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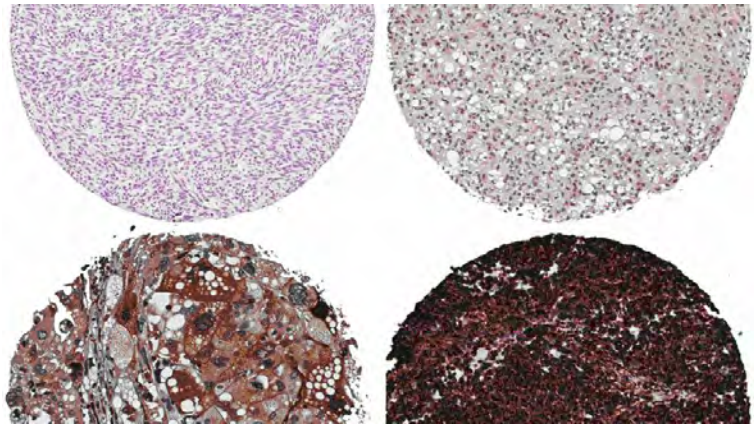
Molecular prognostic markers in soft tissue sarcomas

A retrospective tissue microarray based study with emphasis on proteins associated with tumor growth and differentiation



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LIST OF PAPERS

I. **Valkov A, Sorbye S, Kilvaer TK, Donnem T, Smeland E, Bremnes RM, Busund LT**. The Prognostic Impact of TGF-β1, Fascin, NF-κB and PKC-ζ Expression in Soft Tissue Sarcomas. *PLoS One.* 2011 Mar 3;6(3):e17507, doi:10.1371/journal.pone.0017507

II. **Valkov A, Sorbye S, Kilvaer TK, Donnem T, Smeland E, Bremnes RM, Busund LT**. Estrogen receptor and progesterone receptor are prognostic factors in soft tissue sarcomas. *Int J Oncol.* 2011 Apr;38(4):1031-40, doi:10.3892/ijo.2011

III. **Valkov A, Kilvaer TK, Sorbye S, Donnem T, Smeland E, Bremnes RM, Busund LT.** The prognostic impact of Akt isoforms, PI3K and PTEN related to female steroid hormone receptors in soft tissue sarcomas. *J Transl Med*, 2011 Nov 22;9:200, doi:10.1186/1479-5876-9-200

LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
BAD	Bcl-2-associated death promoter
CDK	Cyclin-dependent kinase
CD	Cluster of differentiation
CK	Cytokeratin
CT	Computer tomography
DAB	Diaminobenzydin
DAKO	Dakota Manufacturing Company
DFSP	Dermatofibrosarcoma protuburens
DSS	Disease-specific survival
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
ESMO	European Society for Medical Oncology
EWSR1-ETS	Ewing sarcoma breakpoint region 1 - E-twenty six
FAP	Familial adenomatous polyposis
FISH	Fluorescent *in situ* hybridization
FKHR	Forkhead homolog 1 in rhabdomyosarcoma
FNCLCC	Fédération Nationale des Centres de Lutte Contre le Cancer
GSK3	Glycogen synthase kinase 3
Gy	Grey
HHV8	Human herpes virus
HR	Hazard ratio
IMRT	Intensity-modulated radiation therapy
IHC	Immunohistochemistry
Mab	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MFS	Metastasis free survival
MPNST	Malignant peripheral nerve sheath tumor
MRI	Magnetic resonance imaging
MSKCC	Memorial Sloan-Kettering Cancer Center
mTOR	Mammalian target of rapamycin
m TORC	Mammalian target of rapamycin complex 2
NCI	National Cancer Institute
NF-κB	Nuclear factor-kappa B
Non-GIST STS	Non-gastrointestinal stromal tumor soft-tissue sarcoma
OS	Overall survival
p-Akt Ser^{473}	Akt phosphorylated on serin 473
p-Akt Thr^{308}	Akt phosphorylated on threonin 308
Par6	Partitioning protein 6
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PET	Positron emission transmission
PgR	Progesterone receptor
PI3K	Phosphatidylinositol 3-kinase

PIP_3	Phosphatidylinositol trisphosphate
PKC	Protein-kinase C
PNET	Peripheral neuroectodermal tumor
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
RNA	Ribonucleic acid
SIN	Size, Invasion and Necrosis
SMA	Smooth muscle actin
SPSS	Statistical Package for the Social Sciences
SSG	Scandinavian sarcoma group
STS	Soft-tissue sarcoma
TGF-β	Transforming growth factor beta
TMA	Tissue microarray
TNGM	Tumor, nodule, grade and metastasis
UICC	Union Internationale Contre le Cancer
WHO	World health organization

1. BACKGROUND

1.1. Soft-tissue sarcoma

1.1.1. Epidemiology and incidence

Soft-tissue sarcomas (STSs) are malignant tumors arising from nonepithelial extraskeletal tissue of body except from the reticuloendothelial system, glia and supporting tissue of various parenchymal organs [1]. They comprise a group of more than 50 histological entities [2]. The term sarcoma does not, unfortunately, indicate the likehood or rapidity of metastasis. It is therefore important to further qualify that as "well differentiated" or "poor differentiated" based on histological features [1].

STSs are rare tumors with an estimated annual incidence around 30 new cases per 1,000,000 of population annually [3-6]. They comprise only 0.5-1% of all cancer types [7]. In children, the incidence of STS is relatively higher, at 1-3%, but cancer is not a common disease of childhood. Like other malignancies, STS becomes more common with increasing age, with 65 years being the median age of diagnosis [4,5,8]. The age-related incidence vary among the different histological subtypes, with embryonal rhabdomyosarcoma occurring almost exclusively in children, synovial sarcoma affecting young adults, while pleomorphic high grade sarcoma, liposarcoma and leiomyosarcoma dominates in the elderly (Figure 1) [1].

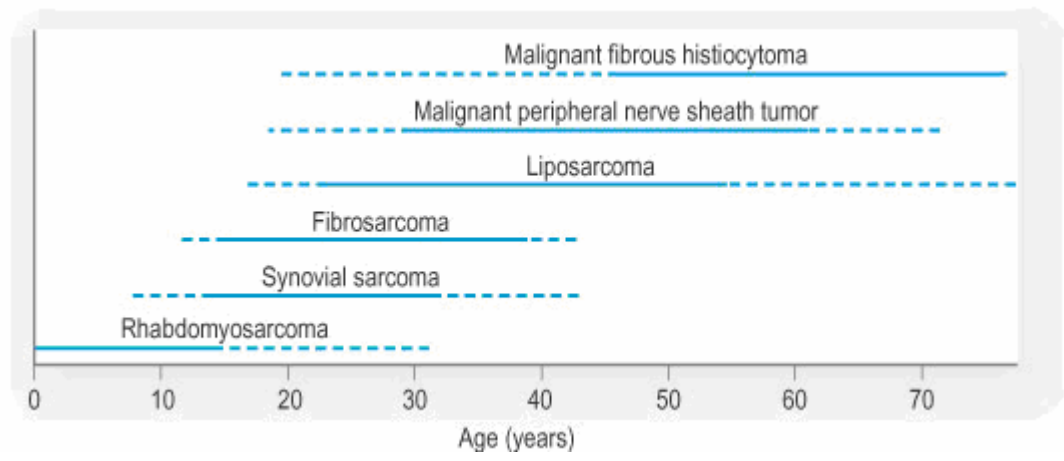


Figure 1. Approximate relation of age to incidence of various types of sarcoma. *From Weiss SW, Goldblum R: Enzinger & Weis's Soft Tissue Tumors, 5th edn. Philadelphia: Mosby, Elseiver Inc; 2008[1] Permission obtained from Elseiver Inc.*

The age-adjusted incidence rates of STSs in Norway have shown a slight increase the last 50 years since registration started (Figure 2), recorded at 3,2 in 2009 [5].

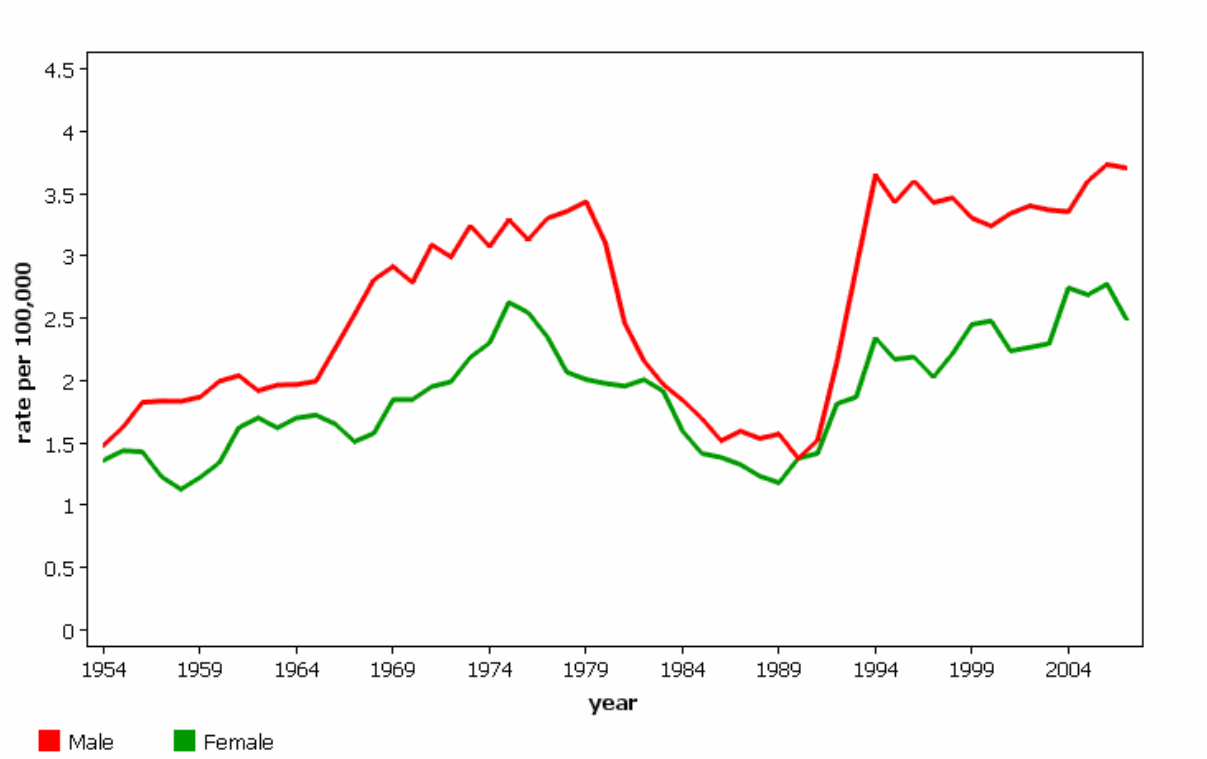


Figure 2. Age-adjusted incidence rates of STSs in Norway, 1954 to 2004. *From NORDCAN: Cancer Incidence, Mortality, Prevalence and Survival in the Nordic Countries, Version 5.0. Association of the Nordic Cancer Registries[8]. Permission obtained from The Cancer Registry of Norway.*

For the Russian Federation, this figure was 2,3 for 2007, but specifically in Arkhangelsk region, where our research material was partly gathered from, it was 3,6 per 100,000 [4].

