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MORPHOLOGIC AND GENETIC FEATURES OF SARCOMAS: IMPROVING DIAGNOSIS AND EXPLORING BIOMARKERS

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MORPHOLOGIC AND GENETIC FEATURES OF SARCOMAS: IMPROVING DIAGNOSTICS AND EXPLORING BIOPMARKERS

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ABSTRACT

Sarcomas are malignant tumors of soft tissue and bone origin. They are a family of rare but diverse neoplasms which pose clinical challenges due to their heterogeneous properties. In order to increase our understanding of sarcoma biology, we are continuously reviewing clinical cohorts regarding morphological presentation and underlying genetic changes. This thesis focuses on translational research from improving diagnostics to exploring biomarkers in select sarcoma subtypes.

In **Paper I** we investigated fine needle aspirations of synovial sarcomas (SS) in order to identify common characteristics. Samples from SS displayed similar features such as abundant oval, round or spindle-shaped cells with pericapillary formation and pink background stroma. All tumors also harbored the pathognomonic fusion gene *SYT-SSX*, which can be detected by sequencing and is recommended to verify a diagnosis of SS.

In **Paper II** we investigated FNAs of clear cell chondrosarcomas (CCCS) in order to identify common characteristics. Samples from CCCS displayed similar features such as abundant clear cells with round nucleus and prominent nuclei, which separated it from its most common differential diagnosis chondroblastoma, a benign tumor. Due to the rarity of CCCS we also conducted a literature review and found that CCCS was more common in patients >25 and chondroblastoma more common in patients <25 years of age. There is however a significant overlap and a clear cutoff age cannot be established.

In **Paper III** we investigated the role of *TERT* promoter mutation in chondrosarcomas (CS). We found that *TERT* promoter mutation (C228T) was common in CS and was significantly correlated with higher tumor grade, increased risk of metastasis and tumor-related death, as well as a more aggressive course of disease. We also found that CS undergo branching evolution, as patients can have a wild-type primary tumor and a mutated metastasis or vice versa. As *TERT* promoter mutation status is easily detected by sequencing it can be useful as a prognostic biomarker.

In **Paper IV** we investigated the expression of PD-L1 in three common sarcoma types: liposarcoma (LS), undifferentiated pleomorphic sarcoma (UPS), and CS. We found that PD-L1 expression was most common in UPS, rare in LS and very rare in CS, but it did not correlate to metastasis or death from disease in any sarcoma subtype.

LIST OF SCIENTIFIC PAPERS

I. **Y Zhang**, S Wessman, J Wejde, E Tani and F Haglund de Flon. Synovial sarcoma diagnosed by fine needle aspiration cytology and molecular techniques during 10 years. *Cytopathology*. 2019;30(5): 504-509.

II. **Y Zhang**, Z Alagic, E Tani, M Skorpil, P Tsagkozis and F Haglund de Flon. Clear-cell chondrosarcomas: Fine-needle aspiration cytology, radiological findings, and patient demographics of a rare entity. *Diagnostic Cytopathology*. 2021; 49(1): 46-53.

III. **Y Zhang**, Y Chen, C Yang, N Seger, A Hesla, P Tsagkozis, O Larsson, Y Lin and F Haglund de Flon. *TERT* promoter mutation is an objective clinical marker for disease progression in chondrosarcoma. *Modern Pathology*. 2021;34(11): 2020-2027.

IV. **Y Zhang,** Y Chen, A Papakonstantinou, P Tsagkozis, C Linder Stragliotto and F Haglund de Flon. Evaluating PD-L1 expression in undifferentiated pleomorphic sarcomas, liposarcomas and chondrosarcomas. *Biomolecules*. 2022; 12(2):292.

CONTENTS

LIST OF ABBREVIATIONS

CS	Chondrosarcoma
CCCS	Clear cell chondrosarcoma
CT	Computer tomography
DFS	Disease free survival
DNA	Dioxyribonucleic acid
FFPE	Formalin-fixed paraffin-embedded
FNA	Fine-needle aspiration
HE	Haematoxylin and Eosin
IC	Immune cells
ICC	Immunocytochemistry
ICI	Immune checkpoint inhibitor
IHC	Immunohistochemistry
LS	Liposarcoma
MFS	Metastasis-free survival
MGG	May-Grünwald Giemsa
MPNST	Malignant peripheral nerve sheath tumor
MRI	Magnetic resonance imaging
NOS	Not otherwise specified
OS	Overall survival
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
SS	Synovial sarcoma
SSG	Scandinavian Sarcoma Group
TC	Tumor cells
TERT	Telomerase reverse transcriptase
TILs	Tumor-infiltrating lymphocytes
TMB	Tumor mutation burden
UPS	Undifferentiated pleomorphic sarcoma

1. INTRODUCTION

1.1 A BRIEF INTRODUCTION TO SARCOMAS

By the 19th century an array of scientific discoveries deepened the understanding of cells as the building blocks of tissue in complex organisms, and of tumors arising from normal tissue. Further on, cells were divided into two major groups: epithelial, such as the lining of the gastrointestinal system or the skin, and non-epithelial, such as soft tissue, bone, hematopoietic tissues and neuronal tissues. Thus, physicians begun to distinguish sarcomas from other neoplasms. In the article "Historical Note on Bone and Soft-Tissue Sarcoma" LF Peltier states: "Alexis Boyer (1757-1833) first used the term osteosarcoma to describe bone tumours, and in 1818 Astley Cooper (1768-1841) separated bone tumours into two groups – intramedullary and extramedullary. The French pathologist J.C.A. Recamier (1774-1852) was the first to distinguish between primary and metastatic bone lesions. Rudolf Virchow (1821-1902) first separated the sarcomas from other cancers and defined them as a variety of tumours originating in non-epithelial and non-hematogenous tissues."[1]

Sarcomas are rare malignant non-epithelial tumors deriving from mesenchymal cells, with embryonal origins from the mesoderm. The mesenchymal cells constitute soft tissues such as connective tissue, fat and muscle, and bone. They account for approximately 1% of all malignant tumors in adults, but up to 20% of all malignant tumors in children. With over 80 subtypes recognized by the WHO, sarcomas are heterogeneous with considerable variations in tumor behavior as well as clinical and pathological features.[2,3]

There is no hereditary component to most sarcomas, but a few syndromes have genetic predispositions for developing sarcomas. One example is Neurofibromatosis type I (NF1), also called Recklinghausen's disease, which is characterized by development of nerve sheath tumors and a heightened lifetime risk to develop malignant peripheral nerve sheath tumor (MPNST). [4–6]

1.2 DIAGNOSING SARCOMAS

The rarity of sarcomas warrants a multimodality approach for early detection including clinical assessment, radiology imaging and pathology assessment. International guidelines recommend centralizing patients with suspect sarcoma to sarcoma referral centres at select

university hospitals, where the medical investigation, diagnostics and subsequent treatment are performed by physicians specialized in sarcomas (in Sweden the referral centres are called "Sarkomcentrum"). This approach has been proven to reduce the need for re-excisions and the risk of local recurrence, as well as reducing loss-of-function due to surgery when possible.[7,8]

1.2.1 Clinical presentation

Clinical presentation of sarcomas varies due to their heterogeneous nature, and the subtypes relevant to this thesis will be discussed more in-depth in later sections. Generally soft tissue lesions exhibiting any features such increase in size, size > 5 cm, localization in deep tissue or pain should raise suspicion for malignancy.[8,9] Bone sarcomas tend to more frequently debut with pain which may or may not be correlated to physical activity, and more rarely as pathological fractures. It is recommended that any persisting, non-mechanical bone pain should be assessed with an imaging modality.[7,9]

1.2.2 Radiology

The recommended imaging modality for sarcomas is magnetic resonance imaging (MRI), which uses strong magnetic fields and radio waves to excite hydrogen nuclei. The radio frequency signals emitted by the atoms are detected and processed in order to visualize different tissues (Fig. 1). This modality offers the highest detail and is also especially useful for distinguishing fat tissue, which is important in assessing soft tissue sarcomas.[10,11]

MRI does not involve the use of radiation, which distinguishes it from the plain radiograph (X-ray) or computer tomography (CT). X-ray machines produce electromagnetic radiation; while the radiation passes through soft tissue it is absorbed by dense materials such as bone, which can then be visualized. A CT simply take X-ray images from multiple angles which are then combined into a more detailed image.

While the X-ray or ultrasound might be the first exam upon suspicion of soft tissue sarcoma, it should always be followed by MRI or CT. A plain X-ray can provide valuable information in the initial assessment of primary bone sarcomas, but upon suspicion of malignancy an additional exam, preferably MRI, should be performed in order to evaluate any intramedullary or soft-tissue components of the tumor.[7,8,11]

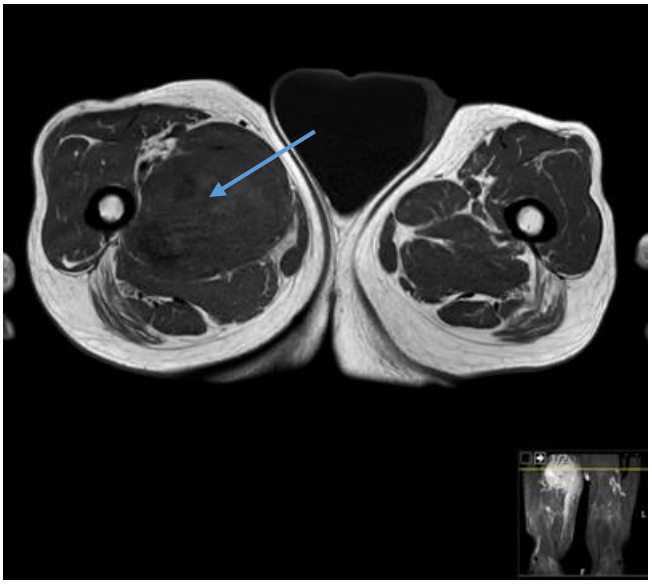

Figure 1. Magnetic resonance imaging (T2-weighted, transverse plane) of a patient with undifferentiated pleomorphic sarcoma (arrow) in the thigh. Image adapted from Zhang, Biomolecules, 2022.

1.2.3 Pathology

The pathologists' role is to evaluate the available tissue, macroscopically (when possible) and microscopically in order to provide a diagnosis. In recent years this also encompasses the evaluation of prognostic markers – the overall outcome – and predictive markers – information about therapy response – using either immunocytochemistry (ICC), immunohistochemistry (IHC) and/or molecular techniques.

