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The background of the cover is a microscopic image showing several large, irregularly shaped cells stained with a bright blue dye. The cells have a granular appearance and some darker, more concentrated blue areas, possibly representing nuclei or specific organelles. The overall tone is a vibrant cyan/blue against a light background.

Immunological Aspects of Soft Tissue Sarcoma and Melanoma

Correlation to prognosis and treatment

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Immunological Aspects of Soft Tissue Sarcoma and Melanoma

Correlation to prognosis and treatment

Helena Nyström



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DOCTORAL DISSERTATION

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Abstract: Knowledge about the interplay between tumor cells and the tumor microenvironment is essential for improved prognostication and treatment of malignant tumors. The aim of this thesis was to study the tumor microenvironment in Soft Tissue Sarcoma (STS) and brain metastases of Melanoma. Furthermore, a protocol for a clinical phase II study was developed.

In paper I and II the tumor microenvironment in STS is characterized using immunohistochemistry. The aim of **paper I** was to link presence of tumor associated macrophages, hypoxia and neovascularity to outcome in high grade leiomyosarcomas and undifferentiated pleomorphic sarcomas. We found that HIF-1 α , a marker for hypoxia, predicted higher incidence of metastasis as well as significantly impaired overall survival. In the HIF-1 α positive group 71% developed metastasis compared with 35% in the HIF-1 α negative group. In **paper II** we aimed to characterize the immune landscape in a cohort of 134 STS including leiomyosarcomas, liposarcomas and synovial sarcomas with correlation to prognosis. The most frequently observed immune cell type was CD163+ macrophages, with high expression noted in 49% of the tumors. Immune cell infiltration, both macrophages and T-cells, were significantly more prevalent in genetically complex leiomyosarcomas and liposarcomas than karyotype simple synovial sarcomas. CD20+ B-cells was noted in only 14% of the tumors, with no difference between the histotypes. Survival analysis including only 113 high grade tumors showed that B-cell infiltration was associated with improved overall survival. In **paper III**, we explored the differences in the immune microenvironment in brain metastases of Melanoma compared to extracranial disease manifestations using immunohistochemistry and GeoMx digital spacial profiling. Twenty-two patients were included. Although the sample size was small, we noted a more immune-excluded microenvironment in brain metastases. Long- term survivors showed increased immune infiltration and T-cell activation. Moreover, surprisingly, corticosteroids were associated with a more immune-rich tumor microenvironment.

Paper IV is a protocol for a clinical phase II study exploring the combination of a PD-1 inhibitor retifanlimab, in combination with a FGFRinhibitor, pemigatinib in advanced dedifferentiated liposarcoma. The study, PERELL, is a single arm, open label, multicenter study aiming to include 33 patients in Sweden Norway and Denmark.

Key words: Soft Tissue Sarcoma, Melanoma, Brain metastasis, Tumor microenvironment, Prognostic factors, clinical trial

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Immunological Aspects of Soft Tissue Sarcoma and Melanoma

Correlation to prognosis and treatment

Helena Nyström



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List of Papers

Paper I

Nyström H, Jönsson M, Werner-Hartman L, Nilbert M, Carneiro A Hypoxia-inducible factor 1 α predicts recurrence in high-grade soft tissue sarcoma of extremities and trunk wall. *Journal of Clinical Pathology*. 2017 Oct;70(10):879-885

Paper II

Nyström H, Jönsson M, Nilbert M, Carneiro A. Immune-cell infiltration in high-grade soft tissue sarcomas, prognostic implications for tumor-associated macrophages and B-cells. *Acta Oncologica*. 2023 Jan;62(1):33-39

Paper III

Nyström H, Lauss M, Hedner C, Ellfors E, Nodin B, Jirstrom K, Nielsen, K, Hafstrom A, Ingvar C, Siesjö P, Bengtsson J, Isaksson K, Jönsson G, Carneiro A. Immune landscape of Melanoma Brain Metastases. *In manuscript*

Paper IV

Nyström H, Aggerholm-Pedersen N, Junker N, Papakonstantinou A, Hansson L, Goplen D, Wesche. J, Carneiro C, Boye, K PERELI, a phase 2, open label, multicenter study of PEmigatinib and REtifanlimab in dedifferentiated Liposarcoma. *In manuscript*

Abbreviations

APC	Antigen presenting cell
ASPS	Alveolar soft part sarcoma
B2M	Beta-2-microglobulin
BM	Brain metastasis
CAIX	Carbonic anhydrase 9
CNS	Central nervous system
CSF-1	Colony stimulating factor-1
CR	Complete response
CT	Computed tomography
CTLA-4	Cytotoxic T-lymphocyte associated protein-4
DDLPS	Dedifferentiated liposarcoma
FDR	False discovery rate
FFPE	Formalin-fixed paraffin-embedded
FGFR	Fibroblast growth factor receptor
GCP	Good clinical practise
GLUT-1	Glucose transporter-1
HIF	Hypoxia-inducible factor
HR	Hazard ratio
ICI	Immune checkpoint inhibitor
IHC	Immunohistochemistry
IFN γ	Interferon gamma
IRAE	Immune related adverse event
ISH	In situ hybridization
LDH	Lactate dehydrogenase
LMS	Leiomyosarcoma
LPS	Liposarcoma
MFS	Metastasis-free survival
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
MVD	Microvessel density
NGS	Next generation sequencing
ORR	Overall response rate
OS	Overall survival

PD	Progressive disease
PD-1	Programmed cell death-1
PD-L1	Programmed cell death ligand-1
PR	Partial response
PFS	Progression free survival
RECIST	Response evaluation criteria in solid tumors
SD	Stable disease
SFT	Solitary fibrous tumor
SRS	Stereotactic radiosurgery
STS	Soft tissue sarcoma
SS	Synovial sarcoma
SSG	Scandinavian sarcoma group
TAM	Tumor associated macrophage
TCR	T-cell receptor
TIL	Tumor infiltrating lymphocyte
TLS	Tertiary lymphoid structure
TMB	Tumor mutational burden
TME	Tumor microenvironment
TRAE	Treatment related adverse event
UPS	Undifferentiated pleomorphic sarcoma
WDLPS	Well differentiated liposarcoma

Introduction

In 1891, the surgeon William B. Coley wrote in his paper, *Contribution to the Knowledge of Sarcoma* [1]:

“While early operation gives a possibility of complete cure in a certain number of cases, the large proportion of cases in which fatal and often speedy recurrence follows operation, is sufficient to make the surgeon almost lose faith in his art in the treatment of this dread disease.

There are certain types of sarcoma that seem almost hopeless from the start, and when surgical skill, if called upon, only proves how utterly powerless it is. Is there nothing else that can be done to stay the progress of this disease? This is a question that has long occupied the attention of many of the best minds in the medical world, and at no time has it received as much thought as it does today.”

Today, 132 years later, most sarcoma surgeons and oncologists can probably still relate to this feeling of inadequacy, but also of hope.

William Coley, after having witnessed a case of tumor remission after an erysipelas infection, developed heat-inactivated bacterial toxins and treated cancer patients. Coley reported remarkable success with his toxins and published many papers on the topic, although he was never able to fully understand the mechanisms by which the toxins exerted their effect.

Since then, our understanding of the interplay between a tumor and the immune system has improved greatly and led, in 2011, to a breakthrough with the approval of the first immune checkpoint inhibitor, ipilimumab. While checkpoint inhibitors have revolutionized the treatment of several tumor types such as melanoma or lung cancer, other tumor types, such as soft tissue sarcoma, have not yet experienced the same benefit.

Furthermore, and despite the good results obtained, many patients with melanoma will not respond, or will progress on treatment with immunotherapy. Unfortunately, predictive factors to help select which patients to offer this immunotherapy, with potentially severe side effects, are lacking. As advances in systemic oncological treatments lead to improved survival of patients with advanced stage malignancies, the incidence of brain metastases has increased [2, 3]. No matter the histology, brain

metastases are notoriously hard-to-treat and remain a major challenge in clinical oncology.

It is now widely accepted that the aggressiveness of a malignant tumor is not only determined by the genotype of the tumor cells, but also by their interactions with the tumor microenvironment, which orchestrates the development of the tumor. Deeper understanding the tumor microenvironment in sarcoma, as well as in brain metastases, may provide keys to improve their treatment.

Background

Tumor Microenvironment

The concept of the tumor microenvironment (TME) was first introduced when Virchow proposed a relationship between inflammation and cancer in 1863. Paget further developed this concept when suggesting metastatic colonization to be dependent on organ specific properties, known as the “seed and soil” theory [4].

The TME includes cellular components such as cancer associated fibroblasts, diverse immune cells, stromal and endothelial cells. Non-cellular components of the TME encompass the extracellular matrix, growth factors, cytokines and other signalling mediators [5]. The composition of the TME varies in relation to tumor phenotype and genotype, as the TME is shaped and trained by cancer cells to ultimately facilitate tumor progression, tumor invasion and formation of metastases [6].

The emergence of immunotherapy as a pillar of cancer treatment, and the understanding that response and resistance to immunotherapy are multifaceted, deriving not only from tumor intrinsic factors, but also from the complex interplay between cancer and its microenvironment, have prompted a new interest into the TME (Figure 1).

Tumor immune microenvironment

Immune cells are a critical component of the TME. Tumors become infiltrated with diverse innate and adaptive immune cells that can have both pro- and anti-tumorigenic effects.

Innate immunity is a non-specific first line of defence mechanism. Immune cells of the innate immune system include macrophages, dendritic cells, mast cells, neutrophils and natural killer cells (NK-cells). The main components of adaptive immunity system are lymphocytes, T- and B-cells, antigen specific cells able to form an immunological memory.

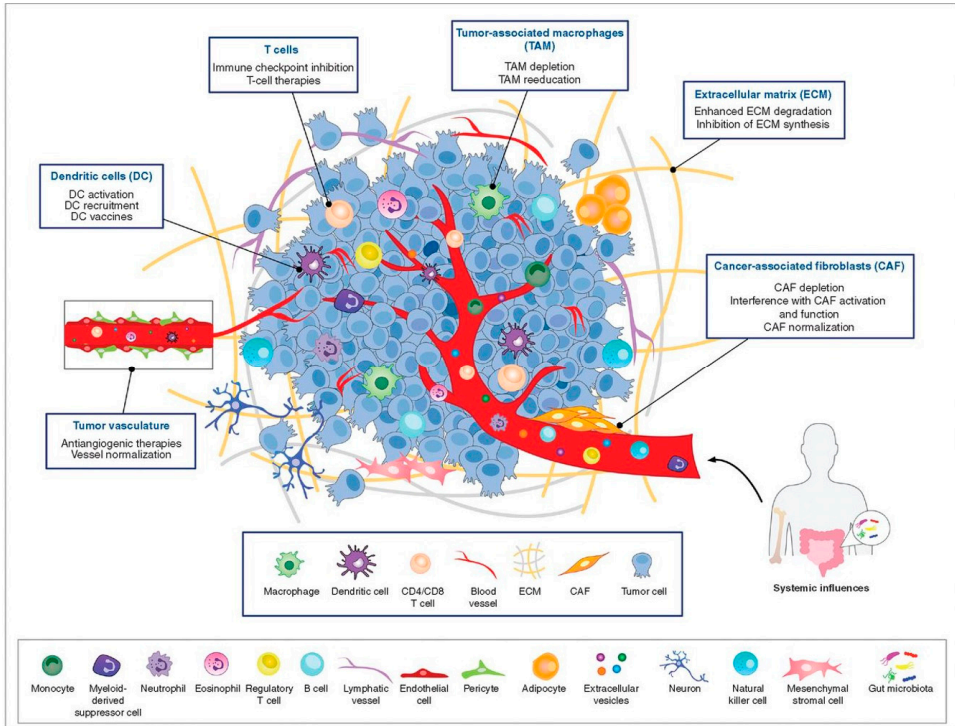


Figure 1. Schematic representation of the tumor microenvironment including infiltrating immune cells such as B- and T-lymphocytes, macrophages, and dendritic cells as well as stromal cells such as cancer-associated fibroblasts. Neovascularization, the formation of novel blood vessel is also an important feature of the TME. All of these cells and features can contribute to tumor progression and influence therapeutic response. Treatment strategies aimed at the TME are highlighted in the blue boxes. *Reprinted from [7] with permission from AACR.*

It is now accepted that the TME plays a significant role in tumor immune surveillance and immunological evasion.

The interplay between the tumor and the immune system is described as cancer immunoediting, a concept introduced by Schreiber and colleagues [8, 9, 10]. The process of immunoediting proceeds through three different phases, elimination, equilibrium and escape [11].

During the elimination phase, the innate and adaptive immunity collaborate to recognise and eliminate tumor cells. This phase is marked by recognition of tumor cells by cells of the innate immunity, release of damage-associated molecular patterns and tumor antigens, but also induction of chemokines in the TME and production of interferon gamma (IFN γ), which further activate the immune system. Furthermore, activation of adaptive immunity, leads to recruitment and infiltration of TME by tumor specific lymphocytes.

During the equilibrium phase, tumor cells surviving elimination can coexist with the antitumor response in a state of dynamic tumor dormancy. Adaptive immunity, CD4⁺ and CD8⁺ T-cells, interleukin-12 and IFN γ are essential in this phase. During this stage, mechanisms to evade the immune response are developed by the tumor modelling the establishment of a suppressive TME ultimately leading to escape.

During the escape phase, tumor cells acquire insensitivity to immunologic processes (detection/elimination) and begin to expand, allowing tumor progression. These progressing cancer cells are usually poorly immunogenic and highly immunovasive [11]. In this phase, processes such as down regulation of major histocompatibility complex class 1, leading to loss of antigen presentation and impaired immune recognition and upregulation of inhibitory immune signals such as expression of immune checkpoint proteins (PD-L1, TIM-3, LAG-3, VISTA etc) contribute to the escape from the immune response. Furthermore, immunosuppressive cells, such as Tregs and myeloid derived suppressor cells are recruited in this phase.

Understanding the processes underlying cancer immunoediting provides the framework for understanding immunotherapy, and how resistance to immunotherapy is developed [12].

Tumor associated macrophages

Tumor associated macrophages (TAM) are derived from blood monocytes and are considered part of the innate immune system. TAMs are attracted to the TME by attractants and chemokines such as transforming growth factor beta and colony stimulating factors [13]. In the tumor, TAMs can phagocyte tumor cells and act as antigen presenting cells (APCs) to activate the adaptive immune response, but TAMs can also contribute to cancer progression through stimulation of angiogenesis and immune response suppression [14]. This dual activity is reflected in the TAM phenotypes, M1- polarized (classically activated, pro-inflammatory) and M2-polarized (alternatively activated, anti-inflammatory, pro-tumorigenic). These phenotypes represent extremes of a spectrum, as macrophages can switch polarization in response to external stimuli.

High infiltration of TAM is generally associated with poor prognosis [15]. TAMs have been shown to accumulate in hypoxic areas in different tumor types [16]. Indeed, hypoxia shapes and maintain M2 macrophage phenotype and TAM in hypoxic niches are known to mediate resistance to anticancer treatment and promote cancer progression.

T-cells

T-cells are the main effector cells of the adaptive immune system. T-cells traffic the lymphatic system and blood stream, and are activated mainly by encountering APCs i.e. dendritic cells, macrophages, or B-cells in lymph nodes. APCs present a group of proteins on their surface known as major histocompatibility complex (MHC).

MHC can be either class I, found on the surface of all nucleated cells presenting endogenous peptides, or MHC class II found on the APC presenting exogenous peptides. The function of MHC molecules is to bind and present peptide fragments on the cell surface for recognition by T-cells.

