells	1.02	0.97-1.08	0.408
PD-1+ / CD3- cells	1.01	0.99-1.02	0.440
PD-L1+ / CD3- cells	1.08	0.89-1.31	0.458

HR (hazard ratio); 95% CI (95% confidence interval); ref. (reference); MFS (myxofibrosarcoma); SS (synovial sarcoma); UPS (undifferentiated pleomorphic sarcoma); LPS (liposarcoma); LMS (leiomyosarcoma).

4.7.3. Prognostic Impact on Overall Survival (OS)

In the univariate Cox regression analysis for OS, advanced patient age (HR: 1.04; 95% CI: 1.02-1.05; *p*<0.001) was the only patient factor significantly associated with worse overall prognosis. Furthermore, immune checkpoint markers including high levels of PD-L1+/CD3- cells (HR: 1.15; 95% CI: 1.02-1.29; *p*=0.021) and low abundance of FOXP3+ cells (HR: 0.98; 95% CI: 0.96-0.99; *p*=0.023) were also associated with worse OS. Large tumour size (HR: 1.04; 95% CI: 1.00-1.08; *p*=0.051) showed a trend towards worse OS prognosis but did not meet statistical significance. No statistically significant associations with OS rate were observed with other patient and tumour factors, immune checkpoint markers or infiltration rates of TIL phenotypes, as shown in Table 19.

Table 18: Multivariate competing risk regression analysis for OS including patient age, tumour size, and infiltration rates of FOXP3+ cells and PD-L1+/CD-3- cells (155). Statistical significance is highlighted as bold *p*-value.

Multivariate Analysis	**Overall Survival**		
	HR	**95% CI**	***p*-value**
Age at surgery (years)	1.03	1.02-1.05	**<0.001**
Tumour size (cm)	1.04	0.99-1.08	0.076
FOXP3+ cells	0.98	0.96-1.01	0.076
PD-L1+ / CD3- cells	1.12	0.98-1.27	0.087

HR (hazard ratio); 95% CI (95% confidence interval); ref. (reference).

As seen from the results of the univariate analysis, advanced patient age (HR: 1.03; 95% CI: 1.02-1.05; *p*<0.001) remained a significant prognostic factor for worse OS in the multivariate model, irrespective of tumour size (HR: 1.04; 95% CI: 0.99-1.08; *p*=0.076), abundance of PD-1+/CD3- cells (HR: 1.12; 95% CI: 0.98-1.27; *p*=0.087), and levels of FOXP3+ cells (HR: 0.98; 95% CI: 0.96-1.01; *p*=0.076).

Table 19: Summarized results of the univariate analysis of prognostic value of patient characteristics, tumour factors, immune checkpoint markers and TIL phenotype abundances for OS (155). Statistical significance is highlighted as bold *p*-values.

Patient Characteristics	**Overall survival**		
	HR	**95% CI**	***p*-value**
Female (vs. male, ref.)	0.90	0.56-1.45	0.667
Age at surgery (years)	1.04	1.02-1.05	<0.001
Adjuvant RTX (vs. no adjuvant RTX, ref.)	0.68	0.42-1.12	0.132
Adjuvant CTX (vs. no adjuvant CTX, ref.)	0.89	0.43-1.86	0.751
Tumour Characteristics	**Overall survival**		
	HR	**95% CI**	***p*-value**
Tumour size	1.04	1.00-1.08	0.051
Lower limb (vs. upper limb, ref.)	0.95	0.55-1.69	0.873
Trunk (vs. upper limb, ref.)	1.35	0.55-3.29	0.511
Deep (vs. superficial, ref.)	0.97	0.55-1.70	0.907
Superficial + deep (vs. superficial, ref.)	1.56	0.76-3.19	0.223
G2 (vs. G1, ref.)	1.40	0.45-4.40	0.566
G3 (vs. G1, ref.)	2.11	0.76-5.85	0.149
R1/2 margins (vs. R0, ref.)	0.68	0.37-1.25	0.215
SS (vs. MFS, ref.)	1.70	0.70-4.13	0.239
UPS (vs. MFS, ref.)	1.73	0.89-4.49	0.108
LPS (vs. MFS, ref.)	0.39	0.15-1.12	0.080
Other (vs. MFS, ref.)	1.41	0.68-2.93	0.356
LMS (vs. MFS, ref.)	1.42	0.62-2.81	0.476