1.2.4 Fine-needle aspiration cytology

Fine needle aspiration (FNA) is an established and widely used diagnostic method where cells from a lesion is aspirated using a fine gauge needle (Fig. 2). The needle can be guided with an imaging modality such as ultrasound or CT to ensure that the sample is representative. The cells are then smeared on a glass slide and evaluated in a microscope by cytopathologists. FNA is a minimally invasive process with low risk of complications, and is often used for preoperative diagnosis.[12,13] Additional ancillary techniques such as ICC and molecular analysis such as polymerase chain reaction (PCR) can also be performed on FNA material in order to increase the diagnostic accuracy.[14]

Most sarcomas have a high risk of recurrence, and tumor cell seeding i.e. the deposit of tumor cells along the needle track or dislodging tumor cells into the circulatory or lymphatic system, is a concern. In order to prevent seeding the aspiration or biopsy site can be marked, and the skin and surrounding soft tissue is then excised during surgery.[15]

1.2.5 Biopsy

Biopsy is the removal of a part of a lesion for microscopic diagnosis and can be performed with several instruments such as a larger gauge needle (core biopsy) or surgical scalpel (open biopsy) (Fig. 2).[16] A clear advantage is the ability to assess different tissues in relation to each other and providing valuable pre chemo- or radiotherapy morphology for comparison when assessing therapy response. In contrast, FNA material cannot be utilized for this purpose as it consists of clusters or single cells.

The risk of tumor cell seeding is also present during biopsy, and as the incision site is larger this method has generally been avoided for lesions suspected of sarcoma due to the high recurrence risk. There is considerable inter-study variation of how common seeding during biopsy really is, and the risk it poses.[17,18] For patients in need of neoadjuvant treatment the advantages and disadvantages of FNA vs biopsy should be individually evaluated.

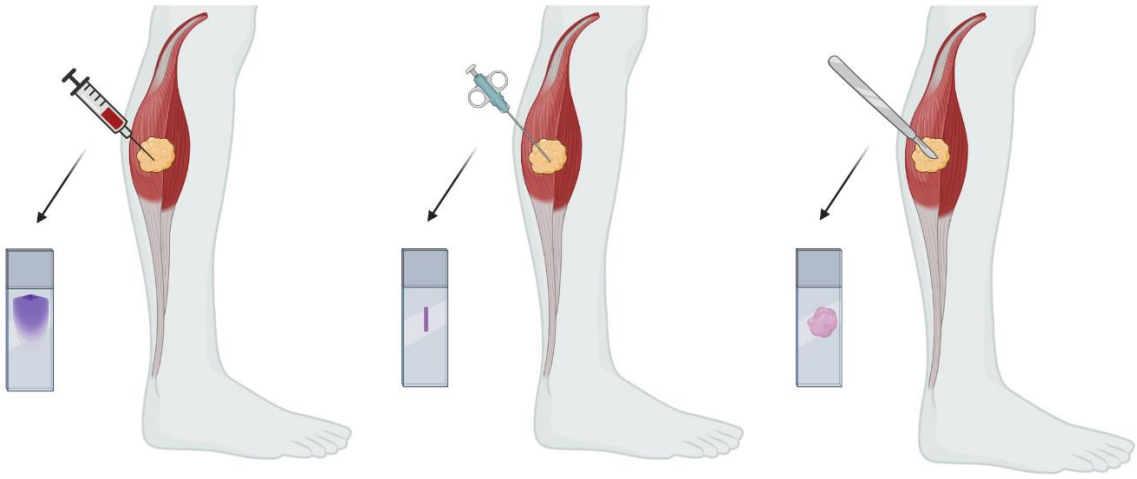

Figure 2. Fine needle aspiration (left) versus core biopsy (middle) and open biopsy (right) of a soft-tissue tumor. Created with biorender.com.

1.2.6 Surgical specimen

Macro grossing is the most essential step in processing surgical specimens. Surgical margins are evaluated by the pathologist, and inked if necessary. The specimen is then fixed with 4% formaldehyde for >24 hours, after which representative areas are sampled. The samples are then processed and embedded in paraffin, and finally sectioned with a microtome to create slides for microscopic evaluation.

Aside from resection margins there are other information provided by surgical specimens, such as evaluation of therapy response and identifying heterogeneous areas like high-grade components in the tumor which could be missed in biopsies.

1.2.7 Immunocytochemistry and immunohistochemistry

Antibodies, also referred to as immunoglobulins, are glycoproteins produced by plasma cells which are a variant of B lymphocytes. The antibody is usually visually represented as a "Y" shaped structure with two binding sites – one on each arm of the "Y" – which can bind to antigens. An antigen is a molecule which can trigger an immune response, and each antigen has surface features called epitopes which specific antibodies bind to.[19]

The use of antibodies to identify tissue expression of antigens has been in use since the 1930's and is now an invaluable technique used in both medical diagnostics and research. ICC is performed on FNA material, and IHC on formalin-fixed paraffin embedded (FFPE) slides. The indirect method (Fig. 3) is most commonly used: the primary antibody binds to the antigen, and a secondary antibody which is conjugated with an enzyme binds to the primary antibody. The enzyme then visualizes the antibody binding site by changing the color of the substrate. This results in a staining which can be evaluated with a light microscope or fluorescent microscope. In pathology ICC and IHC have multiple uses, such as identifying different cell types in order to establish a diagnosis, or to visualize predictive and prognostic markers in tumors.[20]

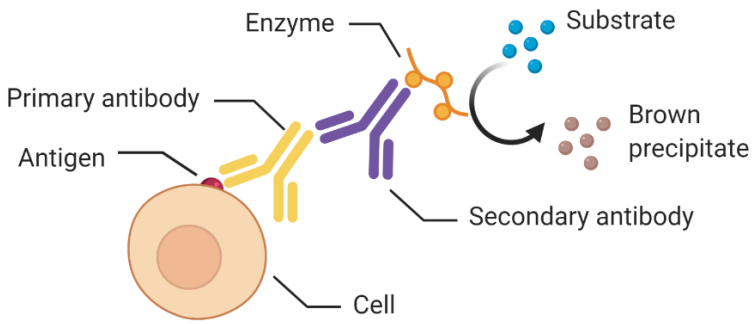

Figure 3. Antigen detection with immunochemistry using the indirect method. Created with biorender.com.

1.2.8 The role of fusion genes

Sarcomas are often driven by pathognomonic fusion genes or larger chromosomal changes and are tumors with low mutational burden, in contrast to carcinomas where point mutations play the larger role in carcinogenesis.[21] Fusion genes are comprised of two separate genes which have joined by chromosomal rearrangement – often translocation, where part of one

chromosome is transferred to another chromosome (Fig. 4).[22] They are not exclusive to sarcomas; the first fusion gene – the Philadelphia chromosome, *BCR-ABL1* – was discovered in chronic myologenous leukemia already in the 1960's by Nowell and Hungerford.[23,24]

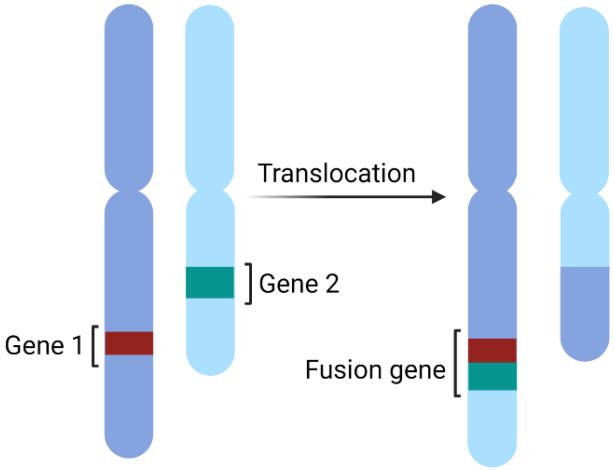

Figure 4. Translocation resulting in two genes originally located on separate chromosomes becoming a fusion gene. Created with biorender.com.

1.2.9 The rise of molecular pathology

Recent years have seen multiple advances in molecular pathology, which has revolutionized the classification of sarcomas. This has led to a considerable shift in how pathologists diagnose sarcomas; over 140 fusion genes are now identified with many of them specific to a certain subtype, such as the *SYT-SSX* fusion gene in synovial sarcoma or *EWS-FLi1* in Ewing's sarcoma.[21]

Sequencing with real-time polymerase chain reaction (PCR) is now a common and available technique in many laboratories, and can be used to detect certain mutations including fusion genes. PCR uses a polymerase enzyme and a primer – a short, single-stranded nucleic acid needed for the initiation of DNA synthesis – as well as temperature regulation in order to replicate the gene of interest in multiple cycles. The amplified DNA is then linked to fluorescent dye, and the increase of fluorescence is detected in real time. A small amount of tissue, for example from FNA material, is usually sufficient for molecular analysis, as the gene copies exponentially increase for every cycle.[25] In addition, New Generation Sequencing (NGS) is becoming more widely available and economically feasible, enabling more exploratory, whole-genome sequencing of tumors.

1.3 TREATMENT

1.3.1 Surgery

The primary treatment for both bone and soft tissue sarcomas is surgical resection with a wide margin.[7,8] The surgeon's expertise is crucial; in sarcomas adjuvant treatment often cannot compensate for a suboptimal excision of the tumor. In extremities one must also consider if amputation is preventable while maintaining an adequate margin, and unfortunately in many cases the surgery will result in loss-of-function for the patient.[26]

The Enneking criteria[27] for surgical procedures (Table 1) is the most widely used system and proposes four categories of resection margins, based on the theory that sarcomas are surrounded by a reactive zone which could contain tumor cells: intralesional, marginal, wide (of which the exact measurement is still debated),[28,29] and radical. Other systems such as the R (residual tumor) classification (Table 1), which is a part of the TNM staging system,[30] is also occasionally used, though R0 encompasses the equivalent of marginal, wide and radical margins. As such, only stating the R stage without a quantitative measurement complicate any future data collection regarding prognosis and resection margins.[29]

Enneking surgical criteria		TNM residual tumor (R) classification	
Intralesional	Excision within tumor	RX	Cannot be assessed
Marginal	Within reactive zone	R0	No residual tumor
Wide	Beyond reactive zone	R1	Microscopic residual
Radical	Whole compartment	R2	Macroscopic residual

Table 1. Categories encompassed by the Enneking surgical criteria and the TNM classification.

The clinical impact of resection margins remains controversial. The largest cohorts published by The Memorial Sloan-Kettering Center in New York found a positive correlation between survival and margin status in soft tissue sarcomas,[31,32] but other published studies with large patient groups failed to either find any correlation with survival,[33–35] or only correlation with local control.[36] Yet another cohort found that R0, regardless of measurement, had a significant impact on survival.[37]

1.3.2 Chemotherapy

Chemotherapy is the use of toxins that interfere with cell growth, either through inhibiting cellular division – mitosis – or damaging the DNA. It can be used as a curative treatment, neoadjuvant (pre-surgery in order to shrink the tumor), adjuvant (post-surgery as a complement)

or palliative. It is a systemic treatment injected into the blood stream, which usually makes tumor localization a non-issue.[38]

Chemotherapy affects both normal and cancer cells. Its side effects primarily stem from the damage to normal cells which divide frequently such as hair follicles, the digestive tract lining or bone marrow. Due to this, a balance in dosage must be found for each patient which optimally is sufficiently high to damage tumor cells while minimizing toxicity.[38] Chemotherapy sensitivity varies considerably between different sarcoma subtypes and is most frequently used on high-grade sarcomas that are highly sensitive, such as Ewing sarcoma or high-grade osteosarcomas.[7,8]

1.3.3 Radiation therapy

Radiation therapy is the use of ionizing radiation to inhibit cell growth by causing DNA damage, most frequently in the setting of cancer treatment. Just as chemotherapy, radiation therapy can be either curative, neoadjuvant, adjuvant or palliative. To minimize damage to normal tissue the radiation beams are aimed at the localized area of and around the tumor – the radiation field – ensuring that the targeted area receive a higher dose.