Binding of the peptide-MHC molecule complex by the T-cell receptor (TCR) is the first required signal for T-cell activation (signal 1). Activation of T-cells further requires presence of co-stimulatory signals (signal 2) leading to activation, increased survival, and proliferation of T-cells. Receptors on APC that can provide this necessary second signal are called costimulatory receptors, members of either the CD28 family of proteins or of the tumor necrosis factor receptor superfamily (Figure 2).

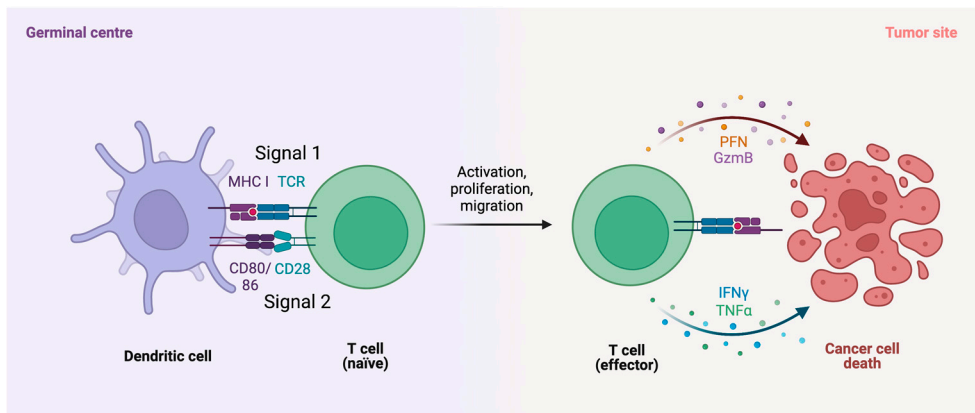


Figure 2 T-cell activation. Process of T-cell activation requires signal 1 MHC-TCR as well as signal 2 a co-stimulatory signal. The activated T-cell can differentiate to a effector T-cell capable of killing tumor cells upon appropriate antigen recognition. Created with BioRender.com

Following activation, the T-cell will differentiate to either a T-helper CD4⁺ cell, or a cytotoxic CD8⁺ T-cell. CD8⁺ cytotoxic T-cells are effector cells, whose main function is to eliminate cells expressing the appropriate antigen. When activated, T-effector cells destroy their target cell by inducing apoptosis [17]. Activation also triggers proliferation of the activated of T-cell.

CD4⁺ T-cells are helper cells, that show a broad range of functions that rely on specialization through functional polarization. These subsets are characterized by different effector functions, defined mainly by the production of distinct cytokines. CD4⁺ T-cells are central coordinators of the innate and adaptive immune response. Regulatory T-cells (Tregs) are a subset of CD4⁺ T-cells critical for control of peripheral tolerance. Tregs suppress anti-tumor immune effector responses in the TME, primarily by promoting an immunosuppressive microenvironment, thus promoting tumor progression.

Increased infiltration of Tregs in the TME have been linked to poor prognosis [18]. CD4⁺ T-cells with a regulatory and activated phenotype are functionally dependent and express FOXP3, which is also commonly used as a marker for this T-cell subset [19].

B-cells and tertiary lymphoid structures

B-cells arise and mature in the bone marrow. Activation of B-cells takes place in secondary lymphoid organs. Once activated, B-cells can present antigens (tumor antigens) to T-cells through the MHC class II pathway or differentiate into antibody-secreting plasma cells and memory cells [20]. In tumors, B-cells are rarely found on their own but, rather, in association with other immune cells (e.g. T-cells, myeloid cells) and mostly within tertiary lymphoid structures (TLS). TLSs reflect lymphoid neogenesis occurring in peripheral tissue upon long-lasting exposure to inflammatory signals mediated by chemokines and cytokines [21]. B-cells are recruited to tumors by local production of lymphoid chemokines [22]. Within the tumor, B-cells engage with the stroma and other immune cells, triggering the formation of high endothelial venules, which in turn stimulate the production of adhesion molecules and chemokines, notably CXCL13, CXCL12, CCL19 and CCL21. These chemokines regulate the development and organisation of TLS, containing B-cells and T-cells zones. TLS have been proposed as a functional equivalent to secondary lymphoid organs, facilitating the recognition of antigens and generation of adaptive immune responses. One of the main effector functions associated with B-cells in TLSs is the production of disease-specific antibodies that can mark antigen-expressing cells for opsonization, complement-mediated lysis, or antibody-dependent cellular cytotoxicity [23]. Presence of TLS has been reported to correlate to improved survival in several tumor types [24]. Presence of TLS has also been correlated with response to immune checkpoint inhibitors [25, 26, 27].

Tumor microenvironment and hypoxia

Hypoxia is considered a hallmark feature of the tumor microenvironment. Hypoxia, defined as an oxygen tension of less than 10 mmHg, arises when an imbalance occurs between the supply of oxygen and its consumption by local cells. In tumors, the presence of a defective vasculature limits the amount of oxygen available in the TME. Tumor cells respond to hypoxia with various adaptations, one of the most important being the activation of hypoxia-inducible factor (HIF) family of transcription factors, mainly HIF-1 α and HIF-2 α [28].

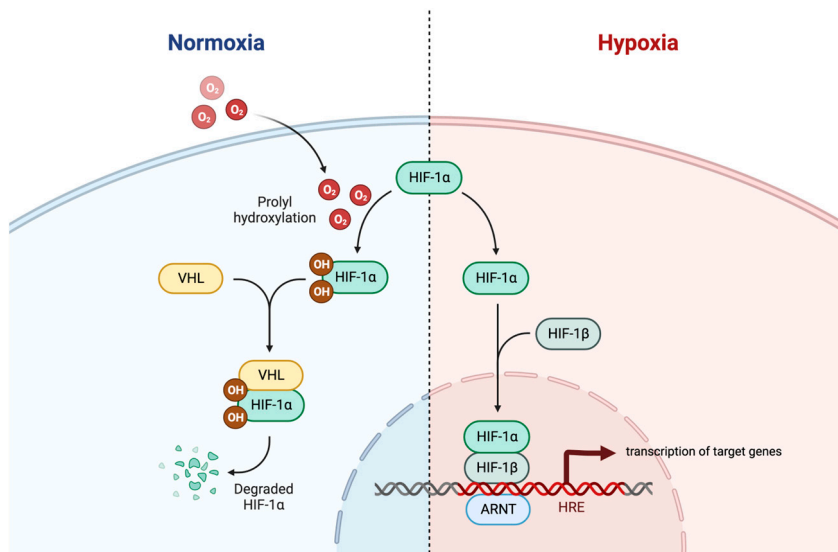


Figure 3. Overview of the hypoxia inducible factor (HIF) pathway. At normoxia, HIF-1 α is hydroxylated and binds to VHL, Von Hippel Lindau, leading to proteasomal degradation. During hypoxia, HIF-1 α binds to HIF-1 β , also known as aryl hydrocarbon receptor nuclear translocator (ARNT). This complex enters the nucleus and binds to hypoxia responsive elements (HRE), resulting in subsequent transcription of target genes. Created with BioRender.com

In normoxic conditions, HIF-1 α is degraded. However, in hypoxia, HIF-1 α translocate to the cell nucleus where it couples with HIF-1 β forming a complex that binds to hypoxia responsive elements in target genes to activate transcription (Figure 3). The genes transcribed are involved in a multitude of processes in the tumor microenvironment and ultimately influence tumor progression and treatment response through mechanisms such as immune escape, increased angiogenesis, and accelerated DNA damage repair [29, 30].

Tumor microenvironment in Melanoma brain metastases

For long the brain has been considered as “immune privileged” a term coined in the 1940’s by Medewar to describe a tissue or organ where the introduction of foreign antigen does not elicit an immune response [31]. This phenomenon has been in part explained by lack of classic lymphatic vessels in the central nervous sytem (CNS), but also by the existence of the blood-brain barrier restricting the passage of molecules into the CNS. Recent studies have however shown that intracranial tumors disrupt the integrity of the blood-brain barrier, making it more permeable [32]. The remodelled barrier allows for facilitated crossing of immune cells and macromolecules from the peripheral circulation.

In the last decade, a lymphatic system has been identified in the mouse brain [33, 34]. Magnetic resonance imaging (MRI) studies have also identified a lymphatic drainage from the brain to cervical lymph nodes in humans, supporting the existence of a lymphatic system in the human brain [35]. This finding supports a possible link between the peripheral and intracranial immune compartments. Indeed, recent studies have shown significant relationship between peripheral and intracranial T-cells suggesting an active crosstalk between peripheral and intracranial immune compartments (Figure 4) [36, 37].

The brain TME includes astrocytes, pericytes and microglia, all brain-specific cells with the possibility to exert stimulatory or suppressive functions [38]. Crosstalk between cancer cells and astrocytes can promote tumor progression through direct stimulation, or through cytokine release and inflammatory mediators [39, 40]. Microglia, tissue-resident macrophages of the CNS and spinal cord and primary immune cells of the CNS, can contribute to metastatic colonization through direct stimulation, or modulation of the microenvironment [41, 42, 43].

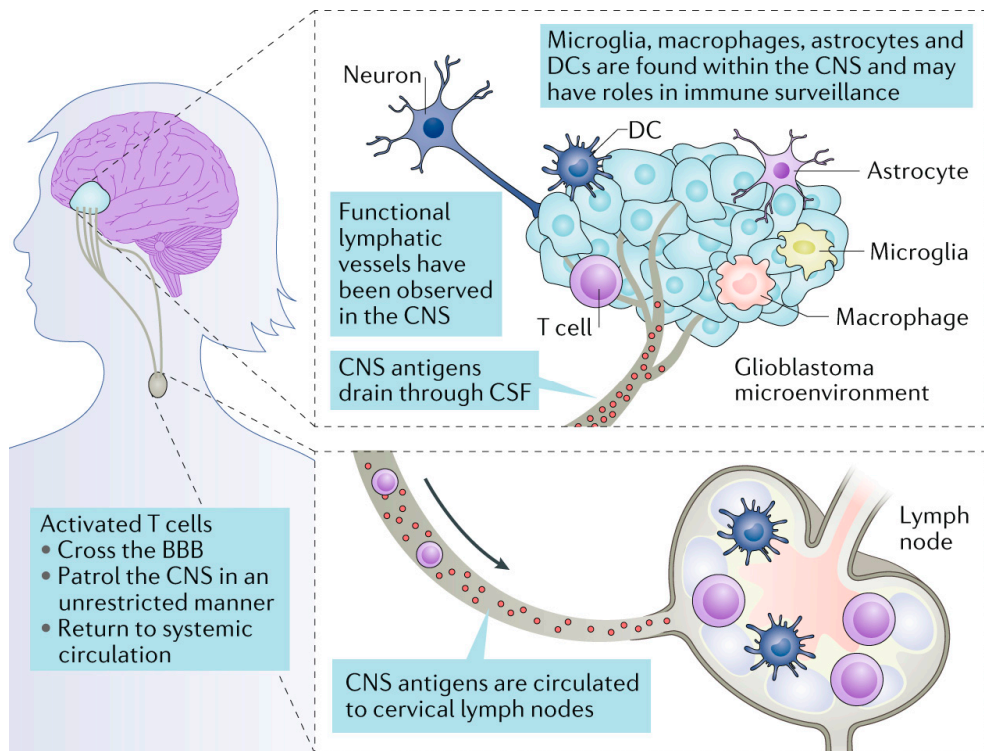


Figure 4. Tumor microenvironment in a brain tumor, visualizing brain-specific features such as functional lymphatic vessels, CNS antigen circulation to cervical lymph nodes and the ability of T-cells to cross the blood brain barrier. Reprinted from Nature [44] with permission from Springer Nature.

Studies have suggested melanoma brain metastases to be a more immunologically cold and immunosuppressed, with less infiltrating T-cells than metastases at extracranial sites [45, 46, 47, 48]. Moreover, further supporting the brain TME as a more immunosuppressed environment are the findings of less T-cell receptor diversity [48] and upregulation of markers of T-cell exhaustion such as PD-1 in brain metastases (BM) [49].

Several studies on cohorts of mixed histology BM have yielded varying results on the prognostic role of T-cell infiltration [50, 51, 52].

Nonetheless, in melanoma BM increased T-cell infiltration has been consistently reported to be associated with improved OS [45, 48, 53, 54].

Although much remains to be learned about BM, current evidence suggests that the brain TME is an immune specialised rather than immune privileged environment.

Tumor microenvironment in Soft Tissue Sarcoma

Immune microenvironment

Sarcomas have generally been regarded as “immune cold”. However, it is becoming increasingly clear that this varies between sarcoma histotypes (Table 1). Studies characterizing T-cell infiltration in STS have reported higher T-cell infiltration in genetically complex sarcomas than in translocation sarcomas, and more abundant CD8⁺ T-cell infiltration than regulatory FOXP3⁺ T-cells. [55, 56, 57, 58]. Data on T-cell infiltration and its association to prognosis have been conflicting. In primary undifferentiated pleomorphic sarcoma (UPS) high densities of infiltrating CD8⁺ and CD3⁺ T-cells were associated to improved outcomes [59]. In synovial sarcoma (SS) one study reported favourable outcome in patients with high CD8⁺ T-cell infiltration [58], whereas another study reported shorter metastases free survival [60]. Several studies have reported no prognostic value of T-cell infiltrates [56, 61].

Most studies reporting CD20⁺ B-cell infiltration using immunohistochemistry have reported sparse staining with positive prognostic association [57, 62]. A study characterizing the immune landscape of STS by analyzing transcriptomic data, identified a subgroup of STS classified as “immune high”. This subgroup showed an elevated expression of B-cell related gene signature that correlated to improved survival and to response to checkpoint inhibitors present in 18% of the STS cohort [25]. Immunohistochemistry revealed that this class of STS is characterized by presence of TLS.

Table 1. Summary of immune biomarker studies in STS. Boxes represent prognostic effect of each biomarker. Green- positive, red-negative, grey-no prognostic effect

STS histology	N	CD4 ⁺	CD8 ⁺	FOXP3 ⁺	CD20 ⁺	CD163 ⁺	PD-L1	ref
Mixed STS	108	Green	Grey		Green			[62]
	203		Red					[61]
	608				Green			[25]
	33+265				Green			[57]
UPS	57		Green				Green	[59]
SS	36	Grey	Green	Red		Red		[58]
	22		Red				Grey	[60]
LMS	149					Red		[63]
	52					Grey		[64]
LPS	56	Grey	Grey	Grey	Grey		Grey	[56]
DDLPS	62					Green		[65]
SFT	113					Red		[65]

UPS-undifferentiated pleomorphic sarcoma, SS- synovial sarcoma, LMS- leiomyosarcoma, LPS- liposarcoma, DDLPS-dedifferentiated liposarcoma, SFT- solitary fibrous tumor

Studies addressing the prognostic value of PD-1/PD-L1 expression in sarcoma have also reported conflicting results. However, a meta-analysis, showed that high PD-L1 was associated with worse overall survival (OS) and worse event free survival [66].

High infiltration of M2-polarised TAM (CD163⁺) has been reported in UPS, leiomyosarcoma (LMS), myxofibrosarcoma and dedifferentiated liposarcoma (DDLPS) [63, 65]. The clinical significance and prognostic value of CD163⁺ TAM are however uncertain. Low infiltration of CD163⁺ TAM were associated to improved prognosis in SS and SFT [58, 65] but not in other STS subtypes.

Taken together, these data suggest that the TME in STS is diverse. Thereby, more research is needed to better understand the impact of the TME on prognosis and response to treatment.