Immune Checkpoint Markers	**Overall survival**		
	HR	**95% CI**	***p*-value**
PD-L1+ cells	1.023	0.98-1.07	0.355
PD-1+ cells	1.006	0.99-1.02	0.481
PD-L1+ / PD-1- cells	1.025	0.96-1.09	0.446
PD-L1- / PD-1+ cells	1.005	0.99-1.02	0.536
PD-L1+ / PD-1+ cells	1.056	0.94-1.19	0.357
FOX3P+ cells	0.977	0.96-0.99	0.023
TIL Phenotypes	**Overall survival**		
	HR	**95% CI**	***p*-value**
CD3+ T cells	0.99	0.96-1.04	0.857
CD3+ / CD4+ helper T cells	1.01	0.88-1.15	0.920
CD3+ / CD8+ cytotoxic T cells	1.01	0.91-1.01	0.991
CD3+ / CD4+ / FOXP3+ Tregs	0.99	0.83-1.18	0.888
PD-1+ / CD3+ T cells	1.04	0.92-1.18	0.525
PD-1+ / CD3+ / CD4+ helper T cells	1.12	0.81-1.56	0.496
PD-1+ / CD3+ / CD8+ cytotoxic T cells	1.08	0.94-1.25	0.258
PD-1+ / CD3+ / CD4+ / FOXP3+ Tregs	0.86	0.05-16.29	0.921
PD-L1+ / CD3+ T cells	1.01	0.93-1.08	0.960
PD-1+ / CD3- cells	1.01	0.99-1.02	0.514
PD-L1+ / CD3- cells	1.15	1.02-1.29	0.021

HR (hazard ratio); 95% CI (95% confidence interval); ref. (reference); N/A (not applicable as not enough variables in group) MFS (myxofibrosarcoma); SS (synovial sarcoma); UPS (undifferentiated pleomorphic sarcoma); LPS (liposarcoma); LMS (leiomyosarcoma).

5. Discussion

The great variability in clinical and histological presentation of STS together with a low overall prevalence of neoplasms arising from the mesenchyme has confronted researchers and experts in this field with difficulties in diagnosis and treatment decision making. With today's available therapeutic approaches for STS, including (neo)adjuvant radiation and/or CTX, limited success is achieved regarding reduction of LR- and DM-risk as well as improvement in OS. Therefore, enhancing the understanding of underlying mechanisms and the origin of STS is urgently in demand. One prominent field gaining the interest of researchers are so-called immune checkpoint markers and cell phenotypes found in the TME. Prognosis of a malignant neoplasm is strongly associated with the host's immune response (138). High infiltration rates and activation of immune cells within the TME have been identified to be a promising indicator of a productive anti-tumour response by the patient's immune system (139). Moreover, molecular switch points known as immune checkpoint markers play an important role in the balance of positive and negative signalling pathways in the immediate surrounding of the tumours tissue. Priming and consequently the activation of T-lymphocytes is a critical process in the adaptive immune response and is regulated by the expression of these immune checkpoint markers and consecutive alteration in pathway activation, such as the PD-1 / PD-L1 signalling pathway. Promising results in the treatment of cancers of different origin such as late-stage melanoma (158–160), lung (161), kidney (162, 163) and bladder cancer (164) targeting these molecular switch points have been reported in recent years (137).

However, expression of PD-1/PD-L1 and markers of the TME together with infiltration rates of various TIL phenotypes have rarely been investigated in STS (144). Therefore, the identification of novel targets for immunotherapy as a personalized treatment approach has to be in focus of research. The results obtained in the study for this diploma thesis contribute to the growing knowledge of the role of immune checkpoint markers as potential prognostic and therapeutic biomarkers in STS.