The incidence of STS is increasing with increasing age and is approximately the same for male and female patients with the exception of a drop in incidence in females during their 60-70 (Figure 3).

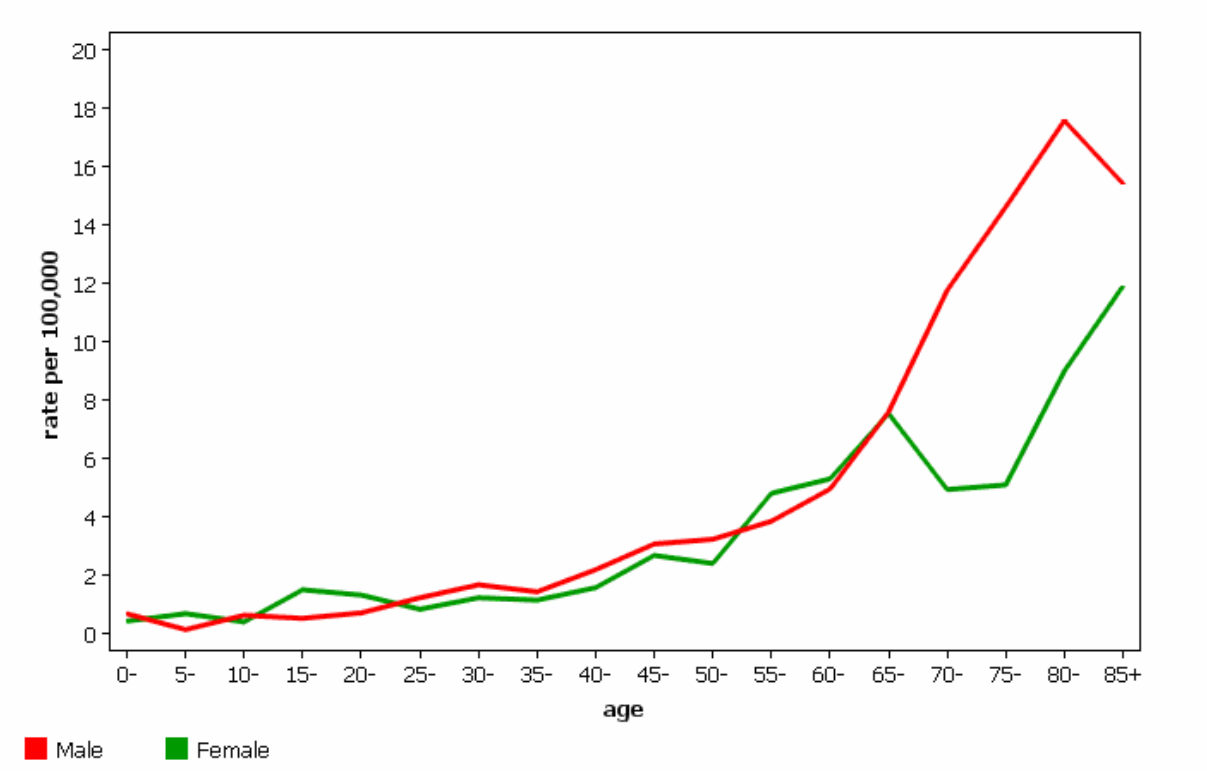


Figure 3. Age-specific incidence rates of STSs in Norway per 100 000, 1954 to 2004. *From NORDCAN: Cancer Incidence, Mortality, Prevalence and Survival in the Nordic Countries, Version 5.0. Association of the Nordic Cancer Registries[8]. Permission obtained from The Cancer Registry of Norway.*

The mortality of STSs remains high at 30-40%, making STS one of the more unfavorable forms of cancer to contract [3-5]. In Norway, the survival has gradually increased in the last 50 years from 30-40% five-year survival during the sixties to a much better 60-70% survival after 1990 (Figure 4). Some of this survival benefit has come from new and better treatment protocols for childhood STSs giving the younger age-groups a better overall prognosis [9]. Even so, the prognosis in the adult population has also increased, maybe because of better and earlier diagnosis through increased awareness in the population and novel diagnostic methods and probably due to implementation of new treatment protocols (Figure 4) [10].

Figure 4. Age-standardized relative survival of STSs in Norway, all ages. *From NORDCAN: Cancer Incidence, Mortality, Prevalence and Survival in the Nordic Countries, Version 5.0. Association of the Nordic Cancer Registries[8]. Permission obtained from The Cancer Registry of Norway.*

1.1.2. Histopathology

STSs are usually classified according to their resemblance to normal mature mesenchymal tissues [1]. However, high grade lesions lose gradually resemblance with the tissue of derivation. Moreover, some sarcomas have no obvious normal counterpart and belong therefore to a class of tumors of uncertain differentiation. Taking in consideration the rarity and variability of sarcomas, they often represent a diagnostic challenge for a pathologist, who in many cases have to give a pathologic diagnosis based on a marginally sized biopsy [2].

According to the current World Health Organization's classification of tumors of soft tissue and bone, there are nine main groups of STSs (Appendix I) [2]. High grade pleomorphic sarcoma, liposarcoma, leiomyosarcoma, synovial sarcoma and malignant peripheral nerve sheet tumors (MPNST) are the most common STS subtypes comprising approximately 75 percent of the annual STS incidence [2,7,8]. Some examples of major STS types are demonstrated in Figure 5.

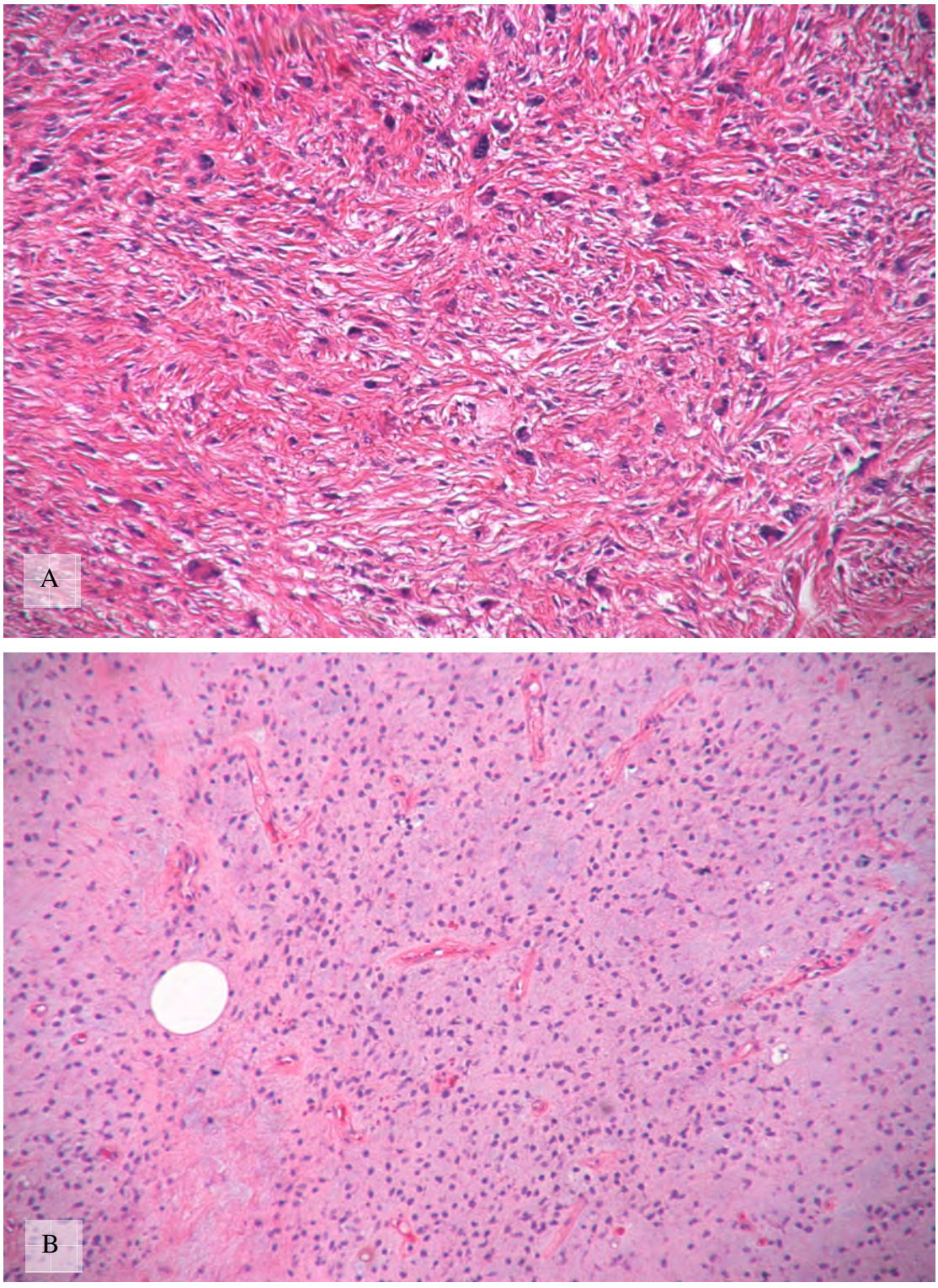
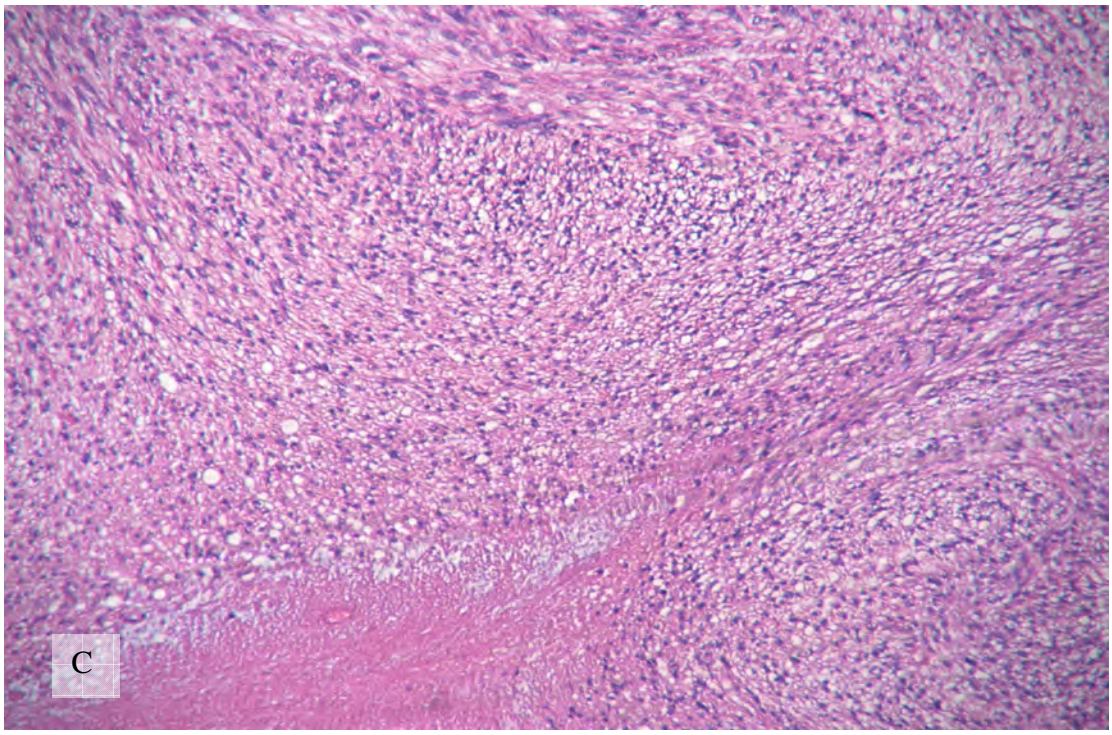