In sarcoma treatment radiation therapy is mainly used in the adjuvant setting as a complement to surgery and/or chemotherapy, particularly in high-grade sarcomas, sarcomas in deeper tissues or unsatisfactory margins.[7,39] Sensitivity to radiotherapy varies between subtypes.[40]

1.3.4 Targeted treatments

In later years targeted treatments such as immune checkpoint inhibitors (ICIs) are increasingly used in carcinomas with established treatment protocols and promising results. While the same cannot yet be said about sarcomas, there are ongoing phase II clinical trials which will be further discussed in 1.8.

1.4 CURRENT CHALLENGES

Despite advances in medicine sarcomas still post several considerable challenges. Due to its rarity and heterogeneity the correct diagnosis is not only crucial but can also be difficult to achieve. While multimodal treatment with radio-chemotherapy and extensive surgery has increased the overall survival, sarcomas remain one of the deadlier malignancies. In recurrent

and metastatic tumors, systemic treatment has limited effectiveness and the development of therapy resistance is not uncommon.[41–43]

1.5 SYNOVIAL SARCOMA

1.5.1 Introduction

Initially named *synovial endothelioma* [44] or *synovioma*[45] due to its histologic resemblance to developing synovium, synovial sarcomas are malignant soft tissue tumors which, despite its name, is of unknown histogenesis and do not originate from synovial cells.[2,46] As one of the more common sarcomas in adolescents with its peak around the 3rd decade of life, SS accounts for approximately 5-10% of all soft tissue sarcomas. SS may arise in any anatomical location – rare primary tumor locations include the heart, liver and prostate – but most frequently in the lower extremities in the vicinity of, but rarely within, large joints.[2,46]

SS are slow-growing and its clinical appearance could mimic a benign lesion. Symptom duration prior to diagnosis range from weeks to years[47] and are often related to tumor size, including swelling, pain, affected range of motion, and signs of nerve compression.[48]

1.5.2 Radiology

The imaging findings of SS in plain X-ray are often non-specific. Small lesions may not be visualized and any aggressive bone invasion is rare, which can lead to misinterpreting the tumor as benign. SS are often described as round or oval soft-tissue masses with juxtaarticular localization. Calcifications may be present and should be interpreted as a warning sign when weighing between a benign differential diagnosis and SS.[47]

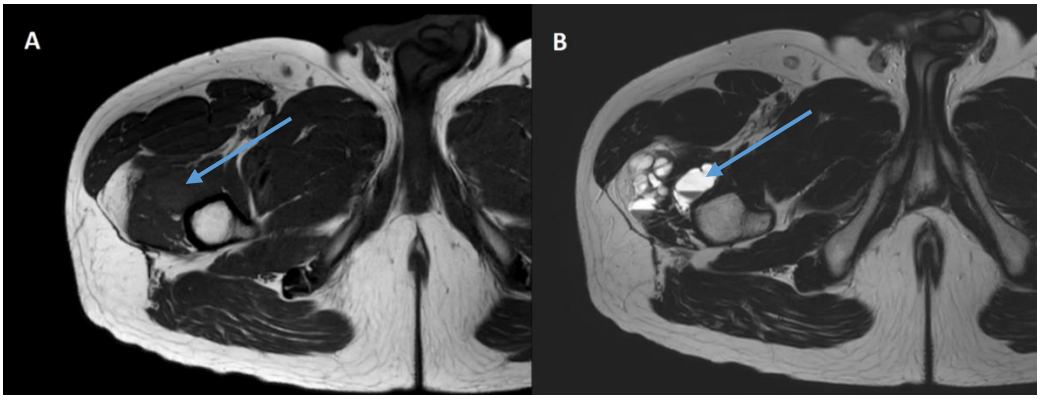

Figure 5. MRI of synovial sarcoma in the hip area. A) T1 weighted, transverse view and B) T2 weighted, transverse view.

As with many soft tissue and bone sarcomas MRI is the optimal modality (Fig. 5). Here SS are generally described as multilobulated, heterogeneous soft-tissue masses. Small tumors may appear well-defined while large tumors are more poorly circumscribed.[47] A "triple sign" has been described by Jones et al. represented by the tumors' heterogenicity with areas of varying low to intermediate to high signal intensity; this is however not specific to SS and can be seen in other soft-tissue tumors.[49]

1.5.3 Morphology

SS is a mesenchymal tumor with three subtypes based on histomorphology. The two main subtypes are monophasic SS, with mesenchymal spindle or oval cells, and biphasic SS which also contains an epithelial component occasionally displaying glandular formation.[2] The third subtype is poorly differentiated SS which is considered a form of tumor progression. In some studies the biphasic pattern seem to have a slightly more favorable outcome[50] while poorly differentiated SS generally has a worse prognosis.[51]

SS display a homogenous cytomorphology in FNA. The smears are highly cellular, comprised of varying oval, spindle and round cells. Other common features are pericapillary formations and a pink background stroma.[14,52–55]

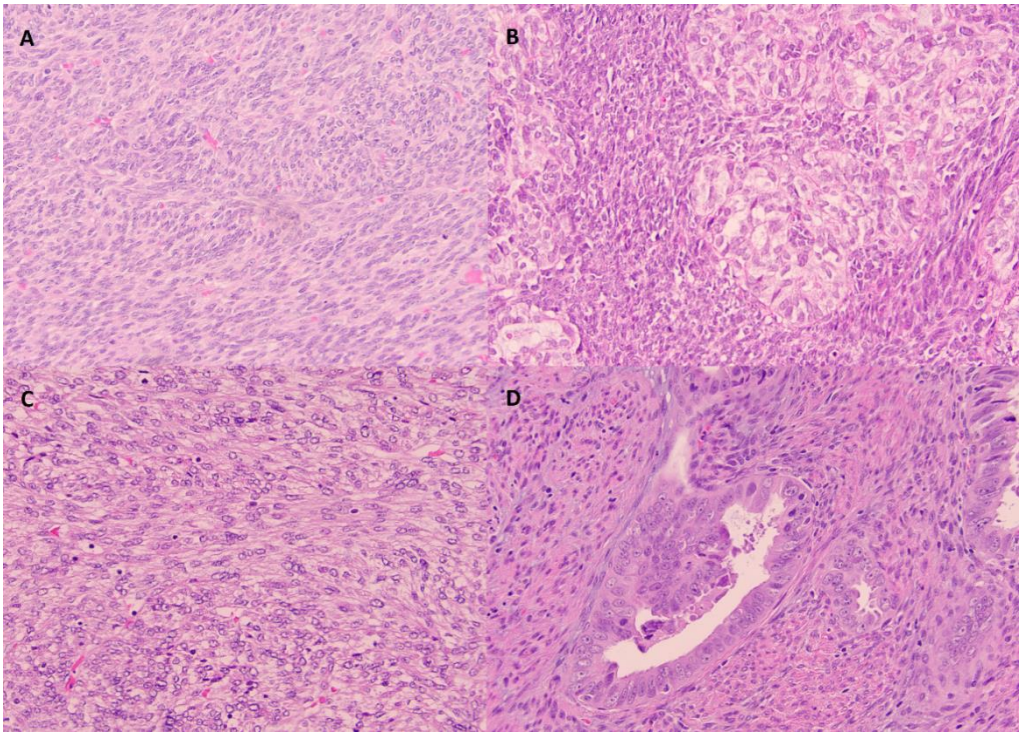

Figure 6. Histomorphology (H&E, 200x) of: A) monophasic SS and B) biphasic SS compared to C) leiomyosarcoma and D) carcinosarcoma.

Mesenchymal tumors pose diagnostic challenges as they often have similar histology, and SS can be difficult to distinguish from other neoplasms (Fig. 6). Possible differential diagnoses to monophasic SS include other mesenchymal or spindle cell tumors such as solitary fibrous tumor (SFT), leiomyosarcoma, spindle cell carcinoma, or MPNST. Histologic similarities with biphasic SS can be found in e.g. carcinosarcomas, and poorly differentiated SS with e.g. rhabdomyosarcoma.[56,57]

1.5.4 Immunochemistry

IHC/ICC analysis of SS frequently show positivity for Vimentin and Bcl2. Biphasic SS may also stain positive for Pan-cytokeratin and CK7.[14,54,58] TLE1 has recently emerged as a new marker for some sarcomas including SS (Fig. 7).[59,60] While none of the abovementioned immunochemistry are specific, the Department of Clinical Pathology and Cytology at Karolinska University Hospital has recently implemented an antibody which targets the SYT-SSX fusion gene (Fig. 5).

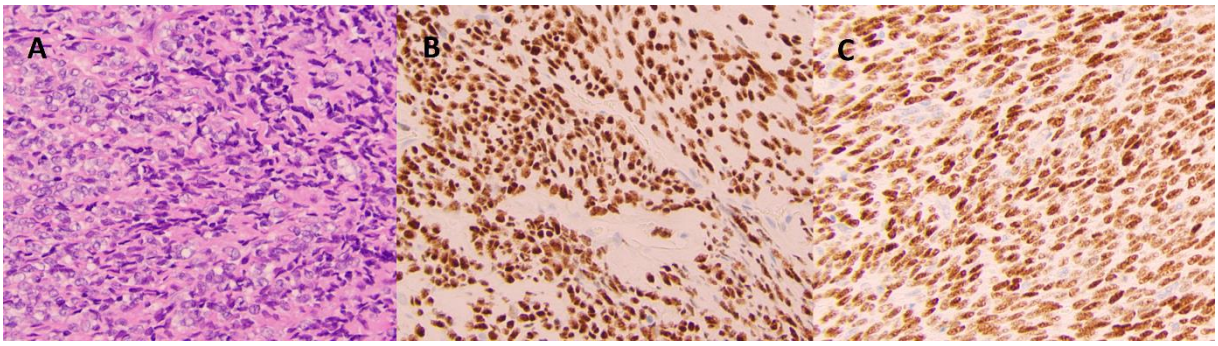

Figure 7. A) Poorly differentiated SS (H&E, 200x) which stains positive for B) SYT-SSX and C) TLE-1 immunohistochemistry.