In the multiplex IHC analyses, immune checkpoint marker expression patterns varied significantly throughout different histological subtypes. In all 192 tumour samples a median cell count for PD-L1+ and PD-1+ cells of 0.82% and 0.57% of all cells, respectively, was found. The density of immune checkpoint expressing cells infiltrating the TME varied among patient subgroups. High abundancies (>1% of total cell count) of PD-L1+ cells were found in 48.4% (n=93) of tissue samples compared to 32.8% (n=63) for PD-1+. Additionally, the co-expression pattern of PD-1+ and PD-L1+ was also assessed, resulting in only 0.01% of total cell count to be positive for both immune checkpoint markers. The most abundant marker, however, was determined to be FOXP3 with a median abundance of 2.60% of all cells and close to two thirds (n=122; 63.5%) of samples showing levels above 1% of FOXP3+ cells.

In the analysis of PD-L1 and PD-1 expression levels by various TIL phenotypes, in 10.4% (n=20) of all assessed tumour samples more than 1% of total cells were CD3+/PD-1+ T cells. PD-L1 expression by CD3+ T cells was found to exceed 1% of all cells in 5.7% (n=11) of samples. However, the highest levels of PD-1+ expression was found in Tregs with 0.15% of cells of total cell count, but 100% (n=192) of samples showing lower levels than 1% of all cells. Assessment of the presence of CD3-/PD-1+ and CD3-/PD-L1+ cells resulted in 24.5% (n=47) and 18.8% (n=36) of samples to show high levels (>1% of total cell count). The expression levels of immune checkpoint markers PD-1 and PD-L1 on TILs in this cohort of STS patients are generally lower in comparison to triple-negative breast cancers (25.1% PD-L1 expression levels on ≥1% of TILs [165]), small cell lung cancers (37.3% PD-L1 and 40.2% PD-1 expression levels on ≥1% of TILs [166]), or gastric cancers (48% PD-L1 expression levels on ≥1% of TILs [167]).

In contrast to previous studies investigating the frequency of PD-L1 expression, dividing STS tumour tissues into PD-L1 positive and PD-L1 negative samples according to a specific cut-off value or the simple presence or absence of fluorescence detection, in this study the actual amount of PD-L1+ cells in percentages of total cell counts were analysed. In literature, determination of the presence of PD-L1 expressing cells in the TME of different histological subtypes results in quite different frequencies for each cohort as seen from the studies by

Movva et al. (50% of sarcoma samples showing PD-L1 expression [168]), *Kim et al.* (65% of sarcoma samples were PD-L1 positive and in 58% of cases, PD-L1 expressing TILs were found [169]), *Paydas et al.* (30% of sarcoma samples [170]), *D'Angelo et al.* (12% of sarcoma samples [150]), and *Boxberg et al.* (6.7% - 40.4% in different histological subtypes [144]). Several decisive factors have to be considered when comparing these differences in PD-L1 expression frequencies: (1) Most important, studies often use different cut-off values for the definition of PD-L1 positive or negative tumour samples. (2) The use of variable IHC assays and antibodies can lead to considerable differences in the staining results. (3) The PD-L1 expression might be highly influenced and altered by preoperative treatment protocols. (4) Most available data on the PD-L1+ frequency in STS were obtained in studies analysing cohorts of low and high-grade sarcomas including many sub-entities and unknown clinical data of neoadjuvant therapies.

Therefore, in this study, percentages of cells expressing immune checkpoint markers PD-L1, PD-1, and FOXP3 of total cell counts assessed by multispectral imaging using DAPI for nucleus staining and cell counting were presented. The quantitative determination avoids loss of information for statistical analysis. Furthermore, as mentioned in the materials and methods section, patients receiving neoadjuvant RTX or CTX were excluded from this study to avoid a distortion of the results by confounding effects.