Hypoxia in STS

Several studies have linked hypoxia to poor prognosis in STS (Table 2).

Different methods, direct or indirect, can be used to assess hypoxia. Direct methods involves insertion of electrodes (Eppendorf method) directly into a tumor to measure pO₂. Indirect methods include tissue-based methods such as immunohistochemistry to assess surrogate markers of hypoxia such as Carbonic anhydrase 9 (CAIX), Glucose transporter 1 (GLUT-1) and HIF-1 α . Gene expression profiling is another indirect tissue-based method to assess hypoxia through monitoring alterations in the expression of hypoxia-associated genes.

Table 2. Summary of trials investigating the prognostic importance of hypoxia in STS

STS histotype	N	Method	Marker	Outcome (p<0.05)	Reference
Mixed STS	22	Eppendorf	pO2	DFS	[67]
Mixed STS	28	Eppendorf	pO2	DFS	[68]
Mixed STS	206	IHC	GLUT-1	DSS	[69]
Mixed STS	47	IHC	CAIX	OS	[70]
Mixed STS	203	IHC	CAIX	DFS	[71]
Mixed STS	49	IHC	HIF-1 α	OS	[72]
Mixed STS	55	IHC	HIF-1 α	OS	[73]
MPNST	82	IHC	HIF-1 α	OS	[74]
Pleomorph STS	89	DNA microarray	Hypoxia gene signature	MFS	[75]
Mixed STS	45	RT-qPCR	3-gene signature	OS	[76]
Mixed STS	55+77	RT-qPCR	15-gene signature	DSS	[77]
Mixed STS	183	RNAseq	24-gene signature	MFS	[78]
Mixed STS	228 + 255	RNAseq	6-gene signature	OS	[79]

MPNST-malignant peripheral nerve sheath tumor, IHC- immunohistochemistry, RT-qPCR- reverse transcription polymers chain reaction, CAIX- Carbonic anhydrase 9. GLUT-1 Glucose transporter1, HIF-1 α hypoxia inducible factor 1 alpha, DFS- disease free survival, DSS-disease specific survival, MFS-metastases free survival, OS- overall survival

Immunotherapy

Efforts to exploit the immune system in cancer treatment can be divided in two main categories, passive and active, based on their ability to (re-)activate the host immune system against malignant cells [80]. Passive forms of immunotherapy, such as tumor-targeting monoclonal antibodies and adoptively transferred T-cells have intrinsic antineoplastic activity and are not dependent of the host immune system. Oppositely, anticancer vaccines and immune checkpoint modulators, exert their effects upon engagement of the host immune system, thus constituting forms of active immunotherapy.

Immune checkpoints are proteins that regulate the activation and function of immune cells, particularly T-cell activation. These pathways are important for maintaining homeostasis and preventing autoimmunity, but they can also be exploited by cancer cells to evade immune surveillance [81]. The two most well-known immune checkpoint proteins are programmed death-1 (PD-1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4).

PD-1 is expressed on activated T-cells, in particular in those that have become exhausted by chronic antigen exposure. When engaged by one of its ligands, PD-L1 and PD-L2, expressed by other immune cells, PD-1 exerts suppressive effects. PD-L1 can also be expressed by tumor cells through upregulation or constitutively.

Binding of PD-1 to its ligands inhibits T-cell activation and promotes T-cell exhaustion [82].

CTLA-4, on the other hand, is expressed on the surface of activated T-cells and competes with CD28, a co-stimulatory molecule on the T-cell surface, for binding to B7-1 (CD80) and B7-2 (CD86), which are expressed on APCs. Binding of CTLA-4 to B7-1 or B7-2 results in inhibition of T-cell activation and proliferation [83].

Thus, blocking of PD-1 or PD-L1 can restore T-cell function and enhance anti-tumor immune responses, while blocking CTLA-4 can enhance T-cell activation and proliferation. Immunotherapy using antibodies targeting these immune checkpoints have become standard of care for several malignancies. Since the mechanism of action of immune checkpoint inhibitors relies on inhibition of physiological tolerance mechanisms, these drugs are often associated off-target effects, so-called immune related adverse events (IRAE).

During the last decade, several other targetable checkpoints such as TIM-3, LAG-3, TIGIT, VISTA, SIRP-CD47 have emerged and are currently being tested in clinical trials [84].

Predictive biomarkers

Despite impressive and durable responses observed in some patients, the majority of patients do not respond to immune checkpoint inhibitors,, as demonstrated by response rates rarely exceeding 15% in most cancer types [85]. Thus, identification of biomarkers of sensitivity and resistance to immune checkpoint blockade are needed.

PD-L1 emerged as an early biomarker to be tested in immunotherapy clinical trials e.g. melanoma, lung cancer and head and neck carcinoma. However, PD-L1 as evaluated by immunohistochemistry has limitations as a predictive tool, as illustrated by analysis of PD-L1 predictive value in 15 different tumor types, unveiling PD-L1 expression to be predictive in only about 30% of cases [86]. Different assays, scoring methods and thresholds, but also variable spatial and temporal expression and regulation of PD-L1 expression, may lie behind these results. Therefore, and despite its clinical indication in some tumor types, PD-L1 expression is not considered an universal predictive biomarker.

Tumor mutational burden, TMB, is representative of the number of non-synonymous DNA mutations and considered to result in increased neoantigen presentation. TMB as estimated by next generation sequencing based techniques, has demonstrated predictive value across different tumor types. Indeed, retrospective analyses have shown higher clinical benefit in patients with tumors with high TMB than in those without high TMB [87].

Defective mismatch repair machinery (dMMR) cause microsatellite instability (MSI) which can result in genetic mutations leading to development of cancer. MSI-

high is associated with a hereditary form of cancer, Lynch syndrome, but can also occur in sporadic tumors, for example in sporadic colorectal cancer. MSI-high leads to high mutational load and creates enrichment in tumor-specific neoantigens. The latter is often accompanied by high lymphocyte infiltration and is highly predictive for response to immune checkpoint inhibitors. NICHE-2 trial in locally advanced colorectal cancer showed impressive responses in 99% of dMMR tumors, of which 67% pathologic complete response. In this trial, patients received a short course of neo-adjuvant treatment with ipilimumab and nivolumab on day 1, and nivolumab monotherapy on day 15, as well as +/- celecoxib daily until the day before surgery [88].

PD-1 inhibitor pembrolizumab has received tissue agnostic approval for tumors with dMMR/MSI-high as well as for tumors with high TMB (>10 mutations per megabase DNA), confirming the predictive value of these markers.

Other factors that have been associated with response to immune checkpoint inhibitors are MHC expression, T-cell receptor diversity, presence of tumor infiltrating lymphocytes, as well as the gut microbiome [89].

No biomarkers for response to checkpoint inhibitors are currently in clinical use in neither melanoma nor in sarcoma.

In soft tissue sarcomas, immune cell infiltration is generally sparse, TMB low and MSI is rare [90].

Based on the results by Petitprez et al. suggesting TLS as a predictive marker for response to immune checkpoint inhibitors [25], the PEMBROSARC study enrolled a cohort (N=30) based on the presence of TLS. Patients were treated with pembrolizumab and low-dose cyclophosphamide and reported overall response rate (ORR) of 30%, and 6-month non-progression rate of 40% [91]. In comparison, in the all-comer cohort of the same study, the ORR was 2.4% and 6-month non-progression rate was 4.9%.

Predictive value of TLS has been demonstrated in melanoma [26, 92] as well as several other solid tumor types [93]

Melanoma

Etiology and Epidemiology

Melanoma is a melanocyte-derived cancer, which most often is found in the skin (cutaneous melanoma) but can occur in all organs harbouring melanocytes, e.g. the ears, the eyes, the mucosal membranes (nose, oral cavity, anorectal mucosa and the

genitourinary mucosa), the central nervous system (leptomeningeal melanoma) and in the gastrointestinal tract.

Melanomas can arise de novo in the skin (about 70%) or have a common nevus or a clinically atypical nevus as a precursor lesion (in about 30%) [94, 95].

In the western countries, incidence of cutaneous melanoma has increased over the past decades. In Sweden, melanoma is the 5th most common malignancy in both men and women with an age adjusted incidence of 43.7/100000 in men and 38.1/100000 in women reported 2020 [96].

UV light exposure is considered the major etiologic factor in the development of melanoma. Multiple studies support an etiologic association between UV irradiation and melanoma and suggest that it mediates its effects by a combination of DNA damage, inflammation, and immune suppression.

Several physical traits have also been linked to increased incidence of cutaneous melanoma. These include blond or red hair, green or blue eyes, presence of multiple (>100) melanocytic nevi, and more than five atypical nevi.

It has been estimated that approximately 10% of melanomas occur in high-risk families with an autosomal dominant inheritance with incomplete penetrance. The most frequent and highest penetrance melanoma susceptibility gene is a germline mutation in *CDKN2A* [97, 98]. *CDKN2A* mutations have been reported in approximately 25% of melanoma-prone families.

Prognostication

The best predictor of metastatic risk is the depth of invasion, measured with an ocular micrometre, from the granular layer of the skin to the base of the primary lesion, as originally described by Breslow. Depth of invasion or so-called Breslow thickness remains an important factor in staging and prognostic stratification. However, other histologic and clinical features have relevance for estimating the risk of metastasis and mortality. These include age, angiolymphatic invasion, mitotic rate, sex, and body site.

In melanoma, the eighth edition of the TNM AJCC staging system is currently the most widely accepted approach to melanoma staging and classification and provides accurate risk stratification essential to guide patient treatment [99].

Primary tumor (Breslow) thickness and ulceration represent important prognostic factors for survival and define T-category strata in primary cutaneous melanoma. The N-category reflects the number and extent of tumor-involved regional nodes. Regional lymph nodes represent the most common first site of metastasis in patients with primary cutaneous melanoma.

Current guidelines recommend sentinel lymph node biopsy in patients with pT >1mm and without clinical or radiographic evidence of regional lymph node metastasis [100].

Metastatic disease is reflected in the M-category, which is defined by the site of distant metastases and the level of serum lactate dehydrogenase (LDH).

Patients with non-visceral distant metastasis (distant cutaneous, subcutaneous, nodal) are categorized as M1a, those with lung metastasis are categorized as M1b, those with non-central nervous system (CNS) visceral metastases as M1c. M1c no longer includes CNS metastasis, and patients with distant metastasis to the CNS with or without any other distant sites of disease are categorised as M1d. M-subgroups from a to d represent increasingly poor prognosis.

Molecular profiles

Genetic alterations in the mitogen-activated protein kinase (MAPK) pathway is central in the development of melanoma. There is evidence of MAPK activation by defined point mutations in at least 70% of melanomas, resulting in constitutive signalling leading to oncogenic cell proliferation and escape from apoptosis.

Typically, cutaneous melanomas are classified into one of four subtypes based on the pattern of the most prevalent mutated genes: mutant BRAF, mutant RAS, mutant NF1, and triple wild type. This last group included melanomas with KIT mutations and focal amplifications and complex structural rearrangements.

Several studies have demonstrated that mutations found in extracranial metastases and BM are concordant [101, 102, 103]. However certain genetic events have been implicated in formation and progression of BM, such as loss of PTEN [104] and upregulation of the PI3K-pathway in BM [101].

Medical Treatment

Immunotherapy

Figure 5 illustrates how the approval of immunotherapies in treatment of melanoma has advanced since 2011. First out was the introduction of ipilimumab, a CTLA-4 inhibitor [105, 106], followed by PD-1 checkpoint inhibitors pembrolizumab and nivolumab [107, 108, 109, 110, 111, 112]. In summary, treatment with PD-1 antibodies give more objective responses, shorter time to response and improved progression free survival (PFS) compared to ipilimumab.

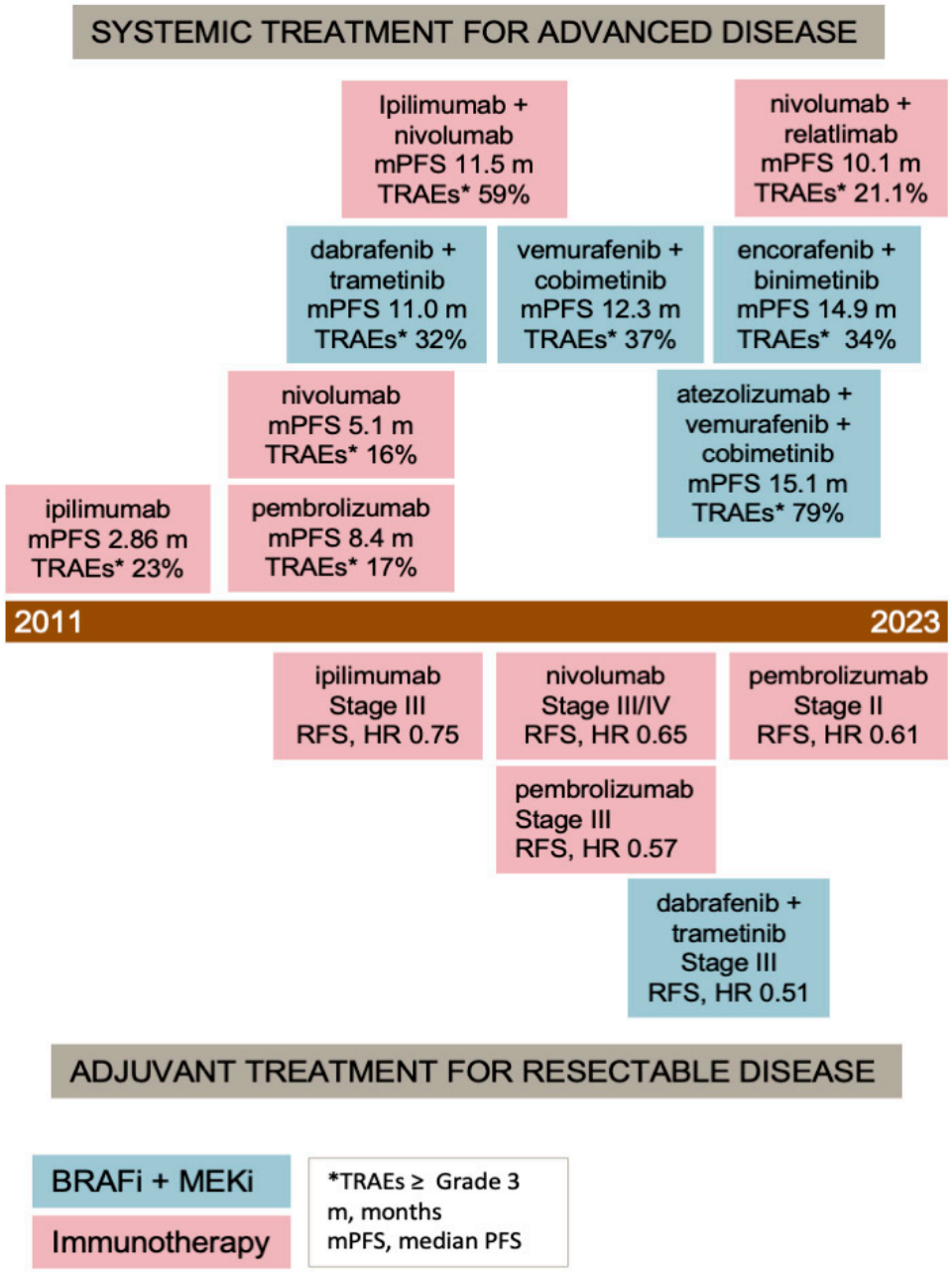


Figure 5. Advances in management of melanoma. Modified from Cancers [113] under the terms of <http://creativecommons.org/licenses/by/4.0> HR- hazard ratio mPFS- median progression-free survival, TRAE- treatment related adverse event

Combination treatment with ipilimumab 3 mg/kg and nivolumab 1 mg/kg increases objective responses, gives more long-lasting PFS but also substantially more toxicity [114, 115, 116]. Flipped dose ipilimumab 1 mg/kg and nivolumab 3 mg/kg does not significantly affect response rates or PFS, but significantly reduces grade 3-4 irAE, 34% compared to 48% [117, 118, 119]. There are no clear-cut guidelines on who to offer combination treatment, but good performance status, high tumor burden, elevated LDH, BRAF-mutation and low PD-L1 expression support the use of combination treatment.