1.5.5 *SYT-SSX* fusion gene

SYT-SSX, t(X;18)(p11;q11) is an oncogenic fusion gene which is pathognomonic for SS. Created through the translocation between the *SYT* (located at chromosome 18q11) and the *SSX1, SSX2* or *SSX4* genes (located at chromosome Xp11),[61,62] its function is not yet fully understood. The fusion protein may affect cell proliferation and gain stem-cell like properties by interacting with transcription factors such as SWI/SNF, an enzyme complex involved in nucleosome rearrangement resulting in easier access to chromatin.[63,64] It was believed that SYT-SSX1 was linked to less favorable outcome,[62,65] but recent data have found no prognostic value depending on SSX gene.[66]

The most accurate method to confirm a morphologic diagnosis of SS is detection of the *SYT-SSX* fusion gene either with PCR or FISH. This is also especially useful when differential diagnoses are involved as the detection rate of *SYT-SSX* in SS ranges from 60-100%.[14,62,67,68]

1.5.6 Treatment

SS are high-grade sarcomas and associated with high local recurrence and metastatic rates. The recurrence rate is reported to be around 40% with adequate and as high as 80% with inadequate treatment. 5-year survival ranges between 36-76% and is related to multiple factors;[47] worse long-time prognosis is associated with higher patient age, larger tumor size (> 5 cm), localization in the head and neck area and the presence of a poorly differentiated component. Metastatic disease is common and reported in up to 50% of the cases, most frequently affecting the lung.[50,69]

The current treatment of localized disease is primarily surgical excision. As SS often arise around large joints loss-of-function can be difficult or impossible to prevent; high recurrence rates are seen in cases with inadequate margins.[70] Radiation therapy is given in a neoadjuvant or adjuvant setting in cases where resection margins are inadequate, but has shown no significant difference in local control in cases resected with wide margins.[71] SS is considered moderately sensitive to chemotherapy, which is used to treat disseminated disease.[72]

1.6 CHONDROSARCOMA

1.6.1 Introduction

Chondrosarcoma is the 2nd most common malignant tumor of bone origin. The peak incidence is at the 6th decade of life, with no difference between genders. The tumor frequently affects long tubular bones such as the femur or humerus, but can arise in any part of the skeleton. While most CS are primary, a subset arises from pre-existing lesions such enchondromas and osteochondromas.[2]

1.6.2 Radiology

The standard X-ray is often the first imaging modality used in investigating suspect CS, which is described as a lucent bone lesion with matrix calcification. The organization and extent of calcification correlates with tumor grade, and a high-grade tumor may display a more irregular pattern.[73,74]

MRI is essential in visualizing tumor extension into the intramedullary space as well as any extraosseous component in the surrounding soft tissue (Fig. 8), which cannot be evaluated on X-ray. In addition, MRI is used to evaluate cartilage thickness in patients with osteochondroma in order to assess any malignant transformation to CS. CT can be used to visualize matrix calcification, which is not always visible in MRI, as well as cortical scalloping – focal bone resorption in the endosteum – which is a sign of slow-growing medullary lesions.[7,73,74]

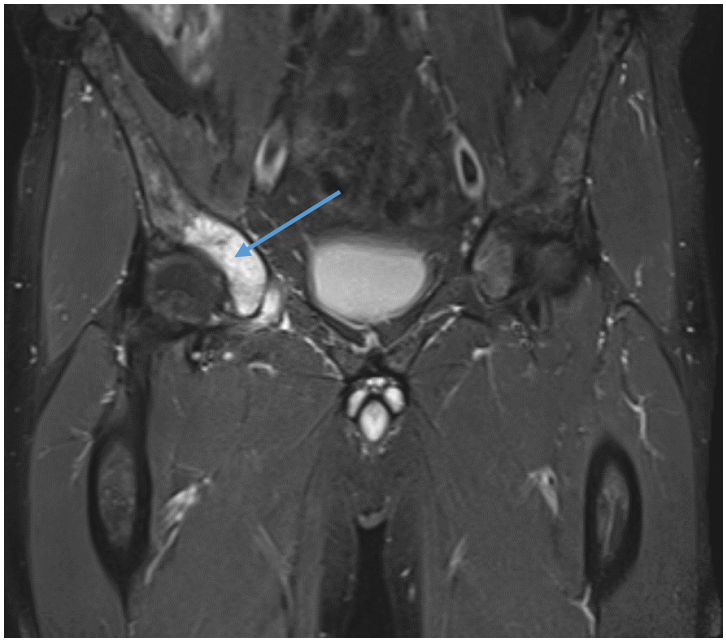

Figure 8. MRI of chondrosarcoma in the pelvic bone (T1 weighted, frontal view).

1.6.3 Morphology

Conventional CS constitute 85% of all cases, with the remaining 15% consisting of rare subtypes such as clear cell chondrosarcoma (CCCS), mesenchymal CS and dedifferentiated CS. Morphologically conventional CS are composed of lobulated cartilage with varying mineralization; the tumor's cellularity, atypia and mitotic rate then determines grading.[2,3]

CS are graded on a scale of 1-3 according to the WHO 2020 classification,[3] which is based on the classification suggested by Mirra et. al. in 1985.[75] Grade 1, also called atypical cartilaginous tumor, is a well differentiated tumor with low cellularity, lobulated growth and abundant cartilage matrix. Grade 1 tumors can be treated with local excision, and the risk of metastasis is low.[7,76,77] In contrast, grade 3 are poorly differentiated, with high cellularity, marked atypia and often abundant mitosis, and associated with aggressive course of disease (Fig. 9).[3] Tumor grading is prone to subjective interpretation with interobserver variation with a Kappa value of 0,44,[78] which is a concern when histological grade influences clinical decision making.

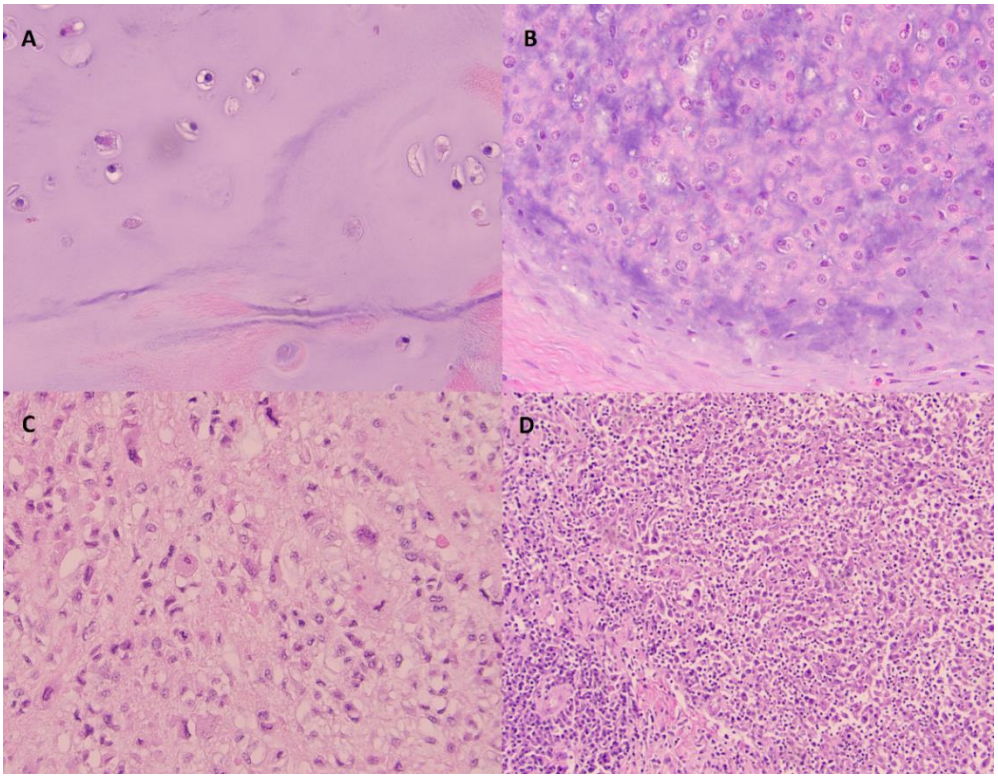

Figure 9. Histomorphology (H&E, 200x) of A) CS grade 1, B) CS grade 2, C) CS grade 3 and D) dedifferentiated CS.

1.6.4 Genetic changes in chondrosarcoma – *IDH* mutations

Activating mutations in isocitrate dehydrogenase 1 (*IDH1*) and *IDH2* genes are frequently found in many cancer types such as glioma and acute myeloid leukemia, as well as in 50-80% of conventional CS. These mutations are likely involved in early tumorigenesis, as they are also found in the majority of enchondromas.[79,80] Syndromes linked to multiple enchondromas and a higher risk for developing CS, such as Mb Ollier and Mb Maffucci,[80,81] are characterized by somatic mosaic *IDH1* and *IDH2* mutations.

IDH1 and *IDH2* are isozymes involved in the catalysation of the oxidative decarboxylation of isocitrate to α-ketoglutarate (α-KG), and the reduction of $NADP^+$ to NADPH. While the precise mechanism behind *IDH* and tumorigenesis isn't completely clear it is hypothesized that mutated *IDH1* and *IDH2* instead consume NADPH in order to convert α-KG to D-2-hydroxyglutarate (D-2HG). Pathways utilizing α-KG are interrupted as structural similarities to D-2HG result in competitive inhibition leading to dysregulation of epigenetic mechanisms.[79] Preclinical studies has shown dysregulated chondrogenesis in IDH-mutant stem cells,[82,83] which could explain its high prevalence in CS.

1.6.5 Genetic changes in chondrosarcoma – *TERT* mutations

Another common mutation in CS is human telomerase reverse transcriptase (*hTERT*) gene promoter mutations. The role of telomerase is to lengthen the telomeres – a non-coding region at the end of chromosomes – by adding a repetitive nucleotide sequence. In somatic cells the telomere region shortens each time a cell undergoes mitosis, and as telomere shortening reaches its critical point the cell becomes senescent and replication ceases.[84] The concept, called the Hayflick limit and demonstrated by Leonard Hayflick in the 1960s, implies that somatic cells can divide between 40-70 times before going into senescence.[85]

Telomerase is normally not active in the majority of human somatic cells – otherwise the cells would be able to divide infinitely. The concept of replicative immortality is one of the cornerstones in the Hallmarks of Cancer (Fig. 10).[86] As such, *TERT* promoter mutations are generally associated with high-grade tumors in multiple types of malignancies.[87,88]

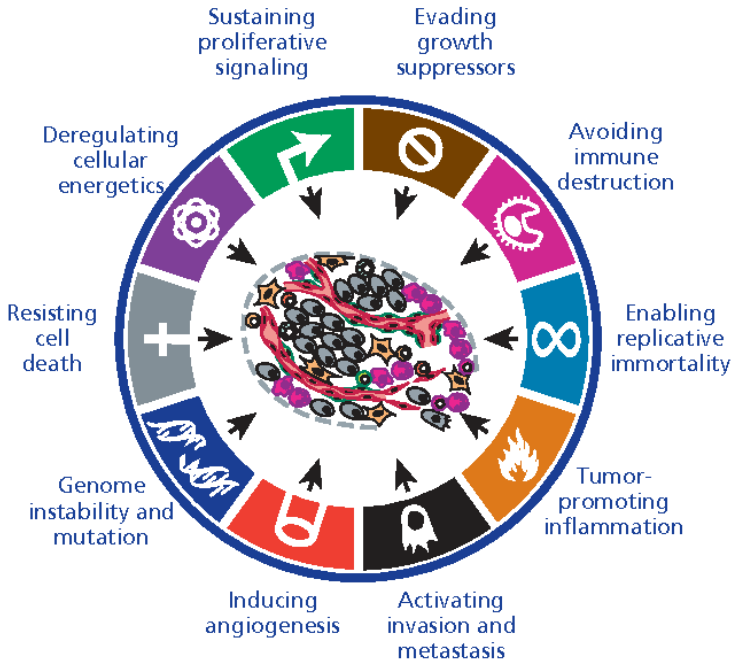

Figure 10. The ten hallmarks of cancer, which are cornerstones for tumorigenesis. Hanahan & Weinberg, Cell, 2011.