Compared to the study from *Kim et al.* with a considerably younger patient cohort (138), the median age in this cohort was 63.5 years and used to split patients into two groups according age (≥63.5 vs. <63.5 years). Comparing the abundance of TIL phenotypes, significant increased amounts of (CD3+/CD4+) helper T cells (0.14 vs. 0.07%; p=0.030) and Tregs (0.06 vs. 0.02%; p=0.010) were observed in older patients compared to the younger group, in line with previous observations (169). Moreover, in older patients (compared to younger patients), significantly higher levels of PD-1 expressing T cells (0.06 vs. 0.01%; p=0.013), helper T cells (0.01 vs. 0.00; p=0.010), and cytotoxic T cells (0.03 vs. 0.00%; p=0.019) were detected. Older age at surgery was the only factor found to show a significant association with worse OS rates (HR: 1.03; p<0.001) also shown in previous reports (171).

Differences in the efficacy of immunotherapeutic treatments of various histological STS subtypes might be explained by expression patterns of immune checkpoints and varying infiltration rates of TILs within the TME. Therefore, the 192 STS samples were divided into six different histological subgroups according to the most prevalent entities included. Significantly higher levels of TILs, including CD3+ T cells, CD3+/CD4+ helper T cells, CD3+/CD8+ cytotoxic T cells, and CD3+/CD4+/FOXP3+ Tregs, in MFS compared to SS, LPS, LMS and "Others" were observed. Furthermore, the second highest infiltration rates of the previously mentioned T-lymphocytes were found in UPS, whereas the lowest levels of TILs were determined in SS. These results are in line with the findings of *Pollack et al.* (172).

Regarding immune checkpoint marker positive TIL phenotypes, the highest levels of PD-L1 expressing CD3+ T cells were found in MFS and UPS with significantly higher infiltration rates in MFS compared to SS, LMS, and "Others". For PD-1+ TILs similar results as for T lymphocyte subtypes were obtained, with MFS showing significantly increased infiltration of the TME by PD-1+/CD3+ T cells, PD-1+/CD3+/CD4+ helper T cells, PD-1+/CD3+/CD8+ cytotoxic T cells, and PD-1+/CD3+/CD4+/FOXP3+ Tregs compared to SS, LPS, and LMS. Again, second highest amounts of all PD-1+ TILs, except for PD-1+ Tregs, were found in UPS, supporting the findings of *Boxberg et al.* (144) and *Pollack et al*. (172) who also reported increased infiltration rates of PD-1 expressing TILs for UPS compared with AS, LMS, and SS.

Concentrating on all cells found in the TME that express immune checkpoint markers, significantly higher levels of PD-L1 expressing cells (PD-L1+ cells, PD-L1+/PD-1- cells, and PD-L1+/PD-1+ cells) as well as PD-1 expressing cells (PD-1+ cells, PD-1+/PD-L1- cells, PD-1+/PD-L1+ cells, and PD-1+/CD3- cells) in MFS compared to LMS were found. Excluding the subgroup of "Others", the highest levels of all PD-L1+ and/or PD-1+ cells were found in MFS compared to SS, LMS, LPS, and UPS. In this STS cohort, an increased infiltration rate of all PD-L1 expressing cell phenotypes (PD-L1+ cells, PD-L1+/CD3- cells, PD-L1+/PD-1- cells, and PD-L1+/PD-1+ cells) in UPS compared to LMS, LPS, and SS was present. These results are also in line with the findings of *Boxberg et al.* (144) and *Pollack et al.* (172). However, abundances of PD-1+ cell phenotypes varied among histological

subtypes and could not confirm the results reported by *Pollack et al.* (172) comparing UPS with WDLPS, DDLPS, LMS, and SS.

As expected and shown in previous observations in ovarian cancer, strong positive correlations between the presence of CD3+ T cells and all other TILs (helper T cells, cytotoxic T cells, and Tregs) were found (173). The only strong positive correlation observed in this STS cohort is between the abundance of PD-1+ cells and FOXP3+ cells. Moreover, there was a moderate positive correlation between the presence of PD-L1+ cells and T cells, helper T cells, and cytotoxic T cells.