Combination of PD-L1 inhibitor with BRAF and MEK inhibitor (BRAFi, MEKi) showed improved PFS at 15.1 months, however with the price of increased toxicity [120] and recently published data showed no OS benefit of the combination [121].

LAG-3 is a CD4 homolog which upon binding to MHC class II results in negative regulation of T cell proliferation [122]. The LAG-3 inhibitor, relatlimab, has in combination with nivolumab shown a mPFS of 10.1 months with favorable toxicity [123].

In the adjuvant setting, treatment with PD-1 inhibitor leads to decreased risk for relapse in patients with stage II and III melanoma [124, 125]. Most current guidelines recommend one year of adjuvant treatment with PD-1 inhibitor for patients in stage IIIB-D. There is no support for use of combination treatment with ipilimumab and nivolumab over monotherapy PD-1 inhibitor in the adjuvant setting [126, 127]. However, promising results have been reported in the neoadjuvant/adjuvant setting, both with the latter combination and with combination relatlimab and nivolumab [128].

Targeted therapies

For patients with BRAF V600 mutation, three combination regimens of BRAF-MEK inhibitors are approved: dabrafenib and trametinib, vemurafenib and cobimetinib, and encorafenib and binimetinib. The combinations are comparable in efficacy, with response rates between 60-70% and mPFS between 11.0-14.9 months [129, 130, 131]. In the adjuvant setting, treatment with dabrafenib and trametinib is approved in stage III melanoma [132].

Optimal choice of first-line therapy with checkpoint inhibitors or BRAFi-MEKi for BRAFV600 mutant metastatic melanoma has been addressed in two clinical trials. Both studies strongly support the use of dual checkpoint inhibition as first-line treatment. The Dreamseq study showed a 20% OS benefit at two years follow up in favor of dual checkpoint inhibition, 72% OS for ipilimumab and nivolumab compared to 52% for dabrafenib and trametinib [133]. The SECOMBIT trial, not powered to compare the different treatment arms, reported two-year OS of 73% for ipilimumab and nivolumab compared to 65% for encorafenib and binimetinib in first line [134].

Brain metastases

Development of BM is frequent in melanomas, clinically evident in 40-60% of metastatic melanoma, in autopsy material as high as 75% [135, 136].

Historically, survival after diagnosis of melanoma BM has been poor, with median survival between 4-6 months [137, 138, 139, 140]. Prior to introduction of targeted therapies and checkpoint inhibitors, treatment of melanoma BM relied on surgery and radiotherapy.

Whole-brain radiotherapy has been reported to improve neurologic symptoms but fails to provide long-term disease control and improve survival (median survival of only 14 weeks) [141], thus whole-brain radiotherapy has limited value and is now rarely used in the management of melanoma BM.

Temozolomide, a chemotherapy agent that crosses the blood–brain barrier, has been used for decades in patients with melanoma BM despite clinical trials demonstrating intracranial clinical response rates of 3–7% [142].

Current management of Melanoma brain metastases

Local Management

Considerations for local therapy (surgery or radiation therapy) include whether the metastases are symptomatic, the number and site of the metastases and the type of systemic drug therapy available and its chance of providing a response in the brain [143].

BM that are symptomatic or generate mass effect at presentation are usually treated with surgery, which can rapidly relieve symptoms and maintain function.

For patients with a single or a small number of melanoma BM (usually up to four with a maximum diameter of three cm), stereotactic radiosurgery (SRS) provides a high rate of local control, comparable to surgical resection. Postoperative SRS to the resection cavity can be considered after complete resection of melanoma BM based on a randomised phase III study. This study, encompassing several tumor histologies including melanoma, showed that addition of SRS to the surgical cavity significantly improved the 12-month local control rate compared with observation in patients with one to three completely resected brain metastases of several tumor histologies, including melanoma [144].

Combining SRS with systemic therapies is appealing. Several retrospective studies have demonstrated improved intracranial response rates, PFS and OS from combination strategies [145, 146]. However, the optimal sequencing of systemic therapy and SRS remains to be established and needs further prospective studies.

Systemic therapies

Following the introduction of modern melanoma treatments, the overall survival from time of diagnosis of melanoma BM has improved.

Recently, a single-institution retrospective analysis reported median OS from melanoma BM diagnosis of 14.4 months in patients treated between 2014 and 2018, as compared to 10.6 months for patients treated between 2009 and 2013 [147].

Originally excluded from clinical trials due to poor prognosis, several recent studies focusing on melanoma BM have demonstrated intracranial responses to ICIs as well as targeted therapies albeit lower than response rates reported for extracranial disease.

The first clinical trial of a PD-1 inhibitor (pembrolizumab) in patients with active brain metastases demonstrated an intracranial response rate of 26% and a 48% overall survival at 24 months[148]. Two subsequent trials, on the combination ipilimumab and nivolumab, have since then confirmed the intracranial activity of ICIs. The CheckMate 204, showed an intracranial overall response rate of 56%, with 26% intracranial complete responses and 30% intracranial partial responses in patients with asymptomatic brain metastases [149]. In the cohort of symptomatic melanoma BM intracranial response rate was lower, 22% [150]. The three-year follow-up showed an overall survival of 71.9% in asymptomatic patients compared to 36.6% in the symptomatic patients [151].

The Australian ABC trial showed similar intracranial response rates of 46% with ipilimumab and nivolumab in previously untreated asymptomatic patients with melanoma BM [152]. Furthermore, a five-year intracranial PFS-rate of 46% was reported [153]. BRAF mutated melanoma BM can benefit from combination of BRAF and MEK inhibitors. In the COMBI-MB trial, combination of dabrafenib and trametinib demonstrated intracranial response rates of 58%, although with a median duration of intracranial response of 6.5 months in asymptomatic patients, and 4.5 months in symptomatic patients [154]. Other trials or small series showed similar efficacy of other BRAF-MEK inhibitor combinations.

Management of melanoma BM has radically changed during the past decade, particularly with the advent of targeted therapy and immunotherapy, along with improved local therapeutic options such as SRS. Treatment with combination immunotherapy can induce clinically meaningful and durable intracranial responses and is often considered as first line therapy in melanoma BM. Still, further studies are needed to determine the optimal sequencing and combination of the different treatment modalities available.

Soft Tissue Sarcoma

Epidemiology and Etiology

Soft tissue sarcomas (STS) are rare tumors, accounting for less than 1 % of adult cancers. In Sweden, the incidence of sarcomas has been relatively stable over time. In 2020 the reported incidence of sarcomas in Sweden was 3.7/100 000 inhabitants, thus corresponding to approximately 370 sarcoma cases [96]. Approximately 2/3 of these are soft tissue sarcomas. However, the actual incidence may be an underestimation, as cases of visceral sarcomas, e.g. gastrointestinal stromal tumors (GIST) may have been classified with associated organ and not reported as sarcomas.

STS are tumors that may show a wide range of differentiation, usually defined as having primarily a mesenchymal origin, although their histogenesis has not been clearly defined. Although no common cell of origin has been identified, transformation from a common multipotent mesenchymal stem cell has been suggested [155, 156, 157].

Most STS are sporadic and have no defined cause. However, in a small percentage of cases, predisposing or associated factors have been identified. Hereditary genetic syndromes such as germline mutations including NF1 (neurofibromatosis), TP53 (Li-Fraumeni) and RB (hereditary retinoblastoma) account for 2-4% of all sarcomas [158]. Environmental exposures, such as chemical carcinogens, ionizing radiation and viral infection have also been implicated in the etiology. [159].

Classification and molecular alterations

Classification of STS is based on pathological features and molecular alterations [160]. Sarcomas are broadly classified in two major groups; those with distinctive cytogenetic aberrations, most often chromosome translocations i.e. karyotypically simple sarcomas or translocation sarcomas, and those with complex karyotypes [161]. Translocation sarcomas represent approximately ¼ of all sarcomas and are characterized, and most often also diagnosed, on the basis of their translocation [162]. Beside from the translocation they tend to have very little other genetic aberrations. The translocations often involve genes encoding for transcription factors or epigenetic modulators resulting in uncontrolled growth [163]. Common translocation STS include synovial sarcoma (SS), myxoid liposarcoma, solitary fibrous tumor (SFT) and alveolar soft part sarcoma (ASPS).

The genetically complex sarcomas typically show extensive chromosomal rearrangements, duplications and deletions and are often copy-number driven. Common STS subtypes in this group include leiomyosarcoma (LMS),

dedifferentiated liposarcoma (DDLPS), angiosarcoma and undifferentiated pleomorphic sarcoma (UPS).

Prognostication

Grading, based on intrinsic qualities of the untreated primary tumor, is one of the most important pieces of information for therapeutic decision. The most commonly used grading system, is the one proposed by the FNCLCC (Fédération Nationale des Centres de Lutte Contre le Cancer) which considers three parameters: tumor differentiation, tumor necrosis, and mitotic count [164]. Each factor is given a score, and the scores are added to determine the grade of the tumor. Grade 1 is considered low-grade, whereas grades 2 and 3 are considered high-grade.

Staging of soft tissue sarcomas is challenging. The AJCC/UICC version 8 is based on primary tumor size and grade, lymph node involvement and distant metastasis. However, it does not consider the fact that the oncologic outcome of STS is strongly influenced by histologic subtype and site of tumor origin [165]. Hence, the AJCC/UICC system is not commonly used in clinical practice. Several nomograms to predict prognosis are available, for example the MSKCC nomogram and the Sarculator [165, 166, 167, 168].

In Scandinavia, the *SING* system is used for prognostication of high-grade soft tissue sarcomas [169, 170, 171]. This system considers tumor size (dichotomised at 8 centimeters), vascular invasion, presence or absence of necrosis, and growth pattern defined as pushing or infiltrative. High-risk tumors are defined by either presence of vascular invasion, or two out of three of the following criteria: tumor size > 8 cm, infiltrative growth pattern and presence of necrosis.

Transcriptomic signatures

Advances in gene expression profiling techniques have led to development of transcriptomic signatures aiming at improved prognostication.

The CINSARC, Complexity Index in SARComas, is currently under clinical evaluation. It is a 67-gene signature, encompassing genes involved in mitosis and chromosomal management. In retrospective studies, CINSARC signature has been shown to predict metastasis better than FNCLCC grading [172, 173, 174]. Furthermore, the CINSARC signature has shown ability to identify poor prognosis within the FNCLCC grade 2 group. This is highly relevant as the grade 2 group is a large, heterogeneous group that would benefit from better risk stratification. Randomized phase III trials utilizing the CINSARC signature to identify high-risk patients for treatment with adjuvant chemotherapy are ongoing (NCT03805022 and NCT04307277).

Genetic grade index, GGI, is a gene expression signature including 108 genes, originally developed for early breast cancer prognostication. However, it has also proved to have prognostic potential in a retrospective cohort of 678 STS [175]. Further validation in clinical trials is needed.

Surgery

Surgery, with wide resection of the tumor, is the mainstay of treatment for all sarcomas and should be performed at a reference centre [176, 177]. Surgical margin is related to risk of local recurrence. Different systems for reporting surgical margins are used. Surgical margins, as defined by Enneking et al [178], are based largely on the macroscopic findings during surgery, and termed intralesional, marginal, wide and radical (compartmental). The R-classification for margins is also used and represent an independent prognostic factor for local recurrence [179]. This system is based mainly on microscopic findings. The R-classification is categorized as R2 grossly positive, R1 microscopically positive or R0 microscopically negative.

Radiotherapy

For STS of extremities and trunk wall, the value of radiotherapy in addition to surgery for local control has been demonstrated in multiple trials [180, 181, 182, 183]. As a result, perioperative radiotherapy is considered standard of care for high grade STS of extremity and trunk wall operated with marginal or intralesional margin. However, no survival benefit has been demonstrated.

Optimal timing, and dosing, of radiotherapy is a matter of debate. In international guidelines, postoperative doses up to 66 Gy are recommended, whereas 50 Gy are recommended preoperatively [184]. However, in the Scandinavian study SSG XX accelerated and hyperfractionated radiotherapy in doses equivalent to 50 Gy were given postoperatively. Local recurrence at five years was reported in 14% of the patients, with low rates of long-term toxicity, although the final results of the trial have not yet been published [185]. Preoperative radiotherapy possibly improves normal tissue sparing, but has been associated with more wound healing complications [186]. In addition, soft tissue sarcomas are heterogenous in terms of radiosensitivity, and therefore there is a risk that preoperative radiotherapy may delay necessary surgery. Postoperative radiotherapy will inevitably include more normal tissue, potentially with significant risk for long-term toxicity. However, available toxicity data is based on dated radiation techniques and long-term toxicity with modern techniques, i.e. rotation techniques or proton therapy, is largely unknown.

Current guidelines from the Scandinavian sarcoma group (SSG) recommend that perioperative radiotherapy should be offered to all high-grade deep-seated tumors,

irrespective of surgical margin, as well as following marginal and intralesional margin surgery irrespective of tumor depth. Perioperative radiotherapy is further recommended to all STS operated with intralesional margin, regardless of malignancy grade.

For retroperitoneal sarcomas, the value of radiotherapy is less certain and the largest reported randomized trial, showed no clear benefit of perioperative radiotherapy over surgery alone [187].

Medical treatment of STS

Neoadjuvant and Adjuvant setting

There is no clear consensus on the benefit of adjuvant chemotherapy in STS as no randomized phase 3 trial has showed a survival benefit of adjuvant chemotherapy. However, most of the trials are now 20-40 years old and should be interpreted with caution as the study populations are heterogenous especially with regards to grade [188]. Two meta-analyses have been published. The first summarizing results from 14 anthracycline based trials, reported an hazard ratio for OS of 0.89 which was not significant ($p=0.12$) [189]. The second, a later, pooled meta-analysis including four additional trials, reported an odds ratio of 0.56 ($p=0.01$) in favor of doxorubicin plus ifosfamide, corresponding to an absolute risk reduction of 11% [190].

A large, randomized phase 3 EORTC trial, often considered a landmark trial for adjuvant chemotherapy in STS, failed to demonstrate a survival benefit of treatment with doxorubicin and ifosfamide [191]. However, it should be noted that more than 50% of the patients included in this trial had low-risk STS when risk-stratified using the Sarculator nomogram [192]. In fact, in high-risk STS identified using the nomogram Sarculator, a post-hoc analysis showed that adjuvant chemotherapy halved the risk of death [192]. The Italian Sarcoma Group published an adjuvant study including only large (>5cm) high-grade STS showing an absolute OS benefit of 13% at two years, increasing to 19% at four years ($p=0.04$) in patients treated with anthracycline and ifosfamide [193]. In summary, these data strongly suggest that correct patient risk stratification is absolutely essential in the adjuvant setting.