In CS the previously described *TERT* promoter mutation at -124 C>T (also called C228T, localized 124 base pairs upstream of the translation start site) has been described in up to 43% of tumors[89,90] and is found to be a negative predictive factor strongly correlating with high-grade tumors, disseminated disease and mortality.[89–91] In tumors with heterogeneous morphology the high-grade areas often harbored *TERT* mutation while low-grade areas

remained largely wild-type.[89,91] It is believed that the mutated sequence is similar to an E twenty-six (ETS) binding motif and hence has the ability to bind the ETS family transcription factor which in turn activates the promoter region (Fig. 11).

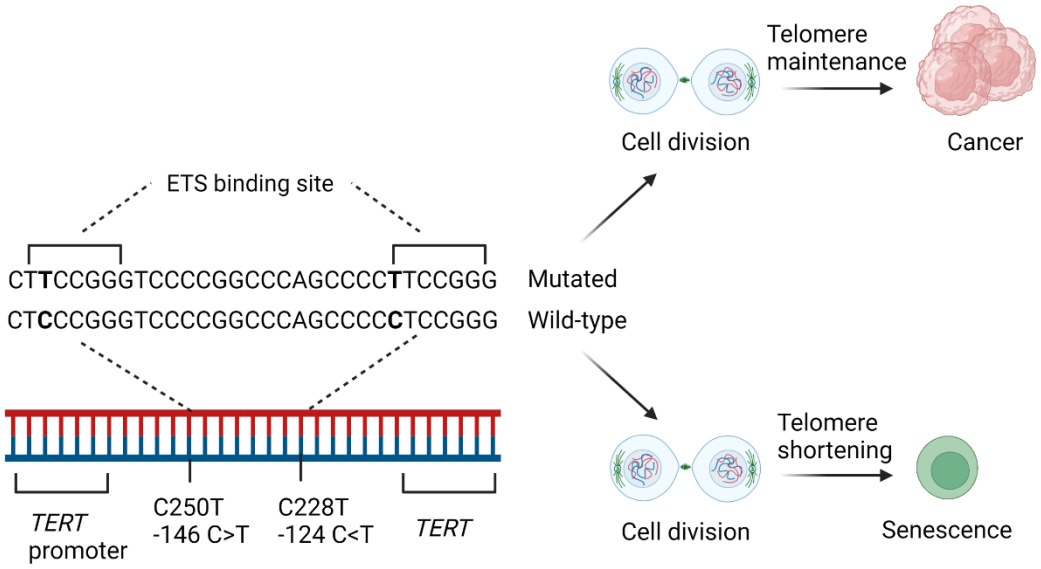

Figure 11. *TERT* promoter mutation creates a novel ETS binding site, leading to activation of the promoter region and transcription of *TERT*. This allows telomere maintenance in cells which usually have none, enabling infinite cell division – replicative immortality.

TERT may not only be a promising prognostic biomarker as studies on CS cell lines have indicated that telomerase inhibition may potentially sensitize drug-resistant CS cells to chemotherapy.[92] There have been multiple Phase II trials of telomerase-targeted treatment and cancer vaccines with varying grade of response, and the main concern is adverse events as a result of telomerase inhibition.[93,94] Currently there are no formally approved telomerase-targeted therapies.

1.6.6 Treatment

The primary treatment for localized disease is surgical resection with wide margins, and there are no efficient therapies for disseminated disease. CS are generally chemo- and radiotherapy resistant.[41,95] Proposed theories include difficulty in drug penetration due to the tumors' hyaline matrix, the presence of a membrane-bound pump called P-glycoprotein which can extract hydrophobic molecules including chemotherapeutic agents from within the tumor cell, and aberrant expression of the anti-apoptotic protein Bcl-2. [96–98] Overall 5-year survival is 75% for

patients with localized disease at diagnosis, but varies greatly depending on tumor grade and is reduced to around 40% for high-grade CS.[99,100]

1.7 CLEAR CELL CHONDROSARCOMA

1.7.1 Introduction

Clear-cell chondrosarcoma (CCCS) makes up 1-2% of all diagnosed CS and is thus a very rare subtype.[101] Common primary tumor localizations are in the long bones, often the proximal femur or humerus. In comparison to CS, CCCS is more common in younger patients, with its incidence peaking around the 3rd and 4th decade of life. The tumor is often slow-growing and it's not uncommon for the patient to exhibit symptoms such as pain during physical activities for multiple years prior to diagnosis.[46,101–103]

1.7.2 Morphology and diagnostic challenges

Histologically CCCS are composed of mature cartilage in a lobular pattern. There is an abundance of the characteristic clear cells, which are also a distinguishing feature in in FNA samples.[104,105]

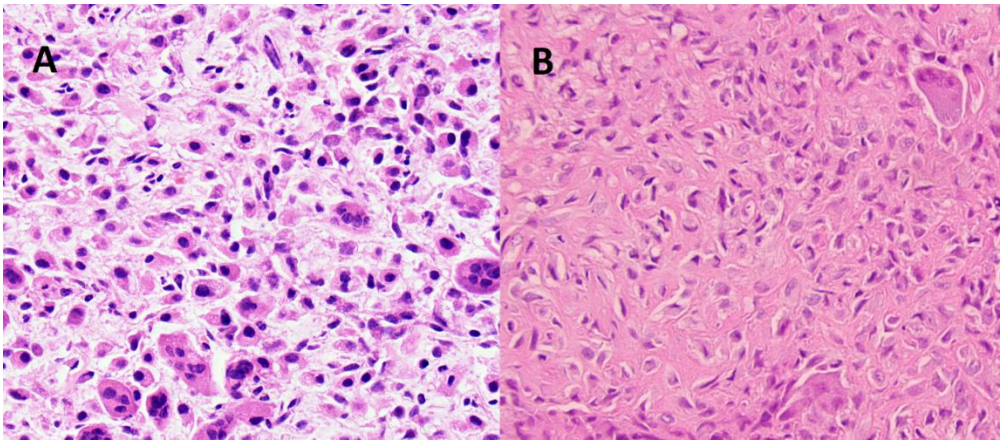

Figure 12. Histomorphology (H&E stain, 200x) of A) CCCS versus B) chondroblastoma.

Chondroblastoma, a benign bone tumor, is the most common differential diagnosis to CCCS (Fig. 12). Masui et. al. found that the immunohistochemstry profile of CCCS share more similarities with chondroblastoma than conventional low-grade CS; both CCCS and chondroblastoma show immunoreactivity for S-100, CD68, MMP-9, PTH-LP, PDGF and PDGF-R.[106]

Aside from morphological similarities the radiologic finds are also similar, with both CCCS and chondroblastoma presenting as epiphyseal lesions with a sclerotic margin (Fig. 13).[74,107–109] The incidence of chondroblastoma is also higher in the adolescent population and there is a considerable overlap with that of CCCS.[110] In addition, CCCS' slow-growing nature is a confounding factor as it could be mistaken as a benign process.[74,107–109]

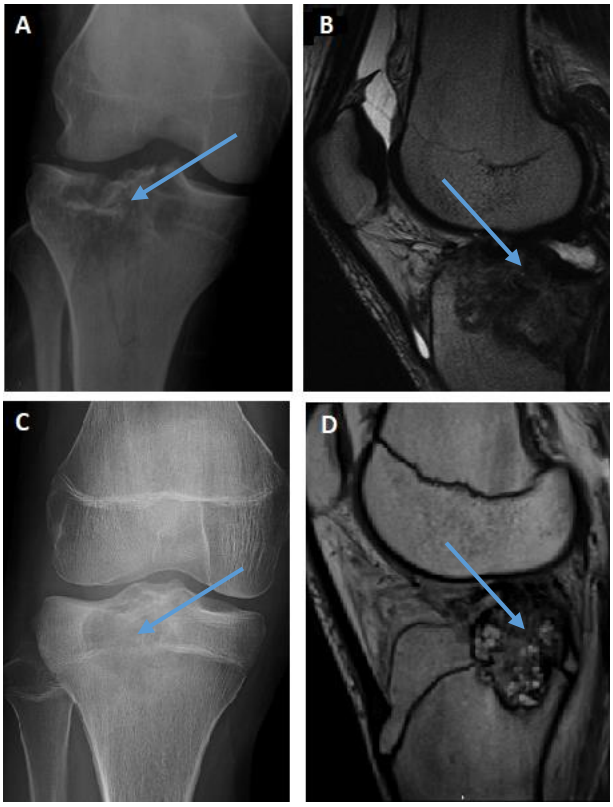

Figure 13. A) Plain X-ray and B) MRI (T2 weighted, sagittal view) of CCCS in the proximal tibia versus C) plain X-ray and D) MRI (T2 weighted, sagittal view) of chondroblastoma in the proximal tibia. Figure 13A and B are adapted from Zhang, Diagnostic Cytopathology, 2021.

1.7.3 Treatment

The treatment for CCCS is, as with many other sarcomas, *en block* resection in order to prevent local recurrence. Local resection with e.g. curettage is sufficient for chondroblastomas, and as such is it vital to distinguish the two neoplasms. While a majority of CCCS are low-grade lesions there is a risk for metastatic disease, and local recurrences are not entirely uncommon. In a study consisting of 17 CCCS patients Klein et. al. found a 30% local recurrence rate and 20% metastatic rate. The 10-year disease-free survival (DFS) was 60% and overall survival (OS) was 80%.[111]

1.8 PROGRAMMED DEATH-LIGAND 1 (PD-L1)

1.8.1 Introduction

The mechanisms underlying tumorigenesis do not involve only the neoplastic cells but also the complex network of interactions in the tumor microenvironment. The tumor microenvironment is the tissue surrounding a tumor – comprised of extracellular matrix, fibroblasts, blood vessels, and immune cells – and its impact on tumor survival, growth, potential targets and therapy response has been the focus of many researchers in recent years. In 2018 the Nobel Prize in physiology or medicine was awarded to James Allison and Tasuku Honjo for their discoveries in immunotherapy, which is now an ever-expanding area with new treatments steadily emerging.