Figure 5. Examples of major STS types. A, Undifferentiated pleomorphic sarcoma; B, Round cell/myxoid liposarcoma. *The present study material, unpublished data. Valkov A.*



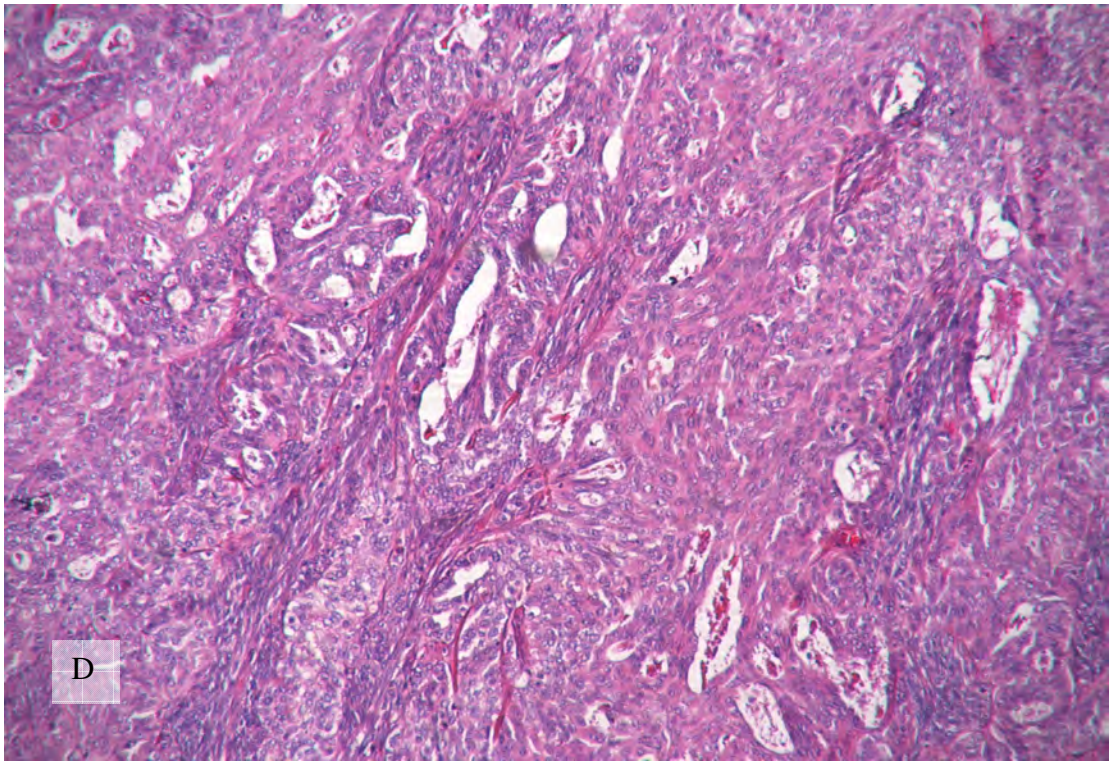
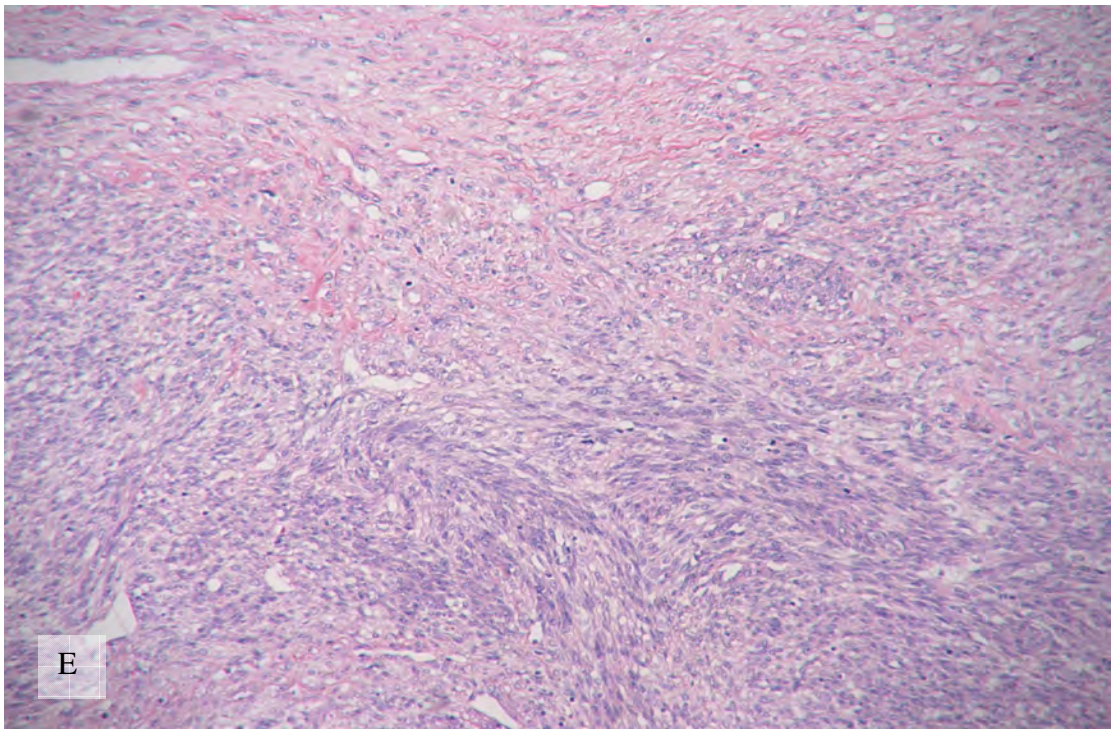


Figure 5 (continued). Examples of major STS types. C, Leiomyosarcoma; D, Biphasic synovial sarcoma. *The present study material, unpublished data. Valkov A.*



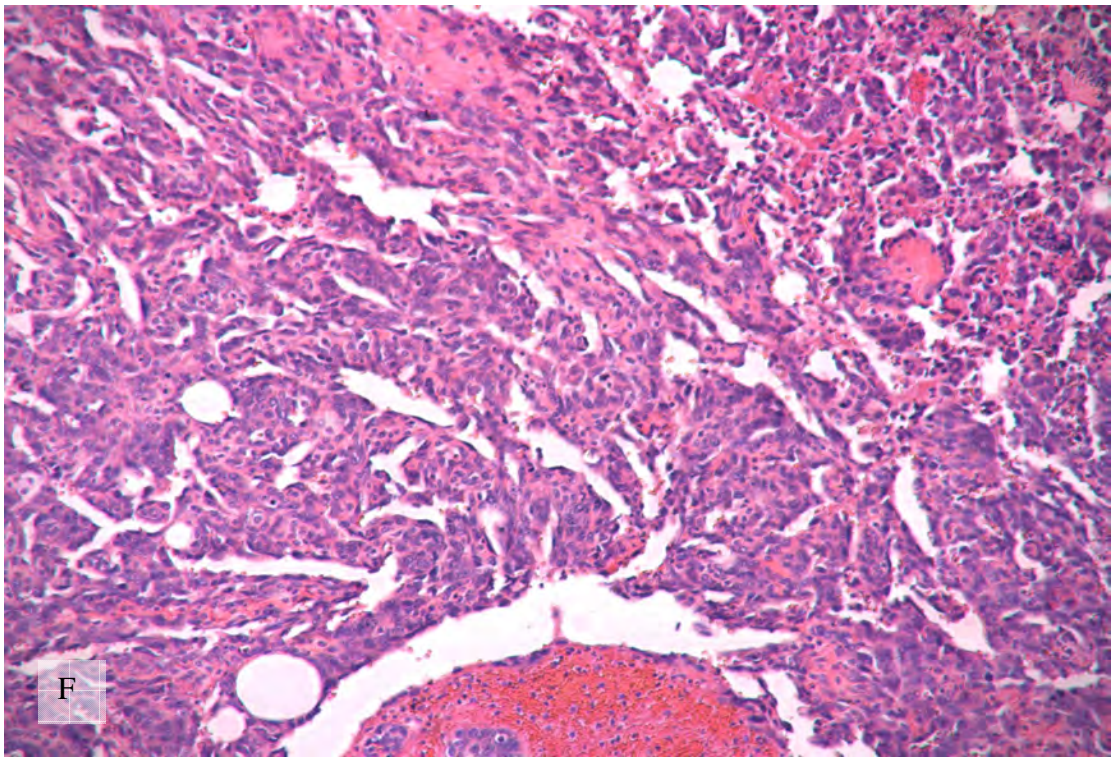


Figure 5 (continued). Examples of major STS types. E, Malignant peripheral nerve sheath tumor (MPNST) and F, Angiosarcoma. *The present study material, unpublished data. Valkov A.*

When conducting studies on STS it appears that some specific sarcomas differ greatly from others and should be excluded/investigated on their own. This is particularly the case for skin-sarcomas, gastrointestinal stroma tumors (GISTs), embryonal rhabdomyosarcoma in children and Ewing/peripheral neuroectodermal tumor (PNET) sarcomas as these have their own tailored treatments [9,11,12].