Furthermore, a positive correlation between high abundances of Tregs and both PD-L1 as well as PD-1 expressing cells was found, confirming the results reported by *Que et al.* (174). This result indicates the downregulation of Treg apoptosis by the activation of the PD-L1/PD-1 signalling pathway (146). Tregs are found to be responsible for balance of immune response amplitude inducing TIL apoptosis and establishing self-tolerance (140, 175, 141). Therefore, high levels were associated with increased negative signals and in consequence with evasion of tumorous tissue from the host's immune system, which may already have mirrored the independent negative prognostic impact of high levels of Tregs with LR risk in this study. Tregs were the only TIL phenotype significantly associated with increased risk of LR (HR: 1.33, p=0.016). In the multivariate model, the significance for higher risk of LR with high levels of Tregs (HR: 1.41; p=0.006) was confirmed, irrespective of resection margins. However, in this study, no significant association between high Treg infiltration and a worse OS could be determined as previously shown by *Que et al.* (174). This implicates that therapeutic regulation of Treg infiltration status might be a promising target to be addressed by ICIs or other immunotherapeutic approaches.

In the univariate Fine and Gray model, no significant association between TIL abundances and immune checkpoint markers with regards to altered DM-risk was found. In the univariate Cox regression analysis, high levels of PD-1+/CD3- cells (HR: 1.15; p=0.021) were significantly associated with worse OS rates. On the other hand, high abundances of FOXP3 expressing cells (HR: 0.98; p=0.023) was significantly associated with improved OS in the univariate analysis. This result is in line with the findings of *Bae et al.* (176) reporting STS patients with improved OS rates showing overexpression of *FOXP3* and other genes associated with the

adaptive immune response on the genetic level. The considerable difference in the obtained results for FOXP3 and Tregs, which are specified using FOXP3 as cellular marker, have been reported previously in other tumour entities (177) and may be explained by the fact that FOXP3 is also found in other infiltrating immune cells, apart from Tregs (178).

In the current diploma thesis, previously known prognostic factors for DM and OS, including patient and tumour characteristics, could be confirmed regarding their prognostic significance for STS (63). In the univariate Fine and Gray model, a significant association between larger tumour size (HR: 1.05; *p*=0.010) and an increased risk of DM was found. Furthermore, a significantly higher risk for metastatic spread was present in case of high grade STS (G3 vs. G1; HR: 4.08; *p*=0.042) and histological subtype UPS compared with MFS (HR: 2.32; *p*=0.020). In this diploma thesis, the majority of STS were classified as G3, with 72.5%, compared to G2 (18.1%) and G1 (9.4%).

In the multivariate analysis, larger tumour size and UPS compared with MFS remained significantly associated with higher risk for DM (HR: 1.06; *p*=0.003 and HR: 2.69; *p*=0.008, respectively), whereas higher grade did not meet statistical significance (G3 vs. G1; HR: 2.69; *p*=0.153). Also, in the multivariate model, LMS was associated with a significantly higher risk for DM compared with MFS (HR: 2.16; *p*=0.031). *Callegaro et al.* also reported a significantly higher risk for DM in LMS as compared with myxoid LPS, whereas no statistically significant difference regarding DM risk was found between MFS and myxoid LPS (179). In the current study, however, no further sub-specification into LPS subtypes was made, owing to the limited sample size available.

6. Limitations

In this diploma thesis, some limitations have to be considered. First, the heterogeneity of STS regarding their presentation and variety in histology combined with low frequency of each histological subtype confronts researchers with difficulties to achieve a representative cohort size. Indeed, there have been more than 100 histological subtypes of STS differentiated so far (2), each exhibiting slightly different biological behaviour, which also includes immune checkpoint