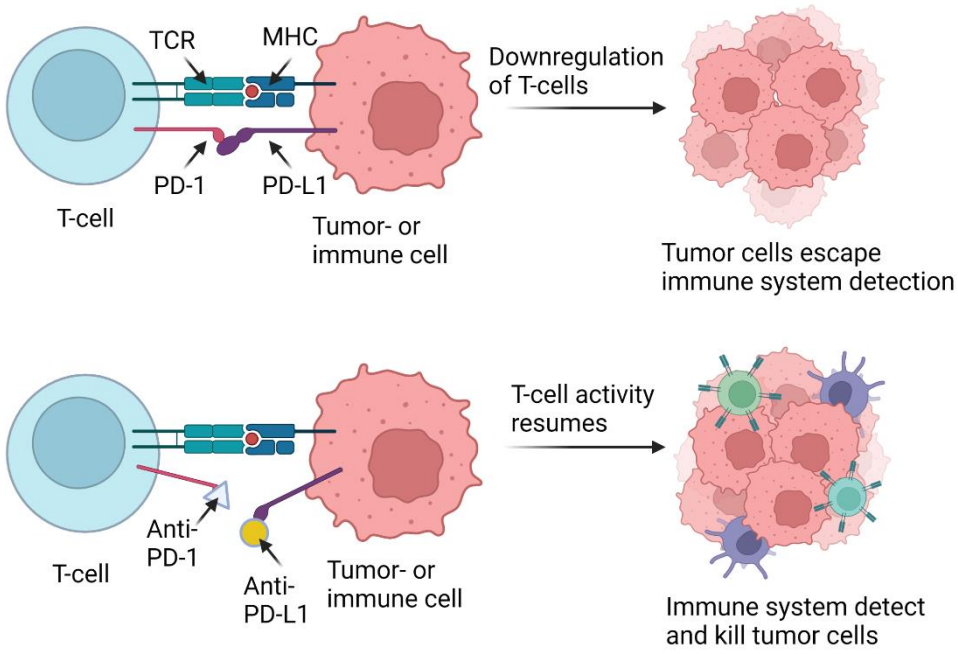

Figure 14. Tumor cells expressing PD-L1 can bind to, and downregulate, T-cells with the PD-1 receptor (top). Blocking PD-1 or PD-L1 leads to resumed T-cell activity, and thus the immune system can identify and attack tumor cells (bottom).

Programmed death ligand 1 (PD-L1) is a type 1 transmembrane protein expressed on antigen cells and a variety of other tissue cells including cancer cells, and Programmed cell death protein 1 (PD-1) is a receptor found on immune cells such as T-, B- and natural killer (NK) cells, dendritic cells and macrophages. The PD-1/PD-L1 pathway regulate the immune system, exercising an immune suppressive effect by inhibiting apoptosis in regulatory T-cells and proliferation of antigen-specific T-cells, enabling tumor cells to evade detection (Fig. 14).[112–114]

Immune checkpoint inhibitors (ICI), with the currently most established ones being PD-1 and PD-L1 inhibitors, are formally approved treatments for an array of solid cancers such as melanoma and non-small cell lung cancer.[115–117] The inhibition of the PD-1/PD-L1 pathway allows the immune system to identify and attack tumor cells. There are multiple formally approved immunoassays for assessing PD-L1 immunoreactivity as well as established cut-off values in both TC and IC for e.g. non-small cell lung cancer, urothelial bladder cancer, and melanomas, and IHC for PD-L1 is often performed as a standard part of the diagnostic work-up.

In certain solid cancers such as renal carcinoma and melanoma a high expression of PD-L1 in TC is significantly associated with worse outcome.[118–121] Aside from assessment of TC multiple variables affect treatment response to PD-1/PD-L1 inhibitors, e.g. PD-L1 expression on IC such as tumor-infiltrating lymphocytes (TIL) or tumor-specific T-cells.[113]

1.8.2 PD-L1 expression in sarcomas

The clinical implications of PD-L1 expression and PD1/PD-L1 inhibitor treatment for sarcomas remain a controversial subject. The predictive and prognostic value is not yet as defined for sarcomas as it is for carcinomas. Due to the many subtypes of sarcomas and its rarity, acquiring sufficient tissue samples and clinical data for cohorts or recruiting patients for phase III trials is a great challenge.

As expected the expression of PD-L1 varies greatly between sarcoma subtypes, but there is also an inter-study variation. In a recent meta-analysis, Veensta et al.[122] found that some sarcomas expressing PD-L1 reactivity are angiosarcoma, chondrosarcoma, osteosarcoma, Ewing's sarcoma, liposarcoma, rhabdomyosarcoma, undifferentiated pleomorphic sarcoma, and synovial sarcoma.[123–125]

There is also a considerable inter-study variation in regard to PD-L1 status in sarcomas and clinical outcome; Zheng et. al. found in a meta-analysis that PD-L1 immunoreactivity was a negative prognostic factor in soft tissue sarcomas,[126] which was corroborated in a study by Bertucci et. al.,[127] while other studies found no significant impact on survival in retroperitoneal liposarcoma[128] and chondrosarcoma.[129,130]

1.8.3 Current utility in sarcoma treatment

While there are currently no formally approved PD1/PD-L1 inhibitors for the treatment of sarcoma, several phase 2-trials are ongoing. SARC028 studied the effects of Pembrolizumab

and saw an objective response in 7/40 patients with soft tissue sarcoma (4 of these with UPS), and in 2/40 patients with bone sarcoma. PD-L1 positive (≥1%) tumors were seen in 3 patients in this study, all with UPS.[131] In Alliance A091401 2/43 patients had an objective response to single-therapy with Nivolumab and 6/42 to Nivolumab + Ipilimumab. The PD-L1 status was not reported, and subtypes showing therapy response included UPS, leiomyosarcoma, alveolar soft part sarcoma, angiosarcoma and myofibrosarcoma.[132] Toulmonde et al. found that 3/50 patients with soft tissue sarcoma showed objective response to treatment with Pembrolizumab + Cyclophosphamide; 6 patients in total had a PD-L1 positive (≥1%) tumor.[133] PEMBROSARC found limited treatment response in osteosarcomas, and the only patient with partial response had a PD-L1 negative tumor.[134]As of now there are no established PD-L1 cut-off values for sarcomas, and IHC for PD-L1 is usually not performed as standard. The recent international recommendation for treatment of sarcomas suggest that ICIs including PD1/PD-L1 inhibitors may be considered in a few select subtypes where first-line therapy has given unsatisfactory results, such as UPS, alveolar soft-part sarcoma, cutaneous angiosarcoma and classic Kaposis' sarcoma.[21,36]

2. AIMS OF THE THESIS

The overall aim of this doctoral project was to improve diagnostic accuracy of select sarcoma types including morphology and ancillary studies, as well as evaluating potential prognostic and predictive markers.

Paper I

Synovial sarcoma diagnosed by fine needle aspiration cytology and molecular techniques during 10 years

To investigate common morphological features and findings from ancillary techniques in FNA samples of synovial sarcomas.

Paper II

Clear-cell chondrosarcomas: Fine-needle aspiration cytology, radiological findings, and patient demographics of a rare entity

To investigate common morphological features in FNA samples and the epidemiology of clear cell chondrosarcomas.

Paper III

TERT promoter mutation is an objective clinical marker for disease progression in chondrosarcoma

To investigate the prognostic significance of *TERT* promoter mutations in chondrosarcoma disease progression.

Paper IV

Evaluation of PD-L1 expression in undifferentiated pleomorphic sarcomas, liposarcomas and chondrosarcomas

To investigate PD-L1 expression in chondrosarcoma, liposarcoma and undifferentiated pleomorphic sarcoma and its prognostic significance, and to see if any clear cutoff values could be established for PD-L1 expression in tumor cells.

3. MATERIALS AND METHODS

3.1 PATIENT COHORTS

All cohorts consisted of patients found by searching the archives of the Department of Clinical Pathology and Cytology at Karolinska University Hospital. Clinical data were retrieved from digital patient records and were available for all patients.

3.1.1 Synovial sarcoma

Paper I is a retrospective cohort consisting of patients with a diagnosis of synovial sarcoma and available FNA material. A total of 38 FNA samples from 35 patients (two patients were sampled more than once) collected between 2006 – 2018 were included. 30 samples were collected from primary tumors, 3 from local recurrences and 5 from metastasis. A majority of patients (33/35) had a corresponding biopsy or surgical specimen which also confirmed the diagnosis.

3.1.2 Clear cell chondrosarcoma

Paper II is a retrospective cohort consisting of patients with a diagnosis of clear cell chondrosarcoma and available FNA material. A total of 7 FNA samples from 6 patients collected between 1992 – 2018 were included. All patients had corresponding biopsy or surgical specimen which also confirmed the diagnosis. All surgical specimens except for one had also been submitted for external second opinion.

Due to the tumors' rarity a literature review was also conducted. Using PubMed search, 237 articles were found to contain the phrase "clear cell chondrosarcoma" and 1400 articles were found containing the phrase "chondroblastoma". Relevant articles containing information on patient age, gender, tumor localization and tumor size were included, resulting in 76 articles about CCCS and 385 articles about chondroblastoma.

3.1.3 *TERT* promoter mutations in chondrosarcoma

In **Paper III**, we identified patients with conventional chondrosarcomas diagnosed between 1994 – 2017. Data from 87 patients in our previous study of *TERT* promoter mutations in a cohort of chondrosarcomas[89] were integrated into the current study, bringing the total to 241 tumors from 190 patients.

3.1.4 PD-L1 expression in sarcomas

Paper IV was based on previously published cohorts consisting of patients diagnosed with conventional chondrosarcoma, liposarcoma and undifferentiated pleomorphic sarcoma diagnosed between 1994 to 2020 and with available FFPE material.[91,135] This resulted in a total of 230 tumors from 214 patients (74 CS, 44 LS and 96 UPS) which were stained with Ventana SP263 and SP142 assays. Due to its low malignant potential grade I CS were excluded. *Ad hoc* staining of PD-L1 (SP263) in the clinical setting was performed on 18 of the UPS cases, and those were not stained with SP142.

3.2 TUMOR SAMPLES

In **Paper I** and **II** FNA samples which were part of the original case workup were evaluated. **Paper III** was comprised of two cohorts of CS patients. Full FFPE slides were used for sequencing in the old cohort.[89] In the new cohort H&E stained FFPE slides were evaluated and high-grade areas were microdissected using a 1 mm punch biopsy; 1-3 punch biopsies were sequenced per patient. In **Paper IV** full slides from CS, LS and UPS were stained for PD-L1.

3.3 IMMUNOCYTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY

In **Paper I** any included ICC were previously performed as a part of the initial case workup. In **Paper IV** IHC for PD-L1 expression (Ventana SP142 and SP263 assays from Roche) was performed according to instructions for Ventana Benchmark Ultra at a clinically certified laboratory (SWEDAC accreditation) at the Department of Clinical Pathology and Cytology, Karolinska University Hospital.

3.4 MORPHOLOGIC ASSESSMENT

All slides were assessed at the Department of Clinical Pathology and Cytology at Karolinska University Hospital by a minimum of two cytopathologists or surgical pathologists in consensus. Assessment were carried out in a group setting in **Paper I** and **Paper II**, and independently in **Paper IV**.

3.5 MOLECULAR ANALYSIS

In **Paper I** molecular analysis by fluorescent in-situ hybridization (FISH), PCR or quantitative real-time PCR (qRT-PCR) had previously been performed on all cases of SS as part of the clinical diagnostic workup. The material used were either FFPE material from surgical specimens or cytological material (cell pellets, fresh frozen unstained slides, cell

suspensions or scrapings of MGG stained slides). The specific gene fusion partner (*SSX1, SSX2* or *SSX4*) was not available in all reports.