1.1.3. Pathogenesis

The pathogenesis of most STS is still unknown [1]. Nevertheless, there are some recognized causes, which are listed below. It is important to mark that, unlike carcinomas, an origin of sarcomas from benign soft tissue tumors is exceedingly rare; virtually the only exclusion is MPNST, frequently arising in neurofibroma in patients with Von Recklinghausen's neurofibromatosis [13].

1.1.3.1. Hereditary sarcoma

A number of syndromes are associated with STS development. Syndromes able to induce STSs are most often due to mutations in tumor suppressor- , growth factor- and growth factor receptor genes and translocations forming new potent fusion-genes and proteins [14]. The list of most common cancer syndromes leading to STS includes Li Fraumeni, neurofibromatosis type I (Von Recklinghausen's) and type II, familial adenomatous polyposis (FAP)/Gardner, Retinoblastoma, Werner, Lynch syndromes and tuberous sclerosis/Burneville disease among others [14]. This list will undoubtedly lengthen with increasing understanding of the molecular underpinnings of mesenchymal neoplasia [1].

1.1.3.2. Environmental factors

Among the environmental factors implicated in the development of STSs, trauma is most frequently mentioned. It is now clear, however, that trauma often seems to be an event that merely calls attention to the underlying neoplasm, though there are relatively many well documented reports of STS plainly linked to trauma [1,15]. Other factors include asbestosis relating to mesotheliomas [16], uptake of chlorophenols, especially belonging to dioxin group [15,17,18], vinyl chloride, clearly associated with hepatic angiosarcoma [19,20], and radiation

exposure resulting in rare cases of postradiation sarcoma, which in the majority of cases is represented by pleomorphic undifferentiated sarcoma [21]. In addition, there is an increased risk of subsequent sarcoma in survivors of childhood cancers such as leukemia, retinoblastoma, Wilms's tumor, Hodgkin's lymphoma and neuroblastoma [22,23].

1.1.3.3. Oncogenic viruses and immunologic factors

Kaposi's sarcoma is closely linked to infection with human herpes virus 8 (HHV8). However, very few healthy individuals infected with HHV8 develop Kaposi's sarcoma, but in immunocompromised individuals many of those with previous HHV8 infection will develop Kaposi's sarcoma [24,25]. There is also a large body of literature, supporting the role of Epstein-Barr virus in the pathogenesis of leiomyosarcoma in patients with suppressed immunity [26,27]. In Stewart-Treves syndrome, angiosarcomas can arise in the setting of chronic lymphedema secondary to radical mastectomy [28,29], which is often explained by the loss of regional immune surveillance.

1.1.4. Diagnostics

Most patients with suspected sarcoma present with a growing, painless extremity lump. Pain is reported in only about one third of cases. Because of mostly painless presentation, the diagnosis of STS is often delayed. Late diagnosis of patients with retroperitoneal sarcomas is especially common because of the large retroperitoneal space, generally slow growth rate, and the tendency of sarcomas to gradually displace rather than to invade adjacent tissues [30].

In Scandinavia, patients presenting with a superficial tumor or lump >5 cm in greatest diameter or deep tumor irrespective of size, should be referred to a sarcoma center as soon as possible and prior to any surgical intervention [31]. All patients with suspected sarcoma are subjected to imaging procedures in order to establish the extent of the tumor and to determine the type of surgical procedure needed. Both normal skeletal x-ray, CT and MRI are used, although MRI gives the best impression of the soft tissues and therefore is the imaging modality of choice [32,33]. In recent years positron emission tomography (PET) scans have become popular and its use has been implemented in the diagnostics for many types of cancer. The role of PET in STS diagnostics is yet to be elucidated and its use is recommended only as

a supplement to MRI [34]. PET-scans are as of today more efficiently used to detect local recurrence after the completed therapy [34].

The necessity of pretreatment biopsy is a topic of discussion due to the risk of possible tumor contamination with further possible recurrence in the needle track after a core biopsy [35]. In Norway, a biopsy is recommended only in cases where initial wide resection is not feasible. However this point is not universally shared, since such recurrences are extremely rare [1]. The biopsy is used to determine the histological type and malignancy grade, and together with imaging procedures also the stage of the tumor.

1.1.5. Prognostic factors

1.1.5.1. Grading

Since the first grading system for sarcomas was introduced by Broders et al. in 1939, a number of systems have been utilized in sarcoma diagnostics [36]. Several parameters have been used to grade sarcomas, such as cellular pleomorphism, cellularity, mitotic index, vascular invasion, tumor necrosis, surgical site, nuclear atypia, histologic type and subtype, tumor size and tumor differentiation [37,38]. The WHO manual on the Pathology and Genetics of Tumors of Soft Tissues and Bone recognizes two grading systems used on STS; the FNCLCC and the NCI grading systems, respectively [2].

The FNCLCC grading system, reviewed in Coindre 2006 [37], is calculated from tumor differentiation, mitotic count and tumor necrosis. Tumor differentiation and mitotic count are given a score from 1-3 and tumor necrosis is scored as 0-2 [1,2,36-39]. The histologic grade is derived from the total score with 2-3 being grade 1, 4-5 being grade 2 and 6-8 being grade 3, Table 1.

Table 1. Definitions of grading parameters for the FNCLCC system.

Parameter	Criterion
Tumor differentiation	
Score 1	Sarcoma closely resembling normal adult mesenchymal tissue (e.g., well-differentiated liposarcoma)
Score 2	Sarcomas for which histologic typing is certain (e.g., myxoid liposarcoma)
Score 3	Embryonal and undifferentiated sarcomas; sarcoma of uncertain type
Mitosis count	
Score 1	0–9/10 HPF
Score 2	10–19/10 HPF
Score 3	≥20/10 HPF
Tumor necrosis (microscopic)	
Score 0	No necrosis
Score 1	≤50% tumor necrosis
Score 2	>50% tumor necrosis
Histologic grade	
Grade 1	Total score 2, 3
Grade 2	Total score 4, 5
Grade 3	Total score 6, 7, 8

From *Weiss SW, Goldblum R: Enzinger & Weis's Soft Tissue Tumors, 5th edn. Philadelphia: Mosby, Elseiver Inc; 2008[1] Permission obtained from Elseiver Inc.*

The NCI grade is derived from the histologic type or subtype and histopathological parameters including necrosis (the most important), cellularity, pleomorphism and mitosis as described by Costa et al. in 1984 and modified in 1990 [40,41].

In a comparative study of 410 patients diagnosed with STS, Guillou et al. found the FNCLCC grading system to be marginally better at predicting metastasis and disease-specific survival (DSS) compared to the NCI grading system [1,38]. However, both systems yielded prognostic groups and are recognized in the WHO manual as suitable for grading STSs [2].

In addition to these well recognized systems both two-, and four-tiered (as in case of SSG) systems exist [38]. In Table 2, a proposed conversion between two-, three- and four-tiered grading systems for STS is presented.

Table 2: Conversion table between different grading systems for Soft Tissue Sarcomas

Two-tiered system	Three-tiered systems	Four-tiered systems
Low grade	**Grade 1**	**Grade 1**
		Grade 2
High grade	**Grade 2**	**Grade 3**
	Grade 3	**Grade 4**

Adapted from *The WHO Classification of Tumors: Pathology and Genetics oft Tumors of Soft Tissue and Bone [2] Permission obtained from WHO IARC.*

The three-tiered systems are considered most suitable for predicting survival and likelihood of treatment response, since they are able to predict the behavior of both low-grade, intermediate-grade and high-grade tumors, which seems to be well defined categories of STSs. Nevertheless, the recently proposed new system, termed SIN by the SSG group, anticipated promising binary stratification which would help to simplify treatment strategy scheme [38,42]. The system uses three factors, namely Size, vascular Invasion and Necrosis in a dichotomous fashion (size < or > 8 cm, and +/– vascular invasion and necrosis). The low-risk group (score 0-1) had an 81% 5-year survival compared to the high-risk group (score 2-3) with a 5-year survival of 32%.

1.1.5.2. Staging

STSs are typically staged according to the tumor, nodule, grade and metastasis (TNGM) system developed by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) as devised by Russel et al. in 1977 and later revised and currently published in the AJCC Cancer Staging Manual 7th edition [43,44]. The TNGM system for STSs includes tumor size, nodal metastasis, malignancy grade and distant metastasis to give a stage ranging from I-IV. The system is designed to include two-, three- and four-tiered grading systems using a conversion table (Table 1). Table 3 summarizes the current TNGM stages based on grades derived from a three-tiered grading system.

Table 3: Clinical staging and survival of Soft Tissue Sarcoma according to the tumor, node, grade and metastasis system

Stage	Tumor	Node	Metastasis	Grade	Definition
Ia	**T1a**	**N0**	**M0**	**G1, GX**	**T1: Tumor ≤5cm in greatest**
	T1b	**N0**	**M0**	**G1, GX**	**dimension**
Ib	**T2a**	**N0**	**M0**	**G1, GX**	**T1a: Superficial tumor**
	T2b	**N0**	**M0**	**G1, GX**	**T1b: Deep tumor**
IIa	**T1a**	**N0**	**M0**	**G2, G3**	**T2: Tumor>5cm in greatest**
	T1b	**N0**	**M0**	**G2, G3**	**dimension**
IIb	**T2a**	**N0**	**M0**	**G2**	**T2a: Superficial tumor**
	T2b	**N0**	**M0**	**G2**	**T2b: Deep tumor**
III	**T2a, T2b**	**N0**	**M0**	**G3**	**N1: Regional lymph node**
	Any T	**N1**	**M0**	**Any G**	**metastasis**
IV	**Any T**	**Any N**	**M1**	**Any G**	**M1: Distant metastsis**
					G: Histological grade

Adapted from *AJCC: Soft tissue sarcoma. In: Edge SB, Byrd DR, Compton CC, et al., eds.: AJCC Cancer Staging Manual. 7th ed. New York, NY: Springer, 2010, pp 291-8.[44] Permission obtained from Springer.*

In 2002, Kattan et al. published the Memorial Sloan-Kettering Cancer Center (MSKCC) nomogram for 12-year sarcoma-specific death in which they utilized a subset of independent prognostic markers to predict the clinical cancer development [45,46]. This approach has later been adapted for several clinical situations (pre-/post-operative, after recurrence etc.) and for specific subsets of patients (specific sites and histology etc.) [47-49]. If developed and used correctly, these nomograms seem to be better able to predict the clinical course of the individual patient than the conventional staging systems [50].