expression and infiltration rates of TILs. Therefore, in this diploma thesis, individuals with adjuvant treatment differences (none, RTX or CTX) have been included to increase the cohort size, achieving a more representative investigation of STS subtypes. Of course, adjuvant therapy should also have an influence on the outcome of patients, which could only be adjusted by the use of a multivariate analysis model, however not excluded. Second, one may argue that even well-known prognostic factors such as age, resection margin, grading and tumour size only showed partially significant results in the present study. However, this may also be explained by the limited number of cases included in the analysis. Third, due to the low incidence of STS and various histologies, the 192 STS samples were separated into 6 different histological subgroups, combining rare histological subtypes upon the category “Others”. As mentioned before, each histology exhibits different biological behaviour and therefore results in the subgroup "Others" should be interpreted with caution, as different histological subtypes (such as AS and SCS) had been analysed jointly. Some entities might show high levels of immune checkpoints or TILs while others express low abundances to even absence. Forth, it may be difficult to directly compare our findings with results from other studies that did not use IHC for the analysis of TIL phenotypes and infiltration rate. Besides IHC, gene expression analysis can be applied for this type of investigation (180). Employing gene expression analysis allows analysis of total mRNA load within the tumours tissue, whereas IHC only detects the functional amount of expressed proteins. However, applying both technologies – i.e. determining infiltration rates and immune checkpoint marker expression at protein and mRNA level – may eventually confirm the herein obtained results and add value. Fifth, when comparing results between different studies it has to be considered that most often varying cut-off values for definition of immune checkpoint marker-positive or -negative tumour samples were used. In the present study, expression levels of immune checkpoint markers and TIL phenotype abundances were determined quantitatively in percentage of total cell count. This approach avoids loss of information for future statistical analysis, while the results may not be applicable to be directly transferred into clinical practice.

7. Conclusion

Immune checkpoint markers are expected to correlate with outcome in patients with STS or with other known clinico-pathogenetic risk factors as seen for specific cancers. Immunotherapy has achieved substantial success in the treatment of patients with malignancies, but its effectiveness in sarcomas remains to be explored.

In the current diploma thesis, a positive correlation between high abundances of Tregs and both PD-L1 as well as PD-1 expressing cells was found. This result indicates the downregulation of Treg apoptosis by the activation of the PD-L1/PD-1 signalling pathway. Tregs are found to be responsible for balance of immune response amplitude inducing TIL apoptosis and establishing self-tolerance. Therefore, high levels are associated with increased negative signals and in consequence with evasion of tumorous tissue from the host's immune system, which is further confirmed by the independent negative prognostic impact. High levels of Tregs were significantly associated with an increased LR risk in this thesis.

However, the strong positive correlation between the abundance of PD-1+ cells and FOXP3+ cells only partially reflect high CD3+/CD4+/FOXP3+ Treg infiltration. While Tregs have been associated with negative outcome, high abundance of FOXP3-expressing cells was significantly associated with an improved outcome. This can be explained by the fact that FOXP3 is also found in other infiltrating immune cells, apart from Tregs.

Considering these findings altogether, it can be assumed that therapeutic regulation of Treg infiltration status might be one of the most promising targets to be addressed by ICIs or other immunotherapeutic treatment approaches. However, due to the positive impact of FOXP3+ cells on OS, the identification of different Treg specific biomarkers is highly demanded for further elucidation of targetability of Tregs in treatment of STS.

8. Key Facts

- Immune checkpoint markers have been reported to correlate with outcome and other known clinico-pathogenetic risk factors in various cancer entities. Immunotherapy has achieved substantial success in the treatment of patients with malignancies, but its effectiveness in STS remains to be explored.

- Expression of PD-1/PD-L1 and markers of the TME together with infiltration rates of various TIL phenotypes have rarely been investigated in STS and are expected to present potential targets for novel immunotherapeutic treatment approaches.

- IHC and multispectral imaging were used to investigate the correlation between presence and abundance of immune checkpoint markers, such as PD-1 and PD-L1, as well as TIL phenotypes, and outcome of patients with STS.

- A positive correlation between high abundances of Tregs and both PD-L1 as well as PD-1 expressing cells was found in the automated cell count of MP-IHC analyses.

- A strong positive correlation between the abundance of PD-1+ cells and FOXP3+ cells was observed.

- The most abundant marker was FOXP3 and a high number of FOXP3+ cells was significantly associated with an improved OS rate.

- High infiltration rates of Tregs were significantly associated with increased risk for LR in both, univariate and multivariate analysis, irrespective of resection margin status.

- Therapeutic regulation of Treg infiltration status might be a promising target to be addressed by ICIs or other immunotherapeutic approaches.

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