In Scandinavia, the SSG XX study, a non-randomized phase 2 study, addressed the benefit of adjuvant doxorubicin and ifosfamide in 150 patients with high-risk STS according to the *SING*-system. In this study, the five-year metastasis-free survival (MFS) was 70.4% and five-year OS was 76.1 %, comparing favorably to historic data [185]. Based on the outcome of SSG XX, current Scandinavian guidelines recommend combination chemotherapy with doxorubicin and ifosfamide in the adjuvant setting in high-grade STS deemed as high-risk according to the *SING*-system (see STS prognostication).

Neoadjuvant chemotherapy has theoretical advantages, however its use in STS is limited mainly due to difficulties in making a correct risk stratification based on a core biopsy [194].

Advanced setting

In the metastatic setting, doxorubicin is considered the single most important drug for most sarcoma histotypes. As single therapy, mOS for doxorubicin range between 12.8- 20.4 months, mPFS range between 4.1- 6.8 months and ORR between 12-20% [195, 196, 197, 198, 199]. Addition of ifosfamide, or other drugs, to doxorubicin remains somewhat controversial but is increasingly used. Even if combination therapies have failed to prove an OS benefit, several combinations have been shown to be clearly better in terms of PFS.

The largest phase 3 study on doxorubicin in combination with ifosfamide versus doxorubicin monotherapy in the metastatic setting showed that combination treatment was associated with significantly longer mPFS, 7.4 months vs 4.6 months ($p=0.003$), as well as significantly increased overall response rate, 26% vs 14 % ($p<0.0006$), however no OS benefit was proven [200]. For LMS, combination of doxorubicin and dacarbazine has, in a retrospective trial, been associated with significantly improved PFS and ORR compared to doxorubicin monotherapy and doxorubicin and ifosfamide [201].

Furthermore, the value of combination chemotherapy is supported by a large retrospective analysis showing a significant impact of combination therapy on OS with a hazard ratio of 0.82 ($p=0.0003$) [202].

Nonetheless, combination regimens have repeatedly been associated with additive and thereby greater toxicity than with single-agent doxorubicin.

In second line treatment, choice of treatment largely depends on STS histotype.

Gemcitabine-docetaxel has shown similar PFS rates to single doxorubicin in first line [199] but with greater toxicity profile. Thus, Gemcitabine-docetaxel is often considered for second-line therapy in foremost, but not restricted to, LMS and UPS.

Trabectedin is a marine-derived anti-neoplastic agent with an dualistic mode of action, acting both on tumor cells by binding to the minor groove of DNA, but also exerting a direct effect on TME by inhibiting TAMs [203]. Trabectedin has shown activity in both LMS and liposarcoma (LPS) [204, 205, 206]. In myxoid LPS response rates of 50% and mPFS of 17 months have been reported in a retrospective trial [207].

Eribulin, a microtubule inhibitor, has demonstrated improved OS compared to single agent dacarbazine in LPS in a study including LMS and LPS [208, 209, 210].

Multi-target tyrosine kinase inhibitor pazopanib was evaluated in STS in the placebo randomized phase 3 Palette trial, showing an improvement in median PFS from 1.6

to 4.6 months ($p < 0.0001$) [211]. Other multi-target tyrosine kinase inhibitors with activity in STS are sunitinib [212], sorafenib [213, 214] and regorafenib [215]

To deal with the rarity of STS, most clinical trials to date have used an all-comers approach. There is however a great heterogeneity among STS subtypes as demonstrated by varying response rates to the same treatment across different STS. Perhaps as a result of this subtype heterogeneity, agents with promising activity in phase 2 trials, have notoriously failed to improve outcome in all-comer phase III trials [196, 198, 216].

Subtype related variations in response rates are also observed in trials exploring immunotherapy in STS. Response rates to PD-1 inhibition typically vary between 10 and 20 %. However, certain subtypes, such as alveolar soft part sarcoma, angiosarcoma and UPS, appear more responsive. In 2022, FDA has given the first approval of a treatment for ASPS, to atezolizumab, a PD-L1 inhibitor. Much focus is currently set on developing combination strategies with checkpoint inhibitors and chemotherapy or small molecule inhibitors, as well as radiotherapy. An overview of selected reported trials with checkpoint inhibitors in STS is presented in Table 3.

Table 3. Summary of selected immune checkpoint inhibitor trials in STS

Treatment	N	ORR %	mPFS (months)	Subtype (RR)	Reference
ICI					
Pembrolizumab	84	18	4.5	UPS 23%, LPS 10%	Tawbi et al. [217]
Ipilimumab+nivolumab	38	16	4.1	UPS 29%, LPS 14.3%	D'Angelo et al. [218] Chen et al. [219]
Durvalumab+tremelimumab	57	12	2.8	ASPS (40%) chordoma (20%) AS (20%) UPS (20%)	Somaiah et al. [220]
ICI + chemotherapy					
Pembrolizumab+cyclofosfamide	57	2	1.4	1 PR SFT	Toulmonde et al. [221]
Pembrolizumab+doxorubicin	37	19	8.1	UPS 67% DDLPS 50%	Pollack et al. [222]
Pembrolizumab+doxorubicin	30	37	5.7	UPS 100% LMS 40% LPS 28.7%	Livingston et al. [223]
Avelumab+ trabectedin	33	13	8.1	LMS (17%)	Wagner et al. [224]
ICI + small molecule inhibitor					
Pembrolizumab+axitinib	33	25	4.7	ASPS (54.5%), non-ASPS (9.5%)	Wilky et al. [225]
Nivolumab+ sunitinib	68	21	5.6	CR AS, PRs in AS, EMC, SS, ASPs	Martin-Broto et al. [226]

AS- angiosarcoma, ASPs-alveolar soft part sarcoma, CR- complete response, DDLPS-dedifferentiated liposarcoma, EMC- extraskeletal myxoid chondrosarcoma, ICI- immune checkpoint inhibitor, LPS-liposarcoma, PR- partial response, SFT- solitary fibrous tumor, SS- synovial sarcoma, UPS-undifferentiated pleomorphic sarcoma

Liposarcomas

Liposarcomas (LPS) represent approximately 20% of STS, making it one of the most common STS subtypes. Further, LPSs are divided in four subgroups that differ in genetic alterations, clinical behaviour, and optimal treatment strategies. Myxoid/round cell LPS are characterized by reciprocal chromosomal translocation t(12;16)(q13;p11), resulting in the *FUS-DDIT3* chimeric gene and are usually responsive to both chemotherapy and radiation therapy. Pleomorphic LPS are genetically complex without pathognomonic alterations. Well differentiated liposarcomas (WDLPS) and dedifferentiated liposarcomas (DDLPS), are usually considered to represent the broad spectrum of one disease, where the WDLPS subtype is regarded as mainly locally aggressive, but usually slow growing and with low metastatic potential. WDLPS can however undergo a process of dedifferentiation, where the tumor cells lose their specialized features and as a result become more aggressive. Indeed, DDLPS have a more aggressive biology, with a high rate of local recurrence and metastatic potential [227]. Genetically, both WDLPS and DDLPS are characterized by amplification in chromosome 12 (12q13-15), harbouring mouse double minute 2 (*MDM2*), cyclin-dependant kinase 4

(*CDK4*), and fibroblast receptor substrate 2 (*FRS2*) genes. These molecular findings have unleashed new therapeutic options targeting the molecular alterations found in WDLPS/DDLPS.

Medical treatment

The backbone in current management of advanced, inoperable WD/DDLPS is doxorubicin, often in combination with ifosfamide. Doxorubicin monotherapy typically results in a PFS of 4 -5 months, whereas combination treatment with doxorubicin and ifosfamide has reported mPFS of up to 12 months [228]. Treatment beyond first line can include eribulin, trabectedin, ifosfamide, gemcitabine and docetaxel, and pazopanib. However, response rates are generally low and there is a great unmet need for effective treatment alternatives in this group of patients. Studies supporting current management of WDLPS/DDLPS are summarized in Table 4.

Table 4. Summary of Clinical trials for WDLPS/DDLPS

Treatment	N	ORR %	mPFS, months	mOS, months	Reference
First line (all retrospective)					
Anthracycline (n=48) Anthracyclinebased combo (n=18) Other(n=43)	109	overall 9 (D+I 22)	4 (D+I 12)	19 (D+I 31)	Stacchiotti et al. [228]
Anthracycline (n=7) Anthracyclinebased combo (n=67) Other (n=10)	84	21	4	29	Livingston et al. [229]
Anthracycline (n=92) Anthracyclinebased combo (n=79) Other (n=36)	208	12	4.6	15.2	Italiano et al. [230]
Anthracycline (n=32) Anthracyclinebased combo (n=16) Other (n=9)	100	17	N/A	9.7	Langmans et al. [231]
Beyond first line					
Gemcitabin-Docetaxel (n=65) Gemcitabin (n=7)	65	9.7	9.2	18.8	Thirasastr et al. [232]
Trabectedin	45	N/A	2.2	N/A	Demetri et al. [205]
Eribulin	31	0	2.0	18.0	Demetri et al. [233]
Pazopanib	41	2.4	4.4	12.6	Samuels [234]

D - doxorubicin, I - ifosfamide, N/A- not reported

Emerging results from recent targeted therapy trials with MDM2 or CDK4/6 inhibitor are summarized in Table 5. Most promising results were obtained with the MDM2 inhibitor BI907828, with a disease control rate in the DDLPS cohort of 89%

with long lasting responses seen among responders [235]. Results with CDK4/6 inhibitors in monotherapy have been less convincing, and different combination strategies are currently being explored, as shown in Table 6.

In addition to the studies presented in Table 5, two studies in WDLPS/DDLPS were recently reported. Sanfilippo et al. in a phase 2 non-randomised study, reported benefit of chemotherapy cabazitaxel in 38 DDLPS reaching an ORR of 8%, a mPFS of 6 months and mOS of 21 months [236]. Gounder et al. reported a large, phase II-III placebo-randomized trial on DDLPS (n=188), exploring the effect of selinexor, an inhibitor of nuclear export, demonstrating an ORR of 2,7 %, a mPFS of 2,8 months and a mOS of 10 months [237].

Table 5. Recently completed early trials targeting MDM2 or CDK4/6 in WD/DDLPS

Treatment	N	ORR %	mPFS (months)	Reference
BI907828 (MDM2)	45 LPS (29 DDLPS 16 WDLPS)	11.1	8.1*	Schoeffksi et al. [235]
Milademetan (MDM2)	53 DDLPS	3.8	7.2	Gounder et al. [238]
Palbociclib (CDK4/6)	60 WD/DDLPS (78% DDLPS)	1.6	4.1	Dickson et al. [239]
Ribociclib (CDK4/6) + Everolimus (mTOR)	21 DDLPS	9.5	3.7	von Mehren et al. [240]
Abemaciclib (CDK4/6)	30 DDLPS	3.3	7	Dickson et al. [241]

*unconfirmed, presented at ESMO 2022. DDLPS- dedifferentiated liposarcoma, LPS-liposarcoma, WDLPS- well differentiated liposarcoma

Ongoing trials in WDLPS/DDLPS including targeted therapies are summarized in Table 6. Several Phase III trials with MDM2 inhibitors are ongoing. Treatment combinations with CDK4/6 inhibitors and PD-1 inhibitors are also being explored, spurred by data suggesting that CDK4/6 inhibitors have an immunomodulatory effect [242, 243, 244].

In summary, the increased understanding of the specific genetic alterations in DDLPS has unfolded a new and exciting field of treatment options/strategies with targeted therapies. Ongoing and future studies will further address treatment strategies exploring possible combinations with immunological therapies.

Table 6. Ongoing clinical trials DDLPS.

Trial name	Status*	Primary outcome and phase
MDM2 inhibitors		
MANTRA - Treatment of Mildematan vs Trabectedin in Patients with advanced DDLPS– NCT04979442)	Recruitment completed Aug-22	PFS (phase III)
Brightline-1: BI907828 vs Doxorubicin (NCT05218499)	Recruiting	PFS (phase III)
CDK4/6 inhibitors		
SARC041-Abemaciclib vs placebo (NCT04967521)	Recruiting	PFS (phase III)
Palbociclib + Retifanlimab (PD1) (NCT04438824),	Recruiting	Best overall response rate (phase II)
Palbociclib + Cemiplimab (PDL1) (NCT05694871)	Active, not yet recruiting	PFS (phase II)

*as per clinicaltrials.gov February 6th 2023. PFS-progression free survival

Aims

The overall aim of this thesis work was to investigate the tumor microenvironment in soft tissue sarcomas and melanoma brain metastasis, **papers I-III**, and to address potential new treatment strategies, **paper IV**.

The specific aims of each paper are listed below:

- To map the tumor microenvironment, linking tumor associated macrophages, hypoxia and neovascularity to outcome in high grade STS (**paper I**)
- To characterize the immune landscape in STS with correlation to prognosis (**paper II**)
- To explore differences in the immune microenvironment in melanoma brain metastases compared to extracranial metastases (**paper III**)
- To design a clinical phase II trial for advanced DDLPS combining a fibroblast growth factor receptor inhibitor (pemigatinib) with a PD-1 inhibitor (retifanlimab) (**paper IV**)

Material and Methods

Patient cohorts

Papers I and II are based on retrospective cohorts of soft tissue sarcomas located in extremities and trunk wall, operated on at the Skåne University Hospital, in Lund (Sweden), between 1979 and 2005.

Paper I included high-grade tumors, corresponding to FNCLCC grade 3, of which 63 were LMS and 10 UPS. Patients with metastases at diagnosis were excluded. No patient had received preoperative treatment.

Paper II included 65 LMS, 47 LPS and 22 SS. Of the 134 included tumors, 113 were high-grade, corresponding to FNCLCC grade 3. All low or intermediate grade tumors were liposarcomas. Patients with metastases at diagnosis were excluded. No patient had received preoperative treatment.

In both papers, clinical data was retrieved from the Scandinavian Sarcoma Group registry, as well as from medical charts.

Paper III is based on a retrospective cohort of 22 patients who had been submitted to neurosurgery for brain metastasis of melanoma at the Skåne University Hospital, in Lund (Sweden) between 2012 and 2019, with available formalin-fixed paraffin embedded (FFPE) tissue from at least one lesion. When available, matched tissue samples from extracranial metastases were also retrieved. For five patients, tissue was available from multiple resected brain metastases. In total, tissue from 28 brain metastases, 13 lymph nodes metastases and 3 extracranial metastases was available for analysis. Clinical data was retrieved from medical charts.

The cohort is probably not fully representative of all melanoma brain metastases, as only selected patients are candidates for neurosurgery. The patients in this cohort were young, had a good performance status and the majority were treatment naïve at the time point of neurosurgery.

Tissue Microarray and Immunohistochemistry

Tissue microarray (TMA) is an important tool in biomarker research. First described by Kononen et al. in 1998, it allows high-throughput examination of protein expression in tissue [245]. Tumor cores, usually of 0.6-2 mm in diameter, are taken from FFPE tissue and transferred to a recipient block, the TMA (Figure 6). The TMA can be cut in thin slices and mounted on microscope slides and further used for, as in our studies, assessment of protein expression. Advantages of the TMA technique include minimal use of antibodies and time-efficient staining and evaluation. Moreover, intra-laboratory variation is minimized as all tissue samples are stained simultaneously. Concerns have been raised on the representativity of TMA, as only a small part of the tumor is examined. However, good reproducibility has been shown when comparing TMA data to data collected from whole tissue sections in STS [246, 247, 248]. One way to increase reproducibility and minimize loss of TMA sections is to use multiple cores from each donor block. In our studies, duplicate cores were used, except for the TMA on synovial sarcomas in which triplicate cores were used.