1.1.5.3. Vascular invasion in STS

Vascular invasion represents a well established prognostic factor in several tumor types, including malignant melanoma, papillary thyroid cancer, endometrial cancer, and testicular cancer. In STS, vascular invasion has repeatedly shown prognostic value [51-53], but it is generally not applied systematically in pathological evaluations. However, it is considered as one of three major prognostic factors in the SIN system, elaborated by SSG [42]. By SSG designation, vascular invasion can be defined as the presence of tumor cells

within any space having an obvious endothelial lining. Such tumor cells have to be either adherent to the luminal aspect of the vessel wall or, if free-floating, associated with adherent fibrin, red blood cells, or leukocytes, Figure 6A.

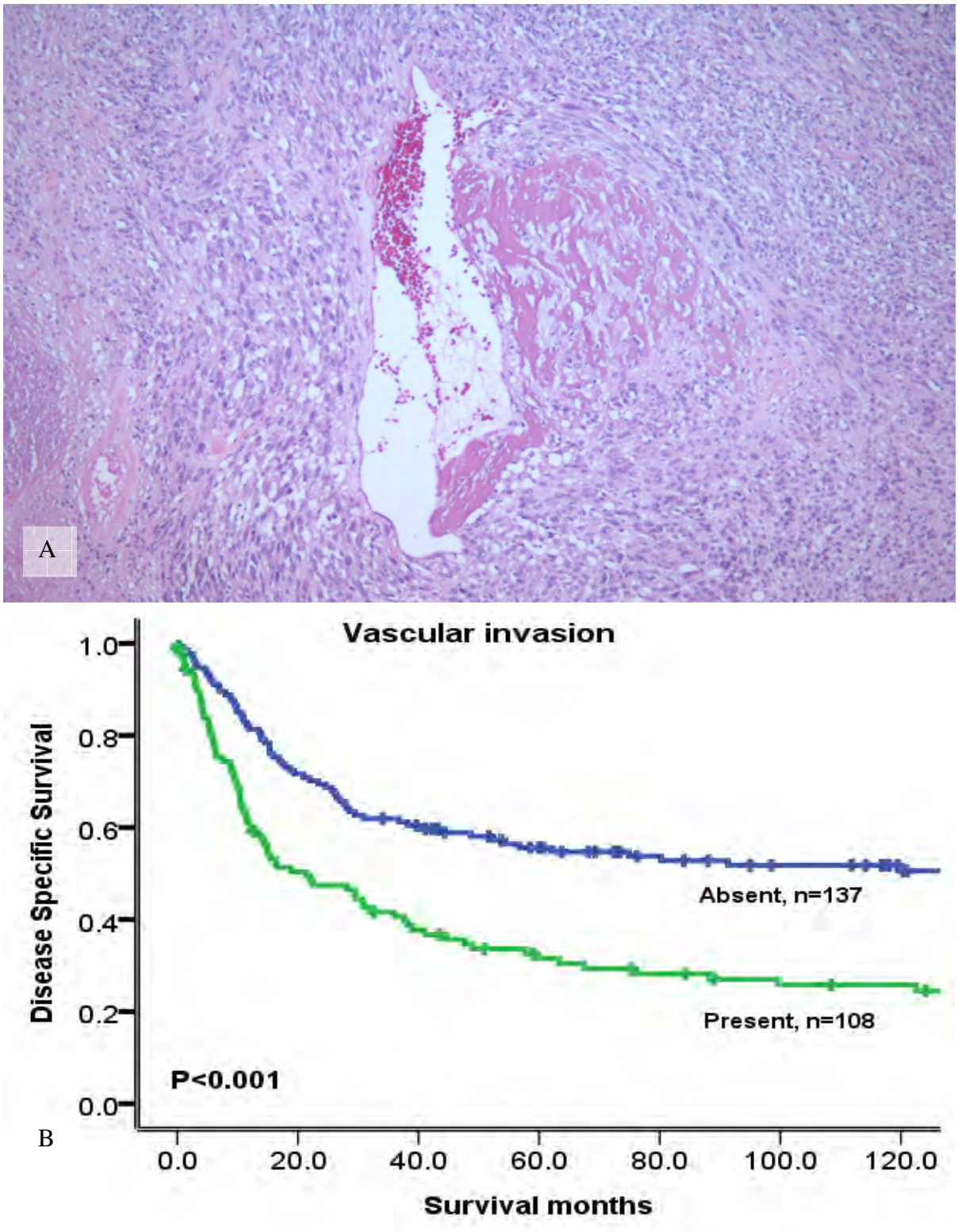


Figure 6. Vascular invasion in STS. A, Tumor cells have direct contact with blood stream in this example of malignant peripheral nerve sheath tumor. *The present study material, unpublished data. Valkov A.*

Consistently, our univariate analysis (Figure 6B) demonstrated that vascular invasion in STS correlated distinctly with higher disease specific mortality rates in the whole patients cohort. In the multivariate analysis, however, vascular invasion was not an independent significant variable as it co-varied with FNCLCC grade.

1.1.5.4. Other prognosticators in STS

Primary tumor location has been previously reported as an important prognostic marker in STS, with head and neck as well as retroperitoneal location greatly increasing STS-specific mortality [46,54].

Traditionally, the specific histopathologic subtype has been considered to be of secondary importance because of the common impression that individual histologic subtypes of comparable histologic grade behave similarly [54,55]. However, several reports have established the independent adverse prognostic significance of specific histologic subtypes [56,57]. Our data could not prove the observation that different high-grade sarcomas possess different biological behaviors.

Several studies suggest that margin positivity is a marker of adverse prognosis. For instance, the MSKCC group reported in 2002 [58] that a positive microscopic margin was associated with a 1.6-fold increase in sarcoma-related death. Our current data further support these observations; in the multivariable analysis, margin positivity was associated with a 2.9-fold increase in STS-related death ($P<0.001$).

Other clinical factors reported as a prognosticator in STS include local and distant recurrence [45], and nodal status [59,60].

Specific molecular prognostic markers may be particularly useful in our epoch of new insight into the molecular biology of cancer. The detection of such markers could potentially be based on high-throughput assays.

1.1.6. Treatment

1.1.6.1. Surgery

Surgery with wide resection margins is the main method of treatment of STS patients [33]. There is ample evidence suggesting that surgery should be planned and executed at a center with expertise in sarcoma surgery. Patients requiring re-excision, after poorly planned

surgery or after finding STS in lesions thought to be benign before surgery, have greater chance for recurrence than patients treated with primary definite surgery [61,62].

Classically, amputation was required for adequate resection margins in extremity STS but in the last twenty years limb-sparing surgery has emerged as a good alternative to amputation with significantly less morbidity for the patients as a result [63,64]. A recent study in extremity STS suggest that for tumors ≤ 3 cm in largest diameter surgery alone is adequate therapy [65]. For larger tumors, and small tumors with marginal or uncertain resection margins, combinations with radiotherapy and/or chemotherapy are common [33].

For trunk, head and neck, visceral and retroperitoneal locations, surgery with wide resection margins is also the treatment modality of choice. However, it is frequently a challenge to obtain wide resection margins for these locations and combinations with other treatment modalities are often warranted [66,67].

1.1.6.2. Chemotherapy

Pre- and postoperative chemotherapy is broadly used in treatment of bone sarcomas [68]. In STS its usage is a bit of a controversy as there have been conflicting reports regarding the effect of such treatment [69]. The "Soft Tissue Sarcomas: ESMO clinical recommendations for diagnosis, treatment and follow-up" assess adjuvant chemotherapy as not standard treatment, but an option in cases of large or high grade tumors [32].

Doxorubicin and Ifosfamide containing regimes are used both for adjuvant and for neoadjuvant treatment of advanced STS [70-72]. Novel drugs as gemcitabine and taxans, among others, are also used [10,73]. Additionally, Trabectidin® was recently approved by FDA for palliative STS treatment [74].

Neoadjuvant chemotherapy is used for inoperable STS to shrink the tumor hopefully leading to a possible wide resection and elimination of subclinical disease [75]. Isolated limb perfusion and hyperthermic isolated limb perfusion are novel techniques available in some cancer centers for the treatment of primary unresectable extremity STS, that renders the tumors operable in up to 40% of the cases, although often at the cost of considerable toxicity [76-78].

1.1.6.3. Radiotherapy

Adjuvant radiotherapy is warranted where initial resection yields uncertain, marginal or intralesional resection margins [79,80]. The dosages are typically between 50 and 75 Gy, and higher radiation doses (63 Gy or more) yield superior tumor control and survival [81]. A rise in complications occurs in patients who receive doses of 68 Gy or more, which provides a therapeutic window for benefit in these patients [81]. For STS of other sites, adjuvant radiotherapy remains controversial [66,82].

Intensity-modulated radiation therapy (IMRT) is an advanced mode of high-precision radiotherapy that utilizes computer-controlled linear accelerators to deliver precise radiation doses to a malignant tumor or specific areas within the tumor. Several studies showed recently that IMRT can be administered safely and with promising efficacy, especially in patients with locally advanced STS [82,83]

Primary radiotherapy is mostly used in cases where surgery is not possible and the effect is difficult to screen as these tumors often have a dismal prognosis [84].

1.2. Molecular-genetic abnormalities in sarcomas

The molecular-genetic background of cancer is a hotspot of nowadays' research. Most of STS carry complex, but non-specific karyotypes, with numerous gains and losses [85], while approximately 15-20% of them, namely Ewing sarcoma, synovial sarcoma, and myxoid/round cell liposarcoma, have specific translocations and relatively simple caryotypes [86]. In addition, a minority of tumors carry specific somatic gene mutations, like c-kit or PDGFR-α mutations in GIST. The target genes, both for the reciprocal translocation fusion products, for instance EWSR1-ETS in Ewing sarcoma, and random mutations, code for hybrid oncoproteins which act as aberrant transcription factors, stimulating several intracellular signaling pathways and resulting in cell proliferation, evasion of growth inhibition, escape from senescence and apoptosis, induction of angiogenesis, invasion and metastasis [87].

The above-mentioned essential mechanisms of carcinogenesis were proposed in 2000 and considerably upgraded in 2011 by Hanahan and Weinberg [88,89]. Each of these mechanisms is regulated by several intracellular signaling pathways which further interact in a complicated cross-talk network. There is, however, growing evidence that certain molecular

aberrations are more likely to influence the clinical behavior of a malignant tumor, including invasion and metastasis.