In **Paper III**, Sanger sequencing was performed as described in our previous cohort of CS patients.[89] To summarize it briefly, DNA extraction was performed using a QIAmp FFPE extraction kit (QIAgen). Sequencing for *TERT* promoter mutation was carried out using PCR using with M13-tagged primers. Chromatograms were manually interpreted using 4Peaks Software version 1.7.1 (Mekentosj).

3.6 STATISTICS

Time to event was defined as the timeframe between date of surgery and date of the event (first known metastasis or death). The Kaplan-Meier method was used to calculate differences in OS and metastasis-free survival (MFS). Two-sided Fisher's exact test and Chi-squared test were used to compare categorical variables. Categorical and continuous variables were compared with Mann-Whitney U test. A *p*-value of <0.05 was defined as statistically significant. Association between clinical features and prognosis was calculated with univariate and multivariate analysis using the Cox regression model in the R package "survival".

In **Paper III**, tumor grade followed the WHO 2020 classification[3] (based on the classification from 1985 as suggested by Mirra et al.[75]) . If more than one histological grade was described in a patient, the highest grade was used for the purpose of statistical comparison.

3.7 ETHICAL CONSIDERATIONS

All studies in this thesis were approved by the local ethical board (*Regionala etikprövningsnämnden Stockholm*, registration number 2013 1979-31).

In this doctoral project we hoped to discover new information which may improve diagnosis and clinical decision-making for sarcoma patients. In order to do this, we investigated morphologic features, tumor-specific mutations and expression of certain biomarkers.

The cohorts presented consist of patients treated at Karolinska University Hospital's sarcoma referral center (Sarkomcentrum). Tissues are acquired from either the initial investigation or from surgical samples; no procedures aside from those which were medically necessary are performed for the purpose of acquiring material. Normal tissue is sampled if it is present in

the surgical specimen. Tumor tissue, as well as normal tissue and blood samples are saved to a Biobank for use in current and future studies within the sarcoma field.

Clinical patient data is collected when available from digital patient records. Only relevant information such as symptoms, surgical/medical treatments, radiology/pathology assessments, disease progression and overall survival is collected.

All patients have received oral and written information from their physician prior to surgery, and have signed a permission form with informed consent. Legal guardians are also informed for patients under the age of 18.

Overall we find that the violation of patient integrity is limited, and the potential benefit of the project clearly outweighs the risks for the patient.

4. RESULTS AND DISCUSSION

4.1 PAPER I

An accurate pre-operative diagnosis of sarcoma is vital in order to offer the patient adequate treatment. SS may arise from any part of the body and poses differential diagnostic challenges with its morphological similarity to other mesenchymal tumors. In our cohort 28/30 primary tumors were correctly diagnosed as SS in the original cytology assessment; the 2 remaining cases were diagnosed as "mesenchymal tumor not otherwise specified (NOS)".

In one of the largest cohorts of SS cytology, we described the tumors' cytomorphology as well as immunocytochemistry and molecular pathology. Our findings were concordant with previously published studies.[52,55] The cytomorphology of SS was homogenous; common features were high cellularity, varying spindle-shaped, round or oval cells, pericapillary formations and pink background stroma (Fig. 15). Biphasic SS often presented with an epithelial, occasionally glandular component in cytology. We did not find any distinct features in cases with poorly differentiated SS in comparison to mono- or biphasic SS, though previous studies had described rhabdoid or Ewing-like morphology.[51,56,57] The current treatment for SS does not depend on subtype.[8]

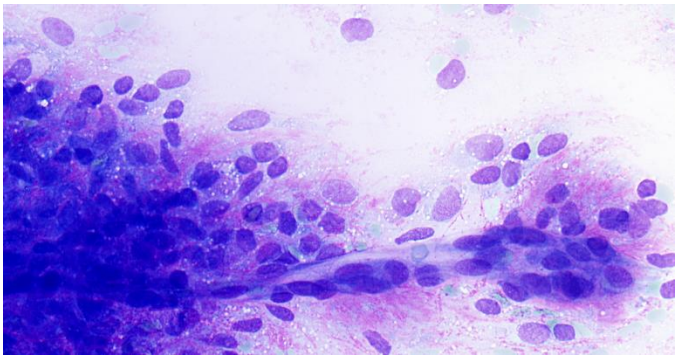

Figure 15. Cytomorphology (MGG, 400x) of SS showing oval tumor cells with pericapillary formation and pink background stroma. Adapted from Zhang, Cytopathology, 2019.

Immunocytochemistry demonstrated that common positive stains were Vimentin, EMA and Bcl-2, which is in-line with previous findings.[54,58,136,137] Biphasic SS often stained positive for pan-cytokeratin (CKMNF116). There are multiple pitfalls: 25% of SS stained positive for S100, which could be difficult to differentiate against nerve sheath derived tumors such as MPNST. CD99 was positive in all cases but is an unspecific marker that is positive in

many tumor types. TLE1 is a more recent marker and therefore only stained in cases after 2015; all stained cases showed varying degrees of positive nuclear staining. It is, however, not specific for SS and is also positive in other mesenchymal tumors such as peripheral nerve sheath tumors both malignant, e.g. MPNST and benign, e.g. schwannoma, as well as liposarcoma and alveolar rhabdomyosarcoma.[59,60]

All SS cases in this cohort harbored the *SYT-SSX* fusion gene; in previous studies this had varied between 60-90% in FFPE material.[62,68] The 100% detection rate in our cohort may indicate an improvement of RNA extraction over the years, or a reflection of higher-quality RNA from FNA samples. While immunochemistry targeting the *SYT-SSX* fusion gene is implemented at our laboratory it is not widely available. A combination of multiple ICC/IHC could be used to diagnose SS, but molecular detection of *SYT-SSX* has proven to be a very sensitive and specific method for both diagnosing SS and rule out potential differential diagnosis.

4.2 PAPER II

CCCS is a tumor with considerable diagnostic challenges, and establishing a learning curve from clinical work is difficult as the tumor is exceedingly rare. None of the 5 patients in this cohort with FNA from primary tumors received a conclusive initial diagnosis of CCCS: 4 received an initial diagnosis of sarcomatous tumor of bone or chondroid origin and the 5th a diagnosis of chondroid tumor NOS. The 6th patient received an initial diagnosis of chondroblastoma, and had multiple local recurrences and metastasis in the following two decades. Diagnosis on biopsy material also proved challenging; one primary tumor was diagnosed as osteosarcoma and another as chondroblastoma, and both were changed to CCCS following external consultation.

In the largest cohort of CCCS we describe the tumors' cytomorphologic and radiologic features, as well as patient demographics. We found the cytomorphology of CCCS to be homogenous with low- to intermediate cellularity and background chondroid matrix. Tumor cells were clustered or single with prominent nucleoli, clear, vacuolated cytoplasm and generally low grade cellular atypia. The finds are similar to previous studies of CCCS and differs from those of chondroblastoma (Fig. 16).[104,138]

Radiology is a fundamental cornerstone in evaluating bone tumors. In our cohort radiology was available for 3 patients, all with osteolytic epimetaphyseal lesions with either a sclerotic

border or a soft-tissue component. In our limited material CCCS cannot be conclusively distinguished from chondroblastoma on radiology alone.

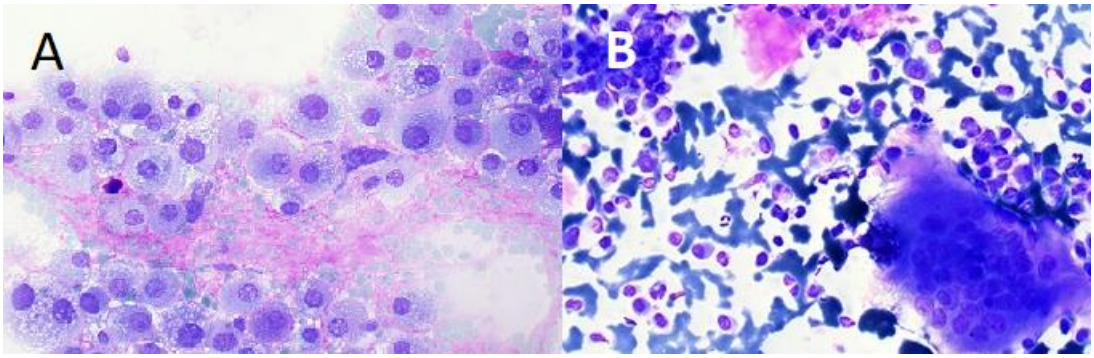

Figure 16. Cytomorphology (MGG, 400x) of A) CCCS showing rounded tumor cells with clear cytoplasm with a chondroid background matrix versus B) chondroblastoma showing cells with less cytoplasm and classic "chicken wire" calcification background. Figure 15B adapted from Zhang, Diagnostic Cytopathology, 2021.

Our literature review found both CCCS and chondroblastoma to skew male-dominant. A histogram was plotted for available patient ages and the age at diagnosis was significantly higher for CCCS than chondroblastoma (Mann-Whitney U, $p < 0.0001$). CCCS was more common in patients > 25 years of age and chondroblastoma in patients < 25 years of age, but there was no clear cutoff at which one could dismiss one diagnosis.

4.3 PAPER III

In the hitherto largest cohort on this topic we aimed to validate the prognostic value of *TERT* promoter mutation in conventional CS by sequencing tumors from 190 patients. The previously characterized -124 C>T *TERT* promoter mutation was detected in 45% of the tumors. In line with previous findings[89] a positive mutation status was significantly associated with higher tumor grade ($p < 0{,}0001$) and shorter MFS ($p < 0{,}001$) as well as DFS and OS (Fig. 17).

Detailed clinical data was available in 36 patients with metastatic disease. *TERT* promoter mutated tumors were associated with a more aggressive course of disease and was identified in 11/13 patients with metastasis at diagnosis or within 6 months of primary surgery, as well as 3/4 patients with a disease transformation (late but multiple metastasis within a short time frame).

We were more successful in identifying *TERT* promoter mutations by sequencing select punch-biopsies of high-grade areas compared to whole tissue sections. In addition, a subset of patients with multiple samples sequenced showed a higher percentage of identified mutations in comparison to those with one sequenced sample, suggesting a significant heterogeneity within the tumors with mutated subclonal populations.

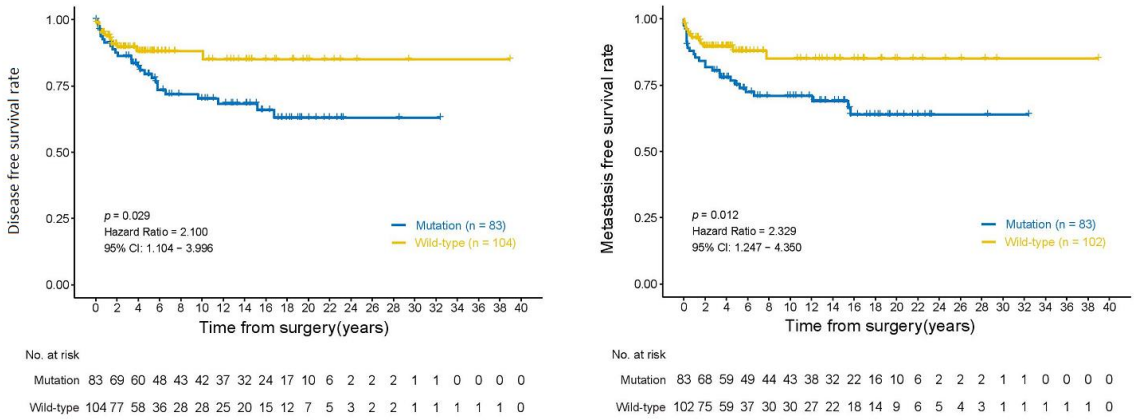

Figure 17. Kaplan-Meier survival analysis of DFS and MFS showing a clear separation between CS patients with *TERT* wild-type and CS patients with mutated *TERT*. Adapted from Zhang, Modern Pathology, 2021.