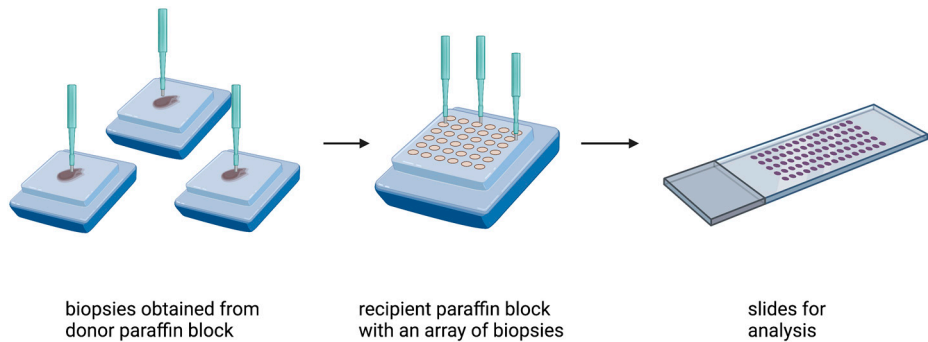


Figure 6. Schematic overview of the tissue microarray technology. Created with BioRender.com

Immunohistochemistry (IHC), the use of antibodies to localise antigens in a tissue, is the backbone of diagnosis and classification of most neoplasms. After the tissue is fixated with formalin, it is dehydrated and placed in paraffin where it can be cut in thin sections. To allow antibodies to react against the target antigen(s), the so-called process of antigen retrieval is performed. The latter involves deparaffination, rehydration and then pre-treatment with heat or microwaves to unveil the antigen epitope(s). A primary antibody is then used, which can be either monoclonal, recognizing only one epitope of the target antigen, or polyclonal, recognizing several epitopes of the target antigen.

The primary antibody can be visualized directly, or as in the indirect IHC technique, a secondary antibody that binds to the primary antibody can be used (Figure 7). The secondary antibody is conjugated to an enzyme labelled with color, visible in light microscope, when a chromogen is added [249].

TMA were used in **papers I-III** for IHC evaluations. IHC stainings used the indirect IHC technique with well validated primary monoclonal antibodies.

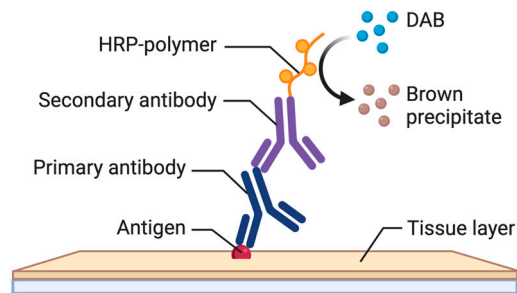


Figure 7. Schematic presentation of the indirect antibody technique. Created with BioRender.com

In situ hybridisation (ISH), is a technique which instead of detecting a protein, as IHC, detects a gene sequence using a complementary sequence (probe). The probe is labelled typically with fluorescent or chromogenic dye which can be detected in microscope. This technique was used in **paper I**, for evaluation of colony stimulating factor-1 (CSF-1) due to lack of reliable antibodies.

Nanostring

In **paper III**, the Nanostring GeoMx Digital Spatial Profiler platform was used. This technology allows measure of the expression levels of multiple proteins in a single tissue or cell sample. In addition to quantification of protein expression it can also provide information on spatial resolution.

Tissue slides, for example TMA, are stained with oligo-conjugated probes and the region of interest (ROI) is defined and selected. Using UV exposure, oligonucleotides are cleaved off the antibody in the ROIs. Released oligonucleotide tags are collected and counted. Read-out is performed using the nCounter[®] Pro Analysis platform (if less than 96 samples) or using the Next-Generation Sequencer[®] platform (if more than 100 samples). The counts can be mapped back to the corresponding tissue location, yielding a spatially resolved digital profile of tags abundance (Figure 8).

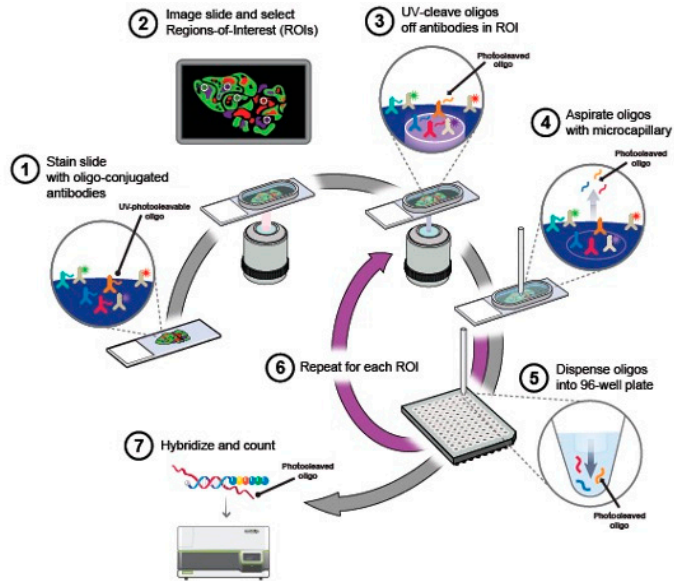


Figure 8. Nanostring GeoMx workflow. Printed with permission from Nanostring Technologies

As part of the Nanostring GeoMx Digital Spatial Profiler workflow, used in **paper III**, TMA slides were stained with antibodies against CD3 (T-cells), CD20 (B-cells), and PMEL17 and S100B (tumor cells) and visualised with immunofluorescence for selection of ROI (Figure 9).

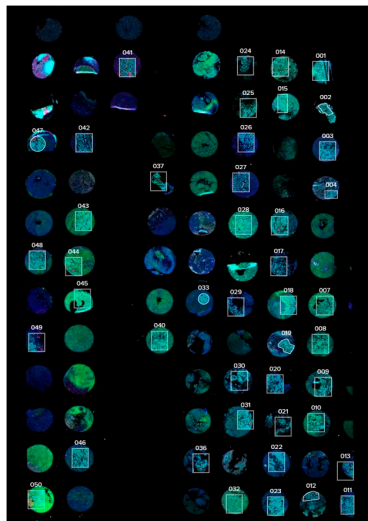


Figure 9. TMA used in **paper III** stained with antibodies for PMEL17 and S100B, CD3 and CD20 visualised with immunofluorescence used for selection of region of interest.

Approximately 50 immune-related proteins were analysed on tumor cells and CD3⁺ cells. CD20⁺ cells were sparse and too few to be analysed. A complete list of analysed proteins is given in Table 7.

Table 7. Protein modules included in the GeoMx profiling. All abbreviations according to www.uniprot.org
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Protein Module	Description	Marker
Immune cell profiling panel	Includes key immuno-oncology targets and markers of immune cell types, including T-cells, B-cells, macrophages, NK cells, epithelia, and stroma. It also includes controls needed to run any GeoMx DSP experiment.	B2M, CD3, CD56, CTLA-4, GZMB, PD-1, CD11c, CD4, CD68, Pan-CK, HLA-DR, PD-L1, CD20, CD45, CD8, fibronectin, Ki-67, SMA <i>Controls: Rb IgG, Ms IgG1, Ms IgG2a, Histone H3, S6, GAPDH</i>
Immune cell typing	Includes an expanded set of cell type markers to more deeply profile immune cell types covered in the Immune Cell Profiling Core and measure additional immune cell types, including T-cell subsets	CD14, CD163, CD34, CD45ro, CD66b, FAPalpha, FOXP3
Immune activation status	Includes additional checkpoint molecules that modulate T-cell activation	CD127, CD25, CD27, CD40, CD80, CD44, ICOS, PD-L2,, PD-1, PD-L1, CD45ro
Immuno Oncology drug targets	Includes drug targets in development within the immunooncology space, including checkpoint molecules and metabolic mediators of immune function.	4-1BB, LAG3, OX40L, Tim-3, VISTA, B7-H3, ARG1, IDO1, STING, GITR, CTLA4
Pan-Tumor markers	Includes markers for detecting EMT or cells of epithelial origin, and an expanded set of targets for detecting specific tumor types, including ER+/HER2+ breast tumors, hematopoietic malignancies, and melanoma	MART-1, NY-ESO-1, Bcl-2, EpCAM, ERBB2/HER2, PTEN, ER-alpha, PR

Statistical Methods

Associations and group comparisons

Associations between categorical and/or categorised prognostic factors were evaluated using the χ^2 test. (**paper I**). One-way Anova was used to compare means between groups (**paper II**). Associations between IHC scores and categorized prognostic factors were analysed using logistic regression in **paper II** and **III**.

In **paper III**, matched-pair analysis extracranial and brain metastases was performed using the nonparametric Wilcoxon matched-pairs signed-rank test. The nonparametric two sample Wilcoxon rank-sum test was used to compare differences between two independent groups, for example unmatched analysis of extracranial and brain metastases. Non-parametric tests do not require assumption of a normal distribution, however, they are less powerful than parametric tests, thus requiring larger differences between groups or larger sample sizes to reject the null hypothesis.

Correlations

Whereas an association simply refers to the presence of a relationship between two variables, a correlation is a specific statistical measure that also quantifies the strength and direction of that relationship. Notwithstanding, it is important to note that a correlation does not imply causation. Spearman's rank correlation coefficient is a non-parametric test used to assess the correlation between variables that are not normally distributed, or when the data is ordinal. In **paper II** and **III**, the Spearman's rank correlation coefficient was used to determine the correlation between the different immune cell markers. A Spearman correlation coefficient between 0.7 and 1 was considered as a strong association, while a correlation coefficient between 0.5 and 0.7 was considered a moderate association, and a correlation coefficient of below 0.5 as a low association.

Survival analysis

Survival analysis is an important tool in oncological research, used to assess effectiveness of treatments, predict outcome, and inform clinical decisions.

The Kaplan-Meier (KM) survival estimate is a method to investigate differences in survival (or time-to-event). The Kaplan-Meier survival estimate is particularly useful when the data is censored, i.e. some patients have not experienced the event of interest by the end of the study or are lost to follow-up, as censoring does not affect the estimate of survival probability [250].

The statistical significance of the difference between groups, i.e. comparison of the survival curves, can be tested using the nonparametric logrank test or the Cox proportional hazards regression. The Cox-regression can be used to analyze a relationship between one or more predictor variables and the time-to-event outcome. The Cox regression model estimates the hazard ratio, which is the ratio of the hazard rate between groups.

Overall survival time was defined as time from diagnosis until end of follow-up or death from any cause. Metastasis-free survival was defined as the time from diagnosis until development of metastases.

In **paper I**, the Kaplan-Meier survival estimate method was used to determine and demonstrate survival proportions, and the logrank test was used to assess statistical significance. Plots of Kaplan-Meier curves were terminated when less than five patients remained at risk. Risk for metastasis was analysed and reported using the cumulative incidence curve estimate of metastasis, handling death of other cause as a competing event. Differences in OS and MFS were evaluated using (cause-specific) Cox regression. Due to non-proportional hazards over time, the HRs generated by Cox regression should be interpreted as mean HRs over the given time period. All tests were two-sided, and effect measures were reported with 95% CI.

In **paper II** differences in MFS and OS were evaluated using the Cox proportional hazards model. In the multivariate analysis, each factor was added to the established prognostic factors.

All statistical testing was performed with a two-sided $p < 0.05$ considered significant.

Statistical analyses were generated using STATA 16.1 (Stata LP, College Station, Texas, USA) except for analysis of GeoMx data in **paper III** and power calculations in **paper IV** (PERELI trial), which were performed using R Core Team (2022). R: A language and environment for statistical computing. (R Foundation for Statistical Computing, Vienna, Austria; URL <https://www.R-project.org/R4.0.5>).

Statistical considerations in clinical trial design

Choice of endpoint(s) in phase 2 clinical trials

In clinical trials, the choice of appropriate endpoints, in particular of the primary endpoint is critical as it determines the success or failure of a trial. PFS and OS are two commonly used endpoints in clinical phase 2 trials.

PFS is defined as the time from inclusion or randomization until objective tumor progression or death, whichever occurs first. The precise definition of tumor progression should be detailed in the clinical trial protocol. PFS is often used in cancer trials because it is a measurable endpoint that can be determined by radiological or clinical assessments.

OS, is defined as the time from inclusion or randomization until death from any cause and is measured in the intent-to-treat population. OS is usually considered a direct measure of the treatment's ability to prolong life and is often considered the gold standard endpoint in oncology trials and a common endpoint in phase III trials [251, 252]. OS may be difficult to interpret if several treatments, with potential impact in OS, are available for the disease being studied.

The choice of the optimal endpoint(s) for a clinical trial depends on the specific disease being studied, the stage of the disease, the type of treatment being tested and the type of trial. PFS is typically used as the primary endpoint in phase 2 trials

because it can reflect tumor growth and be assessed before the determination of a survival benefit, thus providing results faster and its determination is not confounded by subsequent therapy [253]. PFS is considered a surrogate endpoint for OS, denoting that improvements in PFS can indicate improvements in OS. However, there is usually insufficient data to allow a robust evaluation of the correlation between effects on OS and PFS [253].

Response Evaluation Criteria in Solid Tumors (RECIST) is a set of guidelines used to evaluate the changes in tumor size in response to therapy in solid tumors in clinical trials. The size of the tumor is measured using imaging techniques such as computed tomography (CT) or magnetic resonance imaging (MRI). The RECIST criteria provide a standardized method for measuring the size of the tumor and categorizing the response as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD). Thus, the RECIST criteria provide a uniform methodology of evaluating response to treatment in different clinical trials.

Simon's two-stage design

Simon's two-stage design is a statistical method commonly used in clinical phase 2 trials. It is efficient as it allows the study to terminate early if the treatment is unlikely to be effective [254] thereby reducing the number of patients needed to be enrolled in the study and the cost and time of the trial. From an ethical perspective it is also appealing as it can reduce the number of patients exposed to ineffective or harmful treatments.

The sample size and power estimation in the phase II PERELI trial (**paper IV**), is based on the primary outcome PFS only, as PFS allows an objective and timely evaluation of the effect of treatment.

Enrollment will continue until the required sample size has been reached. The null hypothesis that the true PFS rate is 20% will be tested against a one-sided alternative. In stage one, 18 evaluable patients will be accrued. If ≤ 4 patients are progression-free at 24 weeks among these 18 patients, the study will be stopped. Otherwise, 15 additional patients will be accrued in stage two, for a total of 33 patients. The null hypothesis will be rejected if ≥ 10 of the 33 fully evaluable patients are progression-free at 24 weeks. The type I error will be 5% and the study will have 80% power to reject the null hypothesis when the true PFS-rate is 40%.

Ethical considerations

Papers I-III are exploratory studies in retrospective cohorts in patients who have given their consent to biobanking, and performed after approval by the ethical committee, thus not raising any ethical considerations.

When designing the PERELI trial, described in **paper IV**, several ethical aspects were considered. Advanced DDLPS have a poor prognosis. Beyond anthracycline-based first line therapy, the treatment options are sparse, motivating inclusion in a clinical trial in second line or later.

Treatment schedule was decided in discussion with Incyte, based on the pharmacology and toxicity profile of the drugs used in the trial. Even though no additive toxicity has been noticed in trials with pemigatinib in combination with PD-1 inhibitor, toxicity from both drugs can be expected.

The expected toxicity profile associated with immunotherapy, so-called immune related adverse events, is broadly known. Toxicity of pemigatinib, approved in FGFR2 mutated cholangiocarcinoma, is however less known. This limited knowledge determined the design of the trial with a six-week induction phase with monotherapy pemigatinib. This design allows detection and management of pemigatinib toxicity before adding retifanlimab.