1.2.1. Tumor differentiation and EMT

Tumor differentiation is a term used to describe the appearance of malignant tumor. It can be defined as the extent to which a tumor resembles its tissue of origin. Well-differentiated tumors resemble closely their tissue of origin, whereas poorly-differentiated tumors barely resemble their tissue of origin. The lack of differentiation, also called anaplasia, is characterized by a number of histological features, such as pleomorphism, which can be defined as variation in cell size and shape; nuclear hyperchromasia due to abundance of DNA, i.e. polyploidy; and loss of cellular polarity and adhesion [90]. The sense of these changes is to provide the tumor cell by motility, which, in turn, augments tumor's capability to invade and metastasize.

In epithelial tumors, such change in cellular shape from original to spindle, or stellate, associated with loss of polarity and intercellular contact, upregulation of mesenchymal markers with gain of invasive behavior is frequently referred to as epithelial-to-mesenchymal transition (EMT). EMT is defined as a sequence of protein modifications and transcriptional events in response to a certain set of extracellular stimuli leading to a stable, but sometimes reversible, cellular change [91]. This concept, though, is not universally supported [92].

Multiple molecular mediators of EMT have been described in carcinomas [93]. The list of EMT pathways includes nuclear factor-kappa B (NF-κB), AKT/mammalian target of rapamycin (AKT/mTOR) axis, mitogen-activated protein kinase (MAPK), beta-catenin, protein-kinase C (PKC) and others [94]. However, STSs are already mesenchymal tumors, and expression of markers linked to EMT does not support EMT as a biological event in them. Moreover, the markers linked to EMT have clearly defined roles in tumor biology that are distinct from EMT, and the negative impact of these factors on tumor behavior can rather be defined as "dedifferentiation" or "anaplasia" in STS.

1.2.2. Tumor proliferation and growth

Tumor proliferation can be defined as an increase in tumor cell number due to altered balance between growth – antigrowth signalling and/or resistance to apoptosis and

differentiation. Abnormal cell proliferation is necessary, although often insufficient, for tumorigenesis. The rate of tumor cell proliferation depends on the rate of cell division, the fraction of cells within the population undergoing cell division (growth fraction), and the rate of cell loss from the population due to terminal differentiation or apoptosis. This is important since the goal of most current cancer therapy strategies is to reduce the number of tumor cells, to prevent their further accumulation and to be antiproliferative by nature.

The growth fraction of a tumor can be registered by several techniques. The easiest and most used method is the mitotic count under light microscopy, which is incorporated in several STS grading systems, including FNCLCC system [2,38]. Beside the advantages, this method has some drawbacks such as high intra- and interobserver variability and subjective estimation. This can be avoided by the use of immunohistochemical markers of proliferation like Ki-67 or MIB-1 [95,96]. Other methods of measuring the proliferation rate are detection of cells undergoing DNA synthesis [97], flow cytometry to estimate the percentage of cells in S-phase, and the detection of cycle-linked markers.

The transition between cell cycle phases is regulated by checkpoints which, in turn, requires an expression of a variety of proteins, including regulating cyclin-dependent kinases (CDKs), regulatory proteins and transcription factors like Ras oncogene, retinoblastoma tumor-suppressor protein (Rb), and growth factors as platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), insulin-like growth factor (IGF-1) and many others [98-101]. Many of these are known molecular biomarkers and current subjects of research both in epithelial tumors and in STS.

1.2.3. Molecular markers

Molecular markers are biological molecules found in blood, other body fluids, or tumor tissue [102]. They can be divided into diagnostic, predictive and prognostic markers, helping, respectively, to establish more accurate and definitive diagnoses, predict response to specific therapies, and finally, predict survival.

There can be considerable overlap for a marker's role across functional categories. For instance, an immunohistochemical testing of tumor tissue for female steroid hormone receptors can be used both as a diagnostic procedure in differential diagnostics of metastasis and as a predictor of tamoxifen or aromatase inhibitor therapy success in breast cancer. In addition, there is also reported some prognostic value of these receptors in gynecological

cancers [103,104]. Molecular markers may offer great promise in the care of cancer patients especially with respect to individual, tailored cancer treatment [105,106].

1.2.4. Markers of tumor growth, proliferation and differentiation

1.2.4.1. TGF-β

TGF-β is a family of 3 highly homologous proteins, called TGF-β1, TGF-β2 and TGF-β3, which have very similar functions. They all are known to induce G1 arrest in order to terminate proliferation, induce differentiation, or promote apoptosis in normal cells, thus being a natural tumor-suppressive agent. However, in tumorigenesis, this mediator initiates dedifferentiation through activation of SMAD and non-SMAD (DAXX) signalling pathways [107]. This pro-neoplastic action becomes possible through either blockade of the TGF-β pathway with receptor-inactivating mutations, or selective inactivation of the tumor-inhibiting arm of this pathway [108]. Another possibility is TGF-β induced systemic immune suppression [109]. The TGF-β pathway activation has been shown to negatively influence prognosis in both epithelial [110,111] and in mesenchymal bone [112] and soft tissue tumors [113-115]. The most of studies, however, are devoted to one particular STS type, while investigations of TGF-β1 expression by whole-array human STS with concern to impact on survival are not reported.

TGF-β was called the Jekyl and Hyde of cancer [116] for its ability to modulate its action from tumor suppressor to tumor promoter. The factors responsible for this transition remain unclear. The candidates are both tumor-cell-autonomous TGF-β signaling [117] itself, and factors in the tumor microenvironment. Among the latter, inflammatory cells and cancer-associated fibroblasts [109], as well as angiogenetic factors [117], are considered the most potent modulators of TGF-β action.

1.2.4.2. NF-κB

NF-κB is a protein complex that controls the transcription of DNA. There are five proteins in the mammalian NF-κB family which share structural homology with the retroviral oncoprotein v-Rel, and therefore frequently classified as NF-κB/Rel proteins. These are transcription proteins responsible for control of inflammation, regulation of cell cycle and cell proliferation.

NF-kB is constitutively activated in various tumor cells where it promotes cell proliferation, survival, metastasis, inflammation, invasion, and angiogenesis [118]. Its influence on tumorigenesis is rather controversial. Indeed, while the majority of the investigators confirm that this marker augments tumor invasiveness and metastasis resulting in shorter DSS, in a recent study NF-kB p 105 was reported to have a favourable impact on DSS in operable non-small cell lung carcinoma patients [119].

In STS, NF-kB has also been shown to be both a progenitor [120,121] and inhibitor [122] of tumor growth and proliferation. However, all these studies were based on sarcoma cell lines in vitro or animal models. NF-kB expression patterns in native human STS and, more specifically, its impact on survival is not investigated.

1.2.4.3. Regulators of motility and adhesiveness

The process of malignant transformation in epithelial cell is usually characterized by loss of adhesiveness and gain of motility. Fascin and E-cadherin have inverse effects related to cell motility and cell adhesiveness and important factors in the progression and metastasis of cancers [123].

Fascin is an actin-binding protein that is normally found in membrane ruffles, microspikes, and stress fibers at the leading edges and borders of mesenchymal, nervous and endothelial cells. It has a key function in forming the parallel actin bundles that hold lamellipodial and filopodial cell protrusions that are main cellular structures for environmental guidance and cell migration. In intact cells, the actin-binding function of fascin is regulated by to extracellular signals through the activities of PKCα and small GTPases. [124,125]. Fascin has been reported to be overexpressed in sarcomatoid in contrast to conventional non-small cell lung carcinoma [126]. In leiomyomatous tumors of the uterus it was associated with higher malignancy grade [127].

E-cadherin is responsible for epithelial cell junction/adhesion. It is rarely expressed in STS, except for synovial and epithelioid sarcomas, as well as mesothelioma, which naturally express both epithelial and mesenchymal markers.

1.2.4.4. Regulators of cell polarity

Almost all cell types exhibit some sort of polarity, which enables them to carry out specialized functions. Par-6 and PKC-ζ (one of four atypical PKCs) belong to the Par3/Par-6/aPKC polarity complex that governs diverse cell functions such as localization of embryonic determinants and establishment of tissue and organ during the embryonal period. In mature organisms, they are responsible for regulation of cell polarity and the asymmetric division of cells [128]. Both Par-6 and PKC-ζ have been identified as EMT-associated biomarkers [129] and found to enhance proliferation, migration and invasiveness in cell cultures [130,131]. In one rare study on PKC-ζ in real human tumors, Cornford et al. reported that PKC-ζ expression was significantly higher in prostatic carcinomas than in non-neoplastic prostate tissue [132]. In addition, it was shown to have a crucial role at a post-viral entry stage of HHV-8 infection in Kaposi sarcoma [133]. The patterns of Par-6 and PKC-ζ expression and their possible prognostic impact have not been investigated in sarcomas.

1.2.4.5. Female steroid hormone receptors

Estrogen receptors (ER) are a group of mostly intranuclear receptors activated by the hormone 17β-estradiol (estrogen). There are two separate, but highly homologous isoforms of ER, ERα and ERβ, which have completely different tissue distribution [134]. They are encoded by two separate genes, ESR1 and ESR2, respectively. ER, mostly in α isoform, mediates the action of estrogens and is responsible for growth and differentiation of target cells.

Like ER, PgR protein exists as two receptor isoforms (PgR-A and PgR-B), but these are product of the same gene. PgR is considered the ER's antagonist. However, selective ablation of PgR-A in a mouse model, resulted in exclusive production of PgR-B indicating that PgR-B contributes to, rather than inhibits, epithelial cell proliferation both in response to estrogen alone and in the presence of progesterone [135].

These steroid hormone receptors act as ligand-activated transcription factors. There exist several mechanisms with such action, including (1) classic, when transcription starts after receptor-ligand complex binding to the specific response element in the gene promoter, (2) response element – independent pathway via binding to a transcription factor which in turn directly contacts the target gene promoter, (3) ligand – independent genomic action, when different growth factors induce phosphorylation of the hormone receptor followed by

binding to the specific response element in the gene promoter and transcription/translation/protein synthesis, and (4) non-genomic actions, involving extranuclear fraction of hormone receptors [136].

Both ER and, to a lesser degree, PgR are well known predictive markers of endocrine therapy in breast cancer [137,138]. They are also shown to have a slight positive prognostic effect irrelative of endocrine therapy [103]. Steroid hormone receptors are known to be expressed in some extent by soft tissue tumors. In leiomyomatous tumors of the uterus, their expression level correlates inversely with tumor malignancy grade [139,140]. In addition, it was shown effect of hormone-ablation therapy in aggressive intraabdominal fibromatosis [141].