Six patients with metastatic disease had altering *TERT* promoter status between tumors. While the sample size is limited there was a tendency for shorter survival in those with wild-type primary tumor and mutated metastasis compared to those with wild-type metastasis in longitudinal data. The presence of both a *TERT* wild-type and a mutated metastasis was found in one patient with multiple metastatic tumors. These data suggest that CS are capable of developing by branching evolution, and that subclonal *TERT* promoter mutations could be a central event in tumor progression in CS.

Univariate analysis showed *TERT* mutation status, tumor size and tumor grade to be independent prognostic factors for MFS and could be adapted as a nomogram for predicting MFS in CS patients. A calibration plot of predicted probability and actual probability was well-fitting on internal and external validation.

In clinical routine pathology *TERT* promoter mutation status could be useful to identify high-risk patients with increased risk of metastasis. It could also be used for verifying suspected high-grade areas where morphology is inconclusive, or if any suspicious cell populations are neoplastic, rather than tumor associated reactive spindle cell areas frequently observed in CS. Tumor heterogeneity should be considered when selecting areas for *TERT* promoter mutation analysis.

4.4 PAPER IV:

The prognostic value of PD-L1 in sarcomas is still a controversial subject. In this paper we described PD-L1 status in tumor cells and its prognostic impact in a cohort of 230 tumors from 214 patients with CS, LS and UPS using approved IHC assays for diagnostic use. Cutoffs for statistical analysis were set at 0% (negative) and ≥1% (positive) for CS, and 0% (none), <5% (low), 5-9% (intermediate) and ≥10% (high) for LS and UPS.

While there was a strong concordance in the number of positive tumors between the two assays the SP263 assay generally stained a higher % of positive tumor and immune cells in comparison to SP142, which has also been described in previous publications,[139,140] and the two assays cannot be used interchangeably.

PD-L1 immunoreactivity was negative in the majority of CS. Immunoreactivity was more common in grade 3 or dedifferentiated CS, and not observed in any grade 2 CS. We found no significant correlation between clinical outcome and PD-L1 immunoreactivity. A non-significant shorter OS was observed in those with positive tumors, and likely due to high tumor grade.

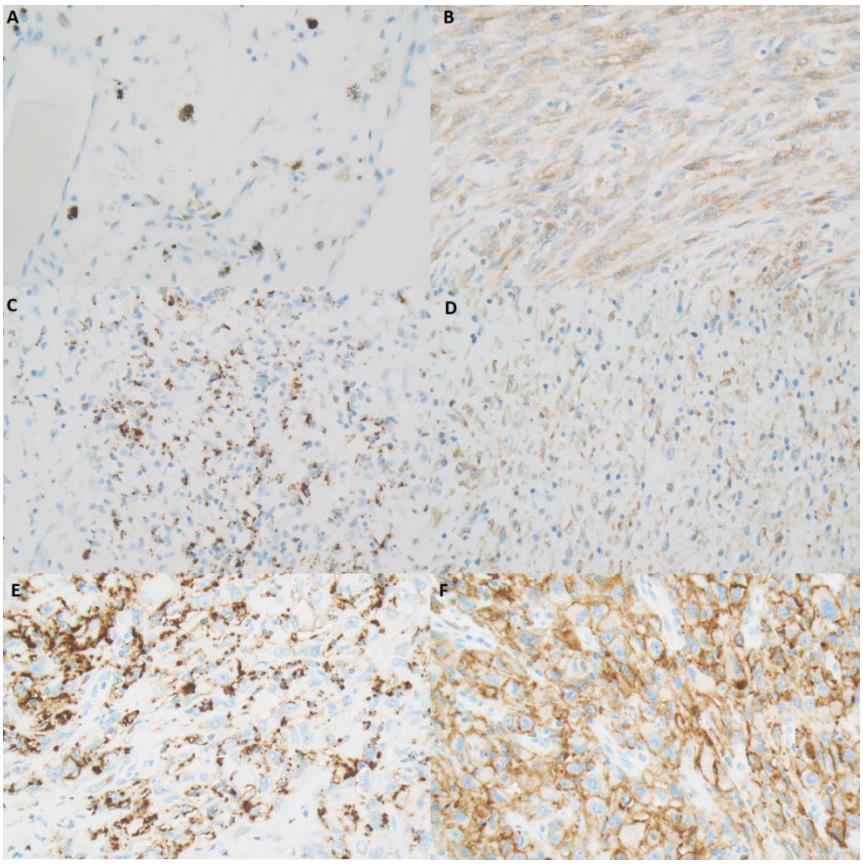

Figure 18. PD-L1 immunoreactivity in tumor cells at 200x. A) CS, SP142. B) CS, SP263. C) LS, SP142. D) LS, SP263. E) UPS, SP142, F) UPS, SP263.

PD-L1 immunoreactivity in LS was generally none-low with SP142 and low-intermediate with SP263. While there was a significantly ($p < 0,05$) shorter MFS and OS in PD-L1 positive cases using the SP142 assay it was not seen in the SP263 assay, nor with univariate analysis.

PD-L1 immunoreactivity in UPS was more common ($p < 0,05$) compared to LS or CS, which is in line with previous studies.[127,141] In phase II trials such as SARC028 and Alliance A091401 PD-L1 immunoreactivity, and treatment response, was more likely seen in UPS,[131,132] and international guidelines recommend that ICIs may be used to treat UPS patients with disseminated disease where first-line treatment has failed.[8,142] We found no significant correlation between clinical outcome and PD-L1 immunoreactivity.

In addition, we extracted gene expression data from The Cancer Genome Atlas (TCGA) on LS and UPS, and found no significant difference in MFS or OS depending on PD-L1 status.

A subset of tumors in all three subtypes had high (>50%) PD-L1 expression in both TC and IC using the SP263 assay. Satisfactory treatment response with PD-1/PD-L1 inhibitors in some patients have been shown in previous studies, and establishing ICI biomarkers could be of value to some sarcoma patients.

As there is a lack of data regarding clinical response to PD-1/PD-L1 inhibitors it's currently not possible to establish a clear cutoff value for PD-L1 immunoreactivity in CS, LS and UPS. While we have not found a usefulness for PD-L1 as a prognostic marker in this study, it must be assessed in the context of therapy response in order to truly value its utility.

5. CONCLUSIONS

Paper I

Synovial sarcomas have distinct cytomorphologic features. As molecular genetic analysis for *SYT-SSX* is essential for diagnosing SS using FNA it should be implemented in laboratories which perform FNA diagnostics of soft tissue tumors on a regular basis.

Paper II

Clear cell chondrosarcomas have distinct cytomorphologic features and should be considered as a possible differential diagnosis especially in older adolescents (>25 years) with suspicion of chondroblastoma. FNA is a useful complement, as radiology and patient age cannot conclusively distinguish CCCS from chondroblastoma.

Paper III

TERT promoter mutation in chondrosarcomas is associated with reduced OS and MFS and seems to be a central event in disease progression. Chondrosarcomas are capable of branching evolution, and patients with disseminated disease and positive *TERT* mutation status had a more aggressive course of disease. *TERT* promoter mutation is easily analyzed and has potential as a prognostic marker in chondrosarcomas.

Paper IV

There was no significant association between PD-L1 immunoreactivity and prognosis in CS, LS or UPS. High (>50%) PD-L1 immunoreactivity in TC with the SP263 assay was seen in a small subset of patients, which could be a group to investigate for targeted treatment with PD-1/PD-L1 inhibitors. There is still a lack of clinical data regarding PD-L1/PD-1 status and therapy response in sarcomas, and as such it is not currently possible to establish any clear cut-off values in TC and IC immunoreactivity. PD-L1 immunohistochemistry is established at many laboratories; for sarcoma patients with metastatic disease and unsatisfactory therapy response from first-line treatment PD-L1 could be considered as a potential prognostic biomarker.

6. FUTURE PERSPECTIVES

6.1 Advances in genetic sequencing

The tremendous advances in genetic sequencing have made the techniques both widely available and economically feasible. The cost of whole-genome sequencing with Next Generation Sequencing (NGS) has decreased massively compared to a decade ago, and more extensive sequencing is increasingly performed as a part of the clinical workup. With techniques such as droplet digital PCR (ddPCR) not only the presence, but also the quantity, of a mutation can be detected. Many are visualizing the opportunity to predict the risk for an individual patient, or to find a personalized treatment regime, on a much more detailed level than ever before. The challenge is no longer acquiring the genetic data, but to interpret it and draw the correct conclusion from the vast amounts of provided information.

6.2 Prognostic and predictive markers

Cancer is ultimately a genetic disease, and the search of accurate prognostic and predictive markers continues. There are challenges: sarcomas inherently have different properties compared to carcinomas, and each subtype may differ from another. A marker which is useful for many carcinomas – for example PD-L1 – may not have a significant predictive value for many sarcomas.

Another major challenge is tumor heterogeneity, both intra-tumoral and in primary versus metastatic tumors of the same subtype. We have seen that *TERT* mutation status is heterogeneous within CS between low- and high-grade areas within the same lesion. The immune system is not static but dynamic and ever changing, including PD-1/PD-L1 expression, which is also discordant in primary vs. metastasis.[143–145] Finding the true prognostic and predictive value of a biomarker may require assessment of more than one area of the tumor, or in cases with disseminated disease, of more than one tumor from the patient.

There are more potential biomarkers to explore, such as tumor mutational burden (TMB), which has shown to be an independent predictor for response to ICIs in certain types of carcinomas.[146,147] Sarcomas, however, generally tend to have a lower TMB on the spectrum of malignancies.[146,148] But quantifying the mutational burden in certain sarcomas may still have value; one potential is the *TERT* promoter mutation where one could investigate if the quantity of mutated alleles have any correlation with tumor grade and prognosis. In addition, more comprehensive sequencing of sarcomas could reveal e.g. previously unknown fusion genes, which could have a prognostic impact as well as improve diagnostic accuracy.

6.3 The future of pathology

As with many other medical diagnostic methods pathology is evolving. Tumor types are increasingly defined from not only their morphology but also their genetic setup. Genetic sequencing is commonly included in the initial diagnostic workup, and the use of whole genome sequencing with NGS in the clinical setting is on the rise. While cyto- and histomorphology will remain a vital cornerstone we move steadily toward molecular pathology as the genetic properties of cancer play an ever larger role in diagnostics and clinical decision making.

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