A CT-scan is scheduled after the induction phase, to allow detection of progression, although the protocol allows patients to continue if deemed to potentially benefit the patient (by the investigator).

Prior to enrollment in the PERELI study patients will give their written consent, after receiving study information by their oncologist. The patients will undergo tumor biopsies, the first at screening (can be replaced by archival tissue), at week 7 before start of combination treatment and at week 15. Blood for correlative studies will be taken prior to each treatment cycle. These study procedures can cause pain or discomfort and tissue biopsy can be associated with a risk for bleeding.

Overall, a major concern during the trial design was to ensure that the declaration of Helsinki and the Good Clinical Practice (GCP) guideline were followed when designing and planning the trial. The trial will be conducted according to ICH E6 (R2).

Results and Discussion

All results herein discussed are presented in detail in the original papers and are therefore only briefly summarized in this section. Paper IV is a clinical trial protocol not yet recruiting. Hence, only the rationale and design of the trial will be addressed.

Paper I

In this study we mapped factors in the tumor microenvironment of STS linking tumor associated macrophages, hypoxia and angiogenesis.

The prognostic impact of a stromal signature derived from macrophage response to colony stimulating factor-1 (CSF-1) was suggested by previous reports [255, 256, 257]. A link between CSF-1 response signature and neoangiogenesis had also been suggested, as well as a prognostic role of hypoxia in STS [258, 259, 260, 261].

These findings prompted us to evaluate the prognostic impact of biomarkers in the TME in STS, linking tumor associated macrophages, hypoxia and angiogenesis: CSF-1, CD16, CD163, HIF-1 α and microvessel density (MVD).

In this cohort of high-grade tumors (LMS and UPS), 56% (41/73) of the patients developed distant metastasis and 22% (16/73) developed local recurrence during follow-up. The relatively high occurrence of distant metastases and local recurrence is thought to reflect the aggressive behavior of high-grade tumors included in the cohort. Post-operative radiotherapy was administered to 45% (33/73) of the patients, whereas only 10% (7/73) patients received adjuvant chemotherapy. Considering current guidelines, if diagnosed today, more patients would have been offered chemotherapy.

Concordant expression of CSF-1, CD16 and CD163 was defined as CSF-response signature and was positive in 31% (22/71) of the tumors. No prognostic impact of macrophage infiltration nor of positive CSF-1 response signature was observed. These findings were rather surprising, given previous published data. One explanation for this could be that our cohort included only grade 3 FNCLCC tumors, whereas in the LMS cohort used by Lee et al. and by Espinosa et al, only \approx 20% (30 of 149) were FNCLCC grade 3 tumors [63, 255]. Moreover, the latter cohort included both gynaecological and non-gynaecological LMS. A subsequent study,

from the same group, in a cohort of LMS more similar to ours, encompassing 52 mostly high-grade non-gynaecological LMS, also failed to confirm the previous findings [64]. Thus, further suggesting that the difference observed may be explained by the cohort composition, and that relevance of CSF-1 response signature may vary between high-grade and intermediate/low-grade tumors.

HIF1 α was positive in 66% (48/73) of the tumors and correlated to necrosis. Multivariate Cox regression identified a prognostic role of HIF-1 α with a hazard ratio of 3.2 for MFS (p=0.004, CI [1.4-7.0]) and a hazard ratio of 1.8 for OS (p=0.05 CI [1.0-3.4]) in multivariate analysis including known risk prognostic factors (size, depth, necrosis and vascular invasion dichotomized as in *SING*).

In the HIF-1 α positive group 71% of the patients developed metastasis compared with 35% of the patients in the HIF-1 α negative group (Figure 10).

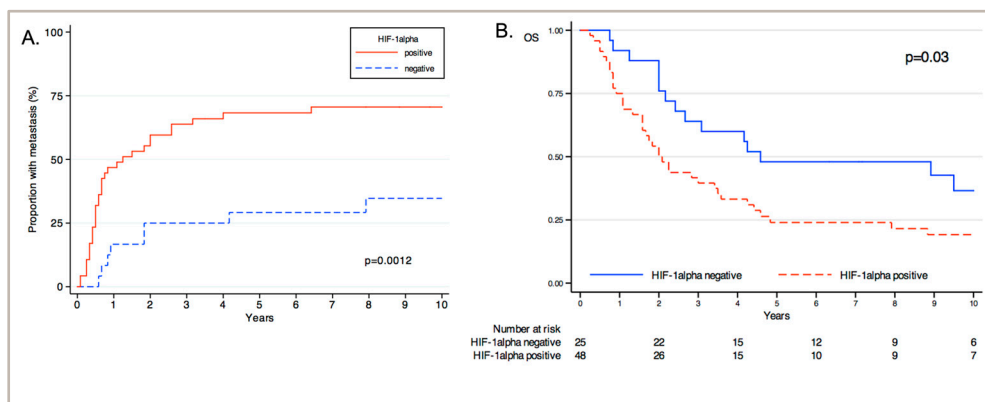


Figure 10. A. Cumulative risk for metastasis **B.** Kaplan- Meier estimates of overall survival in relation to HIF1 α expression

In this cohort, the prognostic effect of HIF-1 α positivity (as evaluated by IHC) on risk for metastases and OS was comparable to well-established prognostic factors currently used, suggesting that HIF-1 α could be incorporated in current STS prognostic models. Still, methodological issues such as lack of uniform criteria for determination of HIF-1 α expression, standardization of IHC antibodies and dilutions need to be addressed before a potential clinical use.

Besides the potential of HIF-1 α as a prognostic biomarker, other clinical applications of hypoxia are currently being explored.

Hypoxia is a hallmark of the TME and considered one of the major causes of resistance to radiotherapy as well as chemotherapy and immunotherapy [262]. Furthermore, hypoxia plays a significant role in shaping and maintaining TAM niches, known to contribute to anticancer resistance.

Hypoxia has proven to be a difficult treatment target [263]. Targeting hypoxia, using hypoxia-activated prodrugs (HAP), has been addressed in clinical trials without major success [264]. As an example, a phase III trial in STS addressed the addition of the hypoxia-activated pro-drug evofosfamide to doxorubicin and failed to show benefit of the combination over doxorubicin monotherapy [198]. It should be noted that no hypoxia biomarker was used for patient selection. HIF-inhibitors are also being explored in clinical studies. In 2021 belzutifan, a HIF-2 α inhibitor, was approved for the treatment of patients with certain types of Von Hippel-Lindau disease-associated tumors. Hypoxia is also being exploited in new imaging techniques such as PET with hypoxia-tracers, that could possibly be used to guide radiotherapy planning with higher doses to hypoxic areas [265, 266].

Paper II

In **paper II**, we explored immune cell infiltration in a cohort of 134 mixed STS, including LMS (n=65), LPS (n=47) and SS (n=22). Of the 134, 113 tumors were high grade (grade 3 FNCLCC). Markers for T-cells (CD3, CD8, FOXP3) B-cells (CD20) and TAMs (CD163) were analyzed on TMA (Figure 11).

The most frequently observed immune cell type in this cohort was CD163⁺ macrophages. High CD163 expression was overall identified in 49% of the tumors (66% of LMS, 46% of LPS and only 9% of SS) and was more frequent in high-grade tumors than in low-grade tumors (53% of high-grade STS compared to 28% of low-grade STS).

Infiltration of CD20⁺ B-cells was sparse and identified in only 14% of STS. No difference in infiltration was observed between the various histotypes. The presence of TLS was not evaluated.

T-cell infiltration, CD3⁺ and CD8⁺ as well as regulatory FOXP3⁺ T-cells, were more prevalent in LMS than in LPS and SS. CD3⁺ and CD8⁺ cell infiltration was more frequently observed than FOXP3⁺ infiltration.

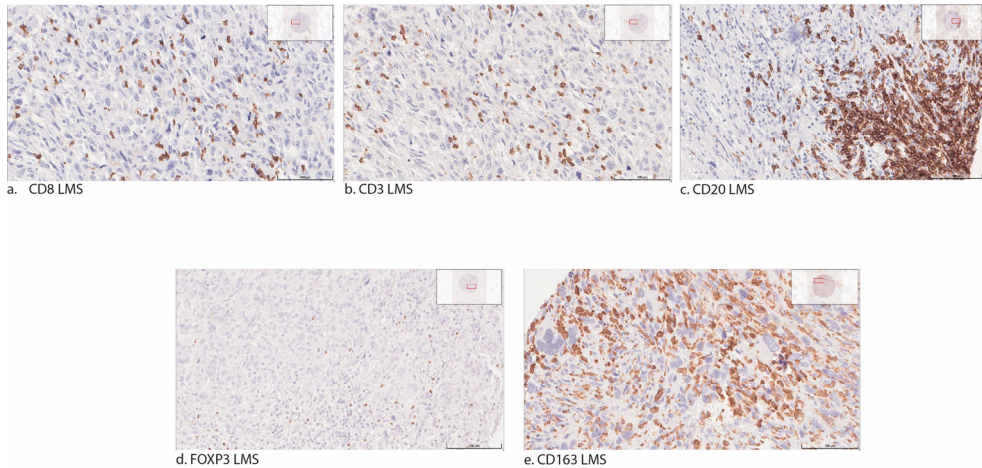


Figure 11. Microscope (x20) photograph of immunohistochemistry for immune cell markers in leiomyosarcoma.

Survival analysis only included the 113 high-grade tumors to allow prognostic consistency. As expected, established prognostic markers, tumor size larger than 8 cm, presence of necrosis and presence of vascular invasion were associated with shorter MFS. Of the immune cell markers, high expression of CD163⁺ macrophages predicted shorter MFS (HR 1.81 p=0.040 CI [1.03-3.19]). When analyzing each histotype separately this trend was strongest in LPSs (HR 3.93 p=0.084 (CI [0.83-18.62])).

Since the survival analysis only included high-grade tumors, the difference observed on the effect of macrophage infiltration cannot be explained by tumor grade, and is therefore more probably related to intrinsic histotype features.

When adjusting for size, necrosis and vascular invasion in multivariate analysis, infiltration of CD163⁺ macrophages did not significantly predict MFS or OS. These results could possibly be explained by the correlation between CD163⁺ macrophages and the known prognostic factors necrosis and vascular invasion (please see paper II, Table 3).

Multivariate Cox regression analysis taking into account known prognostic factors (size, necrosis and vascular invasion) and CD20⁺ B-cells, showed that infiltration of B-cells predicted longer MFS in LMS (HR 0.36 p=0.023 CI [0.15-0.87]). B-cell infiltration was significantly associated with improved OS in the entire cohort (HR 0.35 p=0.003 CI [0.18-0.71]) and this effect was greater in the LMS subgroup (HR 0.26 p=0.002 CI [0.11-0.6]). These results are in line with other published reports [25, 62].

Presence of T-cell infiltration was not found to correlate to prognosis, independently of the T-cell subtype. LMS showed the highest TIL infiltration but also the highest macrophage infiltration, suggesting that the infiltration of T-cells might be counterbalanced by immunosuppressive CD163⁺ TAMs and FOXP3⁺ Tregs.

Taken together these findings motivate further studies of the STS microenvironment and investigation of optimal treatment combinations. Given the immunosuppressive effect of TAMs, treatments targeting TAMs in combination with immunotherapy is interesting and such efforts are ongoing [13]. Furthermore, as noted in the introduction Table 3, multi-therapeutic approaches, such as chemotherapy or small molecule inhibitors in combination with immunotherapy appears as a promising way forward in management of STS.

Although limited by the small sample size, these results also reinforce the existence of histotype specific features (immune infiltration, tumor microenvironment) strengthening the need for histotype specific or histotype stratified clinical trials.

Paper III

In this paper we investigated the immune microenvironment in melanoma BM compared to matching samples from extracranial metastases.

Twenty-two patients with melanoma BM were included in this study. Sixteen (73%) of the were male and six (27%) were female. Median age at diagnosis of brain metastases (BM) was 56.5 years.

In 18 patients, BM were diagnosed at the time of diagnosis of stage IV disease. Of these, nine patients had concomitant manifestation(s) of extracranial disease. And nine patients had intracranial disease only.

At the time of BM diagnosis 17 patients presented with a solitary BM, four patients with three BM and one patient with four BM.

Most of the patients were naïve to treatment and had not received any systemic medical treatment nor radiotherapy at time of BM surgery. Five patients underwent repeated neurosurgeries and, in those cases, tissue from multiple BM were included (Figure 12).

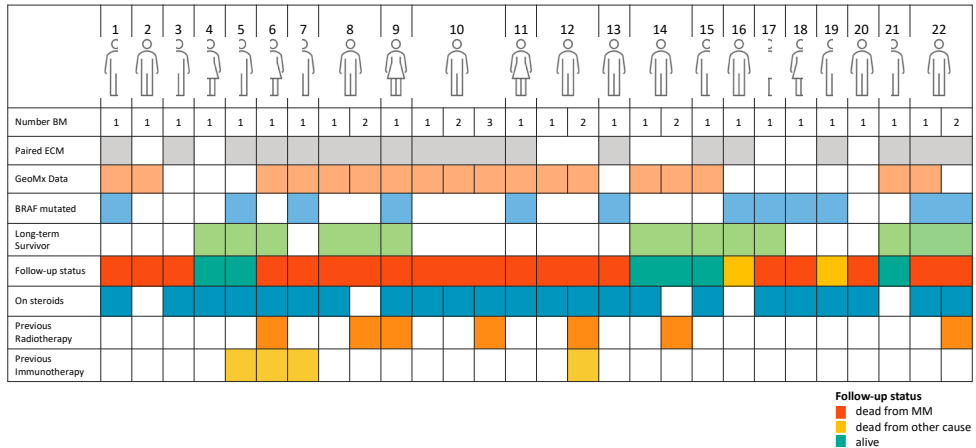


Figure 12. Patient clinical and pathological data (GeoMx data refers to availability of GeoMx data, on steroids refers to treatment with steroids at time of neurosurgery, long-term survivors defined as alive longer than 26.5 months after diagnosis of BM).

We examined infiltration of immune cell populations, CD3, CD8, CD20 as well as tumor cell expression of PD-L1 using immunohistochemistry. Infiltration of CD20⁺ B-cells was sparse but noted in 60% of the tissue cores; no tertiary lymphoid structures were observed. Infiltration of T-cells, both CD8⁺ and CD4⁺, was present in all tissue cores.

Using the Wilcoxon matched-pairs signed-rank test, no statistically significant difference in tumor infiltrating lymphocytes (TIL) was observed between matched samples from BM and lymph node metastases (LNM). CD3⁺ and CD20⁺ showed a non-significant trend towards higher infiltration in LNM. The interpretation of this results is hampered by the small sample size (n=13) and the semi-quantitative evaluation of TIL infiltration which may have impacted the results.

Positive PD-L1 expression, defined as present in more than 5% of tumor cells, was observed in 41% of the tumors. 28% of the evaluable BM (7/25) were PD-L1 positive.

Using the Nanostring GeoMx digital spatial profiler methodology, we further evaluated the expression of 54 immune-related proteins in tumor and tumor infiltrating T-cells.

We first performed a supervised unmatched analysis of protein expression in BM compared to extracranial metastases. This analysis revealed a higher expression of Ki-67 in tumor cells in BM, suggesting that brain metastases are more proliferative than extracranial metastases.

Furthermore, we found markers for T-cell activation (ICOS, CD25 and CD45) to have a lower expression in BM than in extracranial metastases. In addition, we

found lower levels of HLA-DR protein, a MHC class II isotype, in BM (as compared to extracranial metastases suggesting less antigen presentation in BM. No difference between BM and extracranial metastases was detected in T-cell exhaustion markers (PD-1, TIM-3, CTLA-4).