1.2.4.6. PI3K-Akt signaling pathway

The main actors in the PI3K/Akt pathway are PI3K and Akt, performing similar functions, as well as their inhibitor PTEN (phosphatase and tensin homolog deleted on chromosome 10), which is a lipid phosphatase that removes the phosphate group from the 3' position of the inositol ring of PIP_3, thereby blocking Akt activation. Akt is a serine/threonine protein kinase that exists in three highly homologous isoforms, including Akt1, Akt2, and Akt3.

Akt can be phosphorylated at threonine ^{308} and at serine ^{473} for Akt1 or homologous sites for Akt2 and Akt3 by, correspondingly, mTORC2 and PDK2 belonging to the phosphoinositide 3-kinase (PI3K)/Akt pathway. Activated Akt can activate or deactivate its multiple substrates, including mammalian target of rapamycin (mTOR), bcl-2 family member BAD, transcription factor forkhead homolog 1 in rhabdomyosarcoma (FKHR), Mdm2 protein, glycogen synthase kinase 3 (GSK3) and many others, via its kinase activity [142,143]. The PI3K/Akt pathway has been linked to an extraordinarily diverse group of cellular functions, including cell growth, proliferation, differentiation, motility, survival and intracellular trafficking and angiogenesis [144]. Both PI3K and Akt isoforms have been implicated as major players in many types of cancer [145-147].

The PI3K/Akt pathway seems to be more often deregulated in cancer than any other pathway [148]. However, in the literature there is disagreement regarding the prognostic impact of Akt expression. While the majority of studies agree that Akt expression overtly indicates a poor prognosis [149-151], there are several studies showing the opposite effect

[152,153]. Expressions of PI3K/Akt pathway components have rarely been investigated in STSs and there are almost no studies devoted to their prognostic value [154].

Different physiological functions of the Akt family kinases imply that the expression of its isoforms may also have different prognostic impact in cancer. The significance of this variation for the survival of the STS patients is not well investigated and it is not clear whether the site of phosphorylation and the pattern of expression play prognostic roles.

1.2.4.7. Crosstalk among intracellular pathways responsible for tumor growth, proliferation and differentiation

The interplay between pathways responsible for tumor growth, proliferation and differentiation is tightly regulated both spatially and temporally. This gives rise to the remarkable complexity, diversity, and flexibility of the ways the cell function can be performed in order to augment proliferation and (de)differentiation. This has been exemplified by a great number of studies.

The PI3K/Akt action is documented to enhance TGF-β-induced apoptosis and/or cell cycle arrest in multiple types of cells in response to several activating agents [155,156]. Akt activity, in turn, is also shown to increase in response to TGF-β treatment. It seems to be required for several of TGF-β - induced activities, such as cell migration of HER2-expressing breast cancer cells [157], and EMT of normal mammary epithelial cells [158].

Both aberrant expression of Smad4 or disruption of Smad4 activity was shown to decrease the TGF-beta suppression of ER-alpha transactivity in breast cancer cells [159]. The investigation of EMT revealed synergistic roles for TGF-β/BMP signaling pathway components, fascin, NF-κB, Par-6 and PKC-ζ in forming of motile phenotype in cancer cells [160-163].

Female steroid hormone receptors, as it was mentioned earlier, act as growth factors [164] and can be activated in a ligand – independent genomic way. It has been described reciprocal mutual activation of elements of PI3K-Akt pathway and ER [165]. Steroid hormone receptors are also the targets of protein kinase A, MAPK, CDKs, casein kinase, and GCK3 [166,167] in this context. Finally, NF-kappaB activation can influence ER recruitment to inherently inactive ER binding sites [168].

1.3. Tissue microarray

Tissue microarrays (TMAs) represent an powerful technology tool designed to explore molecular targets, on DNA, RNA or protein level, from several tissue specimens assembled in a single microscope slide[169]. This method implies the extraction of small tissue cylinders from donor tissue block to be embedded in a recipient block (Figure 7).

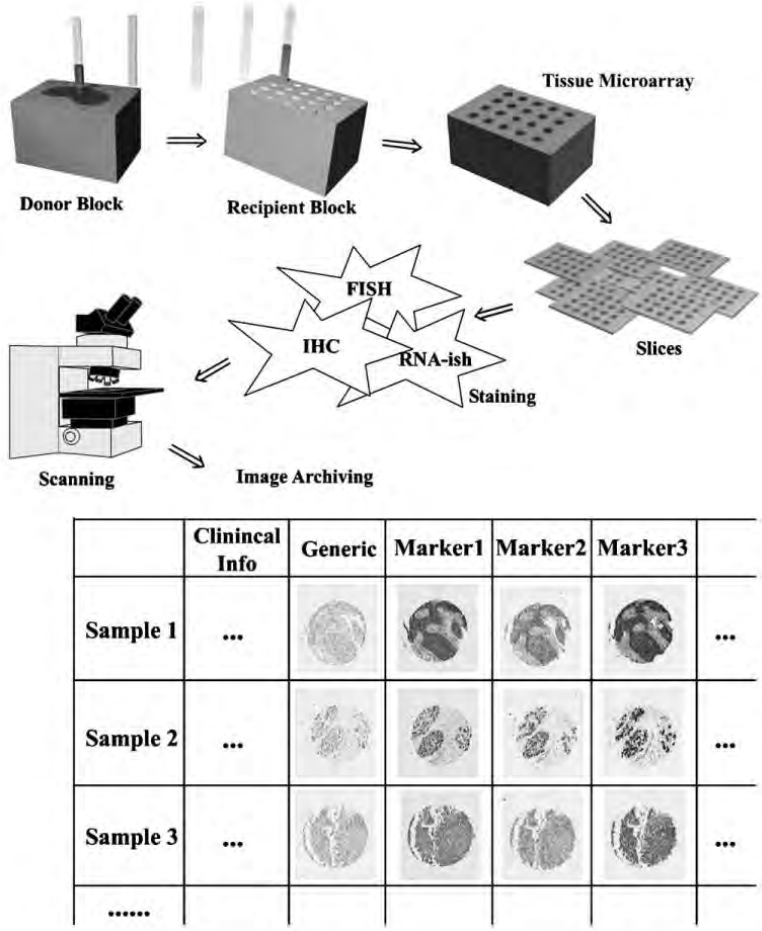


Figure 7: Tissue microarray method. Cores punched from the donor blocks and embedded into the recipient block. The TMA block can then be sectioned and used for various staining methods. Adapted from *Chen W, Foran DJ: Advances in cancer tissue microarray technology: Towards improved understanding and diagnostics. Anal Chim Acta 2006* [170] *Permission obtained from Elseiver Inc.*

This block can then be cut into thin slices available for immunohistochemistry (IHC), *in situ* hybridization etc. Once constructed, one block could potentially yield tissue to several hundred analyzes depending on the thickness of the block [171,172].

The method was first introduced by Battifora 1986 as so called “multitumor (sausage) tissue block"[173] and further modified in 1990 with its improvement, "the checkerboard tissue block”[174]. Although offering significant benefits even at this early stage, the TMA technique was not embraced on a large scale before Kononen et al. devised an instrument able to standardize the TMA construction process in 1998 [175]. Adaptation has also allowed the use of other than paraffinized tissues, including frozen tissue, cell-lines and needle biopsies. This has led to an vast increase of TMA studies and in 2007 nearly 10% of all biomarker studies were conducted using TMA as the principal method of investigation [171].

2. AIMS OF THESIS

The presented thesis was aimed at exploring potential prognostic markers of tumor growth, proliferation and (de)differentiation for non-GIST STSs.

More specifically the aims were to:

- ✓ Elucidate the prognostic significance of dedifferentiation-related factors in tumor cells of non-GIST STSs.

- ✓ Investigate the distribution and prognostic impact of ER and PgR in tumor cells of non-GIST STSs.

- ✓ Evaluate the prognostic impact of the proteins belonging to PI3K-Akt signaling pathway in tumor cells of non-GIST STSs.

3. MATERIAL AND METHODS

3.1. Study population and material

Figure 8 shows the inclusion and exclusion of patients in the different studies. A retrospective archival search for sarcoma diagnosis was done at the University Hospital of North-Norway (1973 – 2006) and the Hospitals of Arkhangelsk County, Russia (1993 – 2004). Regarding the Russian material, there was asked for material from a ten year period due to the fact that the archival system before the chosen time frame was less organized. A total of 959 patients were found (Norwegian, n = 632; Russian, n = 337).

Formalin-fixed and paraffin-embedded specimens from primary tumor tissue were obtained and all biopsies were reevaluated by two experienced pathologists. The tumors were graded according to the FNCLCC system and histologically subtyped according to the World Health Organization guidelines. For the Russian material there were made new slides of all the paraffin blocks. For the Norwegian material new slides were made when necessary. All the biopsies were immunostained with CK, CD117, actin, SMA, vimentin and CD34. Some slides were also stained with S100 when necessary to rule out or confirm a differential diagnosis. Other molecular methods were not considered as necessary for differential diagnostics, but in some cases PCR or FISH were performed in the initial diagnostics. About 10 % of the initial diagnoses were revised due to changing classification systems and appearance of new entities such as GIST. Non-sarcomas, other sarcomas not classified as STS as well as GISTs were excluded: carsinosarcomas (n= 81), dermatofibrosarcoma protuberans (n= 78), GISTS (n = 47), osteosarcomas (n=42), chondrosarcomas (n = 30), Kaposi sarcomas (n=30), endometrial stromal tumors (n=27), benign tumors (n=18), malign mesotheliom (n =11) and other sarcomas/unknown (n=99).

A total of 496 non-GIST STSs (Norwegian, n = 299; Russian, n = 197) were registered. However, 247 patients were excluded due to missing clinical data (n = 86) or inadequate paraffin embedded fixed tissue blocks (n = 161). Thus 249 non-GIST STSs patients (Norwegian, n = 167; Russian, n = 82) were eligible and included in the study.