We then performed a supervised analysis with matched pairs of BM and LNM from the same patient.

Data from nine pairs of metastases (BM-LNM) was available for analysis of tumor cell markers. Higher expression of beta-2-microglobulin (B2M) and IDO1 was found in LNM as compared to BM. This finding is interesting, as loss of B2M has been linked to resistance to immunotherapy. Absence of B2M leads to loss of surface MHC class I expression, which is known to hinder the functionality of the MHC class I T-cell receptor complex and consequently limits tumor control by T-cells [267, 268, 269]. IDO1 has previously been reported to be lower in melanoma metastasis compared to primary melanomas, possibly indicating a less inflamed tumor microenvironment [270, 271].

T-cell markers could be evaluated in seven matched pairs of BM and LNM. Higher levels of both CD3⁺ and CD4⁺ T-cells were observed in LNM compared to matched BM. Again, markers of T-cell activation (CD25, CD45, and ICOS) were higher in LNM whereas no difference in exhaustion markers was observed.

In this cohort, median time from diagnosis of BM to last follow-up was 26.5 months. Patients alive longer than 26.5 months were defined as long-term survivors. We next compared the immune profiles of BM from long-term survivors to those from non-long-term survivors. Of note, this analysis was performed in a very limited sample, including only 10 patients, of which six were long-term survivors.

Interestingly, higher levels of T-cell activation, such as ICOS, CD45, CD40 were found in the T-cells of long-term survivors. Moreover, signs of higher IFN γ response (HLA-DR, B2M and PD-L1), and T-cell presence were observed in tumor cells regions in long-term survivors. IFN γ response is known to play an important role in response to immunotherapy. None of the six long-term survivors had extracranial disease at time of BM neurosurgery, thus the higher immune activation status seen in the long-term survivors does not seem to correlate to extracranial immune activation.

Given the importance of steroids in the management of oedema and its known association with lower response rates to immunotherapy in patients with brain metastases [272], we addressed the potential effect of steroid on the tumor immune microenvironment. To this end, we compared BM specimens from patients under treatment with steroids at the time of neurosurgery with those from steroids-free patients. Only patients naïve to systemic treatment were included in the analysis to allow a homogenous sample set and avoid any potential effects of systemic treatment on the tumor microenvironment.

As steroids are known to be immunosuppressive and lymphotoxic [273], it was surprising to observe higher T-cell infiltration markers and higher levels of immune response markers such as ICOS, HLA-DR and GZMB in specimens from patients with on-going steroid treatment. Given that administration of steroids is associated with inferior response to immunotherapy, it is tempting to speculate that these immune cells do not represent a tumor-specific immune response, but rather represent non-specific bystander lymphocytes. These results require further studies, for example characterization of tumor infiltrating lymphocytes in specimens from patients with ongoing steroid treatment, using single cell profiling.

The results presented should be considered exploratory given the small size of the samples set. Still, our data suggest, in accordance with previous studies, that the tumor microenvironment in BM is immune-infiltrated however with reduced T-cell activation. BM from long-term survivors show signs of increased immune infiltration and T-cell activation, suggesting a more inflamed tumor microenvironment.

Paper IV

The PERELI trial is a phase II single arm, open label, multicenter study exploring the combination of pemigatinib, a selective fibroblast growth factor receptor (FGFR) inhibitor in combination with retifanlimab, a PD-1 inhibitor in advanced dedifferentiated liposarcoma.

It is now appreciated that the tumor microenvironment plays a significant role in tumor immune surveillance and evasion and contributes to, or determines, the therapeutic efficacy of immunotherapy [274]. Targeting the tumor microenvironment in combination immunotherapy, may therefore improve response rates. Indeed, combining immune checkpoint blockade with small-molecule kinase inhibitors and have, in several studies, shown to elicit anti-tumor immune responses and enhance tumor immunogenicity by regulating antigen processing and presentation [275].

In soft tissue sarcomas, amplifications of FGFR, gene fusions involving FGFR, and activating mutations in FGFRs have been shown [276, 277, 278, 279]. Fibroblast Growth Factor Receptor Substrate 2 (FRS2) is an adaptor protein activating downstream signaling cascades (Figure 13).

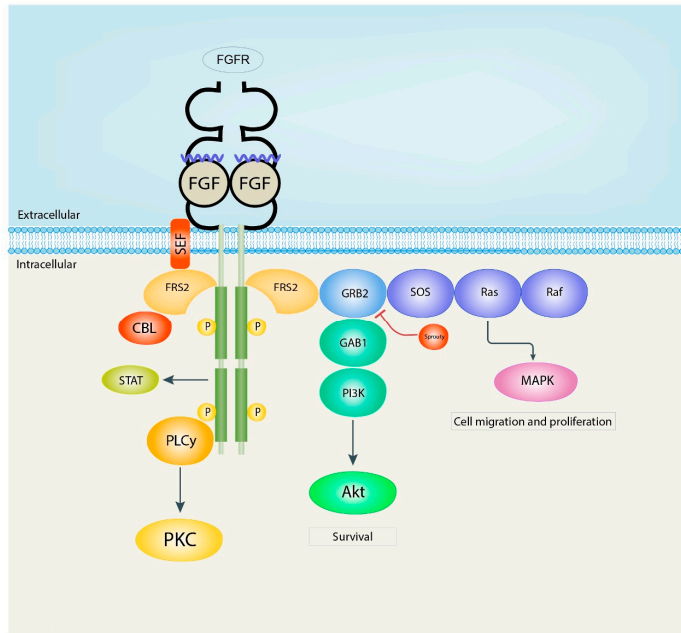


Figure 13. FGFR signaling pathway

The *FRS2* gene is located in the same amplicon as *MDM2* of chromosome 12q13-15, leading to amplification of *FRS2* in >90% of DDLPS [280, 281, 282]. Preclinical studies have shown promising therapeutic effect of pan-FGFR inhibitors in DDLPS patient xenograft models [283, 284, 285], providing a preclinical rationale for exploring FGFR inhibition in patients with advanced DDLPS.

Immunomodulatory effect beyond the possible antitumoral effect, has been suggested also for FGFR inhibitors. Studies have shown that treatment with FGFR-inhibitor and PD-1 inhibitor leads to immunomodulation of the tumor microenvironment, with reduction of immunosuppressive macrophages and regulatory T-cells [286] as well as increase in T-cell infiltration through reactivation of the IFN γ pathway [287].

Activity of immunotherapy in DDLPS has been shown in both the Sarc028 and the Alliance trial [219, 288], as described in paper IV.

Taken together, these data suggest that inhibition of FGFR signaling in combination with immunotherapy could improve response to immunotherapy, and constitute the rationale for this trial.

PERELI is the first study to assess if the selective FGFR inhibitor, pemigatinib, has antitumor activity in DDLPS in combination with immune checkpoint blockade, retifanimab. Translational analyses from tumor samples collected during the

PERELI study are expected to contribute to the understanding of the role of FGFR signaling in DDLPS and the effect of FGFR inhibition on the tumor microenvironment.

Main conclusions

Based on our findings in paper I- III we suggest that

- HIF-1 α is a prognostic marker in STS (**paper I**)
- Tumor associated macrophages are a common infiltrating immune cell type in STS, however with unclear prognostic relevance (**paper I and II**)
- Immune cell infiltration varies between STS subtypes (**paper II**)
- Melanoma brain metastases are more immune excluded than extracranial metastases (**paper III**)

In **paper IV** we hypothesize that

- Inhibition of fibroblast growth factor receptor signaling in combination with PD-1 inhibition can enhance response to immunotherapy and improve treatment in advanced DDLPS.

Future perspectives

The tumor microenvironment is currently seen as a complex ecosystem encompassing tumor cells and non-tumor cells. The cellular composition of the tumor microenvironment is known to vary extensively depending on the intrinsic features of the cancer cells as well as tumor stage and patient characteristics [289].

Data presented in **papers I-III** suggesting that the tumor immune microenvironment is histotype and organ specific, is probably a reflection of these differences.

Furthermore, current therapeutic strategies do seldomly incorporate tumor microenvironment or organ specific approaches. While addressing the tumor microenvironment in STS we found that tumor associated macrophages are the most common infiltrating immune cell type (**paper I** and **II**). These findings require further validation, but noteworthy, tumor associated macrophages have recently emerged as therapeutic targets and several treatment strategies are currently being evaluated in clinical trials. Evaluation of new therapeutic approaches targeting tumor associated macrophages in STS may thus be relevant to consider in the future.

As suggested in **paper III**, melanoma brain metastases seem to have a distinct tumor immune microenvironment compared to their extracranial counterparts. It is possible that the suggested immune-excluded tumor microenvironment of brain metastases impacts on responses to immunotherapy. In the future, therapeutic approaches specifically taking into consideration the tumor immune context of brain metastases may improve outcomes. Specifically, strategies to improve immune infiltration and activation may be worth to investigate.

Tumor microenvironment associated biomarkers are attractive given the role of tumor microenvironment in tumor progression and response to therapy. In **paper I**, we confirmed a prognostic role of hypoxia (HIF-1 α) in STS. Unquestionably, prognostication in STS is a clinical challenge. New biomarkers to help assist in correctly selecting patients for, potentially toxic, therapies are needed. Available evidence of hypoxia as a prognostic marker motivates a prospective translational study to fully address hypoxia's role in STS prognostication.

While deciphering the tumor immune microenvironment seems crucial to achieve better treatment outcomes, defining adequate biomarkers for patient selection and prognostication is essential for development of personalised treatment strategies.

Moving forward from the first rather disappointing trials of immune checkpoint inhibitors in STS, trials exploring combination strategies with chemotherapy or small molecule inhibitors have reintroduced some hope in the field. STS are a heterogenous group of tumors, and increasing evidence suggests that this heterogeneity also applies for the tumor microenvironment in STS. Thus, for future trials to be successful they need to be histotype or biomarker driven.

In line with this, we have designed a clinical phase 2 trial exploring the combination of a fibroblast growth factor receptor (FGFR) inhibitor with a PD-1 inhibitor. The rationale for the study is outlined in **paper IV**.

Together, the works presented in this thesis describe some of the aspects of the tumor microenvironment. Hopefully this work is a small contribution to the characterization of a rather complex and broad field. In addition, we hope that the PERELI trial will contribute to better understanding and improved clinical management of advanced DDLPS.

Populärvetenskaplig sammanfattning

En tumör kan beskrivas som ett helt ekosystem av inte bara tumörceller, utan också blodkärl, stödjeceller, immunceller och signalmolekyler som tillsammans utgör det som kallas för tumörmikromiljö. Genom att förstå hur tumörmikromiljön är uppbyggd och påverkar tumörtillväxt kan vi också hitta nya behandlingsmetoder. Ett exempel på det är immunterapi, dvs att aktivera immunsystemet mot tumören. Behandling med immunterapi har det senaste decenniet visat sig vara effektivt för vissa sorters tumörer, exempelvis malignt melanom, medan andra har liten effekt av sådan behandling, exempelvis mjukdelssarkom.

Mjukdelssarkom är en ovanlig och mycket heterogen sjukdomsgrupp, med över 70 olika subgrupper. Vid spridd sjukdom erbjuds behandling med kemoterapi, men effekten är ofta kortvarig och prognosen dålig. Tumörmikromiljön i mjukdelssarkom är otillräckligt studerad och resultaten varierar beroende på vilken sorts mjukdelssarkom det är som studerats. Ökad kunskap kan bidra inte bara till nya behandlingar, men också till att välja ut rätt patient till rätt behandling.

När en tumör tillväxer är det vanligt att det bildas områden i tumören med låg syresättning, hypoxi. Tumörcellerna måste då anpassa sig till att överleva i dessa förhållanden och hypoxin medför dessutom förändringar i tumörmikromiljön med rekrytering av t ex makrofager, en sorts immunceller. I **studie I** studerade vi med hjälp av immunhistokemi uttryck av proteiner kopplade till hypoxi och makrofager i en kohort av 73 högmaligna mjukdelssarkom. Vi kunde visa att högt uttryck av HIF-1 α , en markör för hypoxi, var kopplat till ökad risk för metastasering. Av de som hade högt HIF-1 α utvecklade 71% metastaser jämfört med 35% av de som hade lågt uttryck av HIF-1 α . Överlevnaden var också sämre för de som hade högt uttryck av HIF-1 α . Makrofager var vanligt förekommande men vi kunde inte notera någon koppling mellan makrofager och försämrad prognos.

I **studie II** fortsatte vi att studera tumörmikromiljön i mjukdelssarkom. I denna kohort ingick 134 mjukdelssarkom. Med immunhistokemi studerade vi infiltration av olika sorters immunceller: T-celler (CD3⁺, CD8⁺ och FOXP3⁺), B-celler (CD20⁺) och makrofager (CD163⁺). Vi kunde påvisa en skillnad i immuncellsinfiltration mellan de olika subtyperna av mjukdelssarkom. Leiomyosarkom innehöll mest T-celler, men också mest makrofager. Det var sällsynt med infiltration av B-celler, bara 15% av tumörerna vi studerade hade det, men förekomst av B-celler var kopplat till förbättrad prognos.

Sammanfattningsvis bidrar dessa studier till ökad kunskap om tumörmikromiljö i mjukdelssarkom.

Malignt melanom, elakartad hudcancer, har visat sig svara särskilt bra på behandling med immunterapi. Vid spridd, metastaserad, sjukdom var den förväntade överlevnaden tidigare kort, nu lever ca 30% av patienterna med spridd sjukdom i mer än 5 år. Spridning till hjärnan är vanligt vid malignt melanom, och då är prognosen sämre.

Kliniska behandlingsstudier med immunterapi har visat att hjärnmetastaser svarar sämre på behandling än metastaser på andra lokaler. I **studie III** samlade vi in tumörvävnad från hjärnmetastaser och lymfkörtelmetastaser av malignt melanom. Studien inkluderade 22 patienter och från dessa hade vi vävnadsmaterial från 28 hjärnmetastaser och 13 lymfkörtlar. Materialet analyserade vi med hjälp av immunhistokemi och GeoMx, ett sätt att mäta uttryck av immunrelaterade proteiner i tumörceller och immunceller. Vi kunde se att hjärntumörcellerna hade högre uttryck av Ki67, ett mått på celledelning, vilket innebär att tumörceller i hjärnan delar sig (tillväxer) snabbare än tumörceller i lymfkörtlar. Vi kunde vidare se att det fanns mer T-celler i lymfkörtlar än i hjärnmetastaser och att de T-celler som fanns i hjärnmetastaser inte var lika aktiverade som de som fanns i lymfkörtlar. Detta är i linje med vad tidigare studier förslagit, att immunmiljön i hjärnan är mer hämmande vilket i sin tur kan bidra till att behandling med immunterapi fungerar sämre. Hos de med lång överlevnad, kunde vi se tecken till att immuncellerna var mer aktiverade, men det var ett litet material och stor osäkerhet i dessa resultat.

Studie IV är ett protokoll för en klinisk fas II studie vid en typ av mjukdelssarkom, liposarkom. Patienter med avancerat dedifferentierat liposarkom kommer erbjudas behandling med immunterapi, retifanlimab (PD-1hämmare), i kombination med en hämmare av fibroblast growth factor receptor (FGFR), pemigatinib. Pemigatinib har förutom en potentiell tumörcellshämmande effekt även en förväntad positiv effekt på tumörmikromiljön. Totalt 33 patienter kommer att inkluderas i denna studie vid sarkomcentra i Sverige, Norge och Danmark.

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