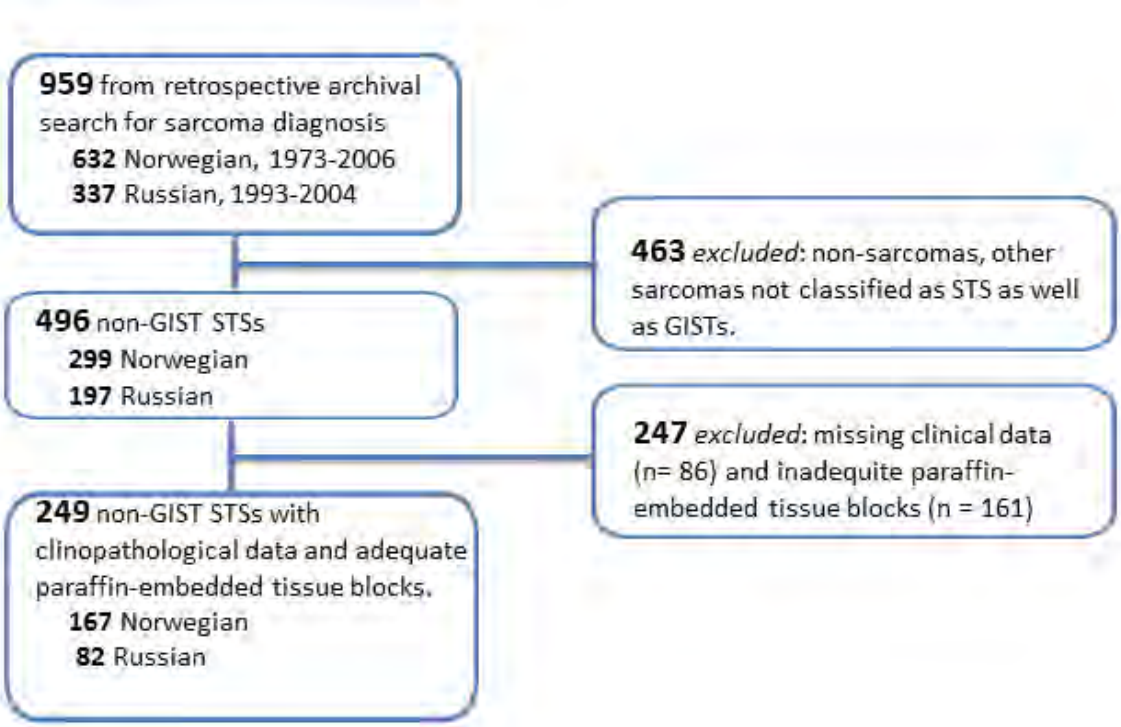


Figure 8: Flow-chart visualizing inclusion and exclusion of patients in the study.

Demographic and clinical data were collected retrospectively and include follow-up data as of September 2009. The minimum follow up for the survivors was 41 months and the median follow-up for the entire patient population was 37.6 (range 0.1-391.7) months.

3.2. Immunohistochemistry (IHC)

3.2.1. IHC procedure

The applied antibodies were subjected to in-house validation by the manufacturer for IHC analysis on paraffin-embedded material. The antibodies used in the study are summarized in Table 4.

Four µm thick sections were deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval was performed exposing slides to microwave heating for 10×2 min at 450 W in 0.01M citrate buffer pH 6.0 for fascin, Akt (Akt1) phosphorylated at threonine^{308} and serine^{473}, Akt2, Akt3, PI3K and PTEN. For steroid hormone receptors, TGF-β1, PKC-ζ, Par-6α, E-cadherin and vimentin, Ventana Antigen Retrieval Cell Conditioning Buffer 1 (Tris/Borate/EDTA Buffer pH8.0) was used for antigen retrieval, with mild regimen (32 min)

for PgR, TGF-β1, PKC-ζ, Par-6α, E-cadherin, and standard regimen (64 min) for ER and vimentin. TGF-β1, PKC-ζ, Par-6α, E-cadherin, vimentin, ER and PgR were stained using Ventana Benchmark XT (Ventana Medical Systems Inc)**,** procedure iViewDAB.

Primary antibodies against PgR, TGF- β 1, PKC-ζ, E-cadherin, ER and Par-6α were incubated at 37°C for 24, 28, 28, 32, 32 and 52 min, accordingly. Primary antibodies against NF-kB, Akt (Akt1) phosphorylated at threonine^{308} and serine^{473}, Akt2, Akt3 and PTEN were incubated overnight at 4°C, while those against fascin and PI3K were incubated correspondingly for 30 and 32 minutes at room temperature.

The DAKO EnVision + System-HRP (DAB) kit was used as endogen peroxidase blocking agent and to visualize the antigens for all stains. This yielded a brown reaction product at the site of the target antigen. As negative staining controls, the primary antibodies were replaced with the primary antibody diluents. All slides were counterstained with hematoxylin to visualize the nuclei. For each antibody, including negative controls, all TMA staining were performed in one single experiment.

Table 4. Schematic overview of the antibodies used in the studies.

Antigen	Dilution	Antibody	Clone	Source
TGF-β1	1:50	Rabbit polyclonal	SC-146	Santa Cruz Biotechnology
Fascin	1:25	Mouse monoclonal	MAB3582	Chemicon International
NF-κB p105	1:50	Rabbit monoclonal	4808	Cell Signaling Technology
PKC-ζ	1:100	Rabbit polyclonal	SC-216	Santa Cruz Biotechnology
Par-6α	1:10	Rabbit polyclonal	SC-25525	Santa Cruz Biotechnology
E-cadherin	Prediluted	Mouse monoclonal	ECH-6	Cell Marque
Vimentin	Prediluted	Mouse monoclonal	V9	Ventana Medical Systems
ERα	Prediluted	Mouse monoclonal	SP1	Ventana Medical Systems
PGR	Prediluted	Mouse monoclonal	1E2	Ventana Medical Systems
phosphoAkt Thr^{308}	1:50	Rabbit monoclonal	244F9	Cell Signalling Technology
phosphoAkt Ser^{473}	1:5	Rabbit monoclonal	736E11	Cell Signalling Technology
Akt2	1:18	Rabbit monoclonal	54G8	Cell Signalling Technology
Akt3	1:8	Rabbit monoclonal	4057	Cell Signalling Technology
PTEN	1:10	Rabbit monoclonal	9559	Cell Signalling Technology
PI3K	1:50	Rabbit polyclonal	4254	Cell Signalling Technology

3.2.2. Scoring

The ARIOL imaging system (Genetix, San Jose, CA) was used to scan the slides of antibody staining of the TMAs. The slides were loaded in the automated slide loader (Applied Imaging SL 50) and the specimens were scanned at low resolution (1.25×) and high resolution (20×) using the Olympus BX 61 microscope with an automated platform (Prior). Representative and viable tissue sections were scored manually on computer screen semi quantitatively for cytoplasmic staining. The dominant staining intensity was scored as: 0 = negative; 1 = weak; 2 = intermediate; 3 = strong. All samples were anonymized and independently scored by two trained pathologists. When assessing a variable for a given core, the observers were blinded to the scores of the other variables and to outcome. All cores from each patient were scored by the two pathologists. Mean score from each individual was calculated separately. Cut-off values with regard to high and low expression were decided as described in chapter 5.1.4.2. For E-cadherin, NF-κB, PKC-ζ, ER and PgR the cut-off points were established at 0. For other markers, high expression of tumor cells were defined as ≥ 0.5 (PI3K and PTEN), ≥ 1.0 (phosphoAkt Ser^{473}) and ≥ 2 (TGF-β1, fascin, Par6, vimentin, phosphoAkt Thr^{308}, Akt2 and Akt3).

3.3. Statistical analysis

All statistical analyses were done using the statistical package SPSS (Chicago, IL), version 16. The IHC scores from each observer were compared for interobserver reliability by use of a two-way random effect model with absolute agreement definition. The intraclass correlation coefficient (reliability coefficient) was obtained from these results. The Chi-square test and Fishers Exact test were used to examine the association between molecular marker expression and various clinicopathological parameters. Univariate analyses were done using the Kaplan-Meier method, and statistical significance between survival curves was assessed by the log rank test. DSS was determined from the date of diagnosis to the time of cancer related death. To assess the independent value of different pretreatment variables on survival, in the presence of other variables, multivariate analyses were carried out using the Cox proportional hazards model. Only variables of significant value from the univariate analyses were entered into the Cox regression analyses. Probability for stepwise entry and removal was set at .05 and .10, respectively. The significance level used for all statistical tests was $P < 0.05$, but in the subgroup analysis the significance level was moved from P=0.05 to P=0.01 due to risk of false positivity.

3.4. Ethical clearance

The National Data Inspection Board and The Regional Committee for Research Ethics approved the study.

4. MAIN RESULTS

4.1. Paper I

This study was designed to investigate the prognostic impact of the dedifferentiation-associated biomarker expression in tumors from patients with non-GIST STS. These markers were shown to induce and/or sustain EMT in epithelial tumors. Interobserver scoring agreement was tested for all markers. The intraclass correlation coefficients were 0.92 for E-cadherin ($P<0.001$), 0.89 for fascin ($P<0.001$), 0.91 for NF-κB p105 ($P<0.001$), 0.86 for Par-6α ($P<0.001$), 0.97 for PKC-ζ ($P<0.001$), 0.87 for TGF-β1 ($P<0.001$) and 0.93 for vimentin ($P<0.001$).

In univariate analyses of the total material TGF-β1 ($P=0.016$), fascin ($P=0.006$), NF-kB p105 ($P=0.022$) and PKC-ζ ($P=0.042$) were significant indicators of shorter DSS, while in subgroup analysis, high TGF-β1 expression was a negative prognostic indicator particularly for undifferentiated pleomorphic sarcoma ($P<0.001$) and for trunk-located STS ($P=0.003$). In the multivariate analysis, high TGF-β1 expression was an independent negative prognostic factor for DSS (HR=1.6, 95% CI=1.1-2.4, $P=0.019$) in addition to tumor depth, malignancy grade, metastasis at diagnosis, surgery and positive resection margins in the total patient cohort.

4.2. Paper II

The estrogen (ER) and progesterone receptor (PgR) regulate growth and cell differentiation upon ligand-dependent and ligand-independent activation. In breast cancer and gynecological tumors their expression are known predictors of endocrine therapy benefits and a favorable therapy-independent prognosis. The study aimed to investigate the distribution and possible prognostic impact of ER and PgR expression in non-gastrointestinal stromal tumor soft tissue sarcomas (non-GIST STSs). Interobserver scoring agreement was tested for both steroid hormone receptors. The intraclass correlation coefficients were 0.92 for ER ($P<0.001$) and 0.96 for PgR ($P<0.001$).

Uterine leiomyosarcomas expressed ER and PgR most frequently and intensively. However, the moderately and especially weakly positive tumors were distributed relatively equally between genders and histological entities. The expressions of ER and PgR correlated strongly with each other ($r=0.206$, $P=0.002$). Fifty-three percent of STSs expressed at least one of the steroid hormone receptors.

Neither ER nor PgR showed significant prognostic impacts on DSS by analysing the whole cohort. Though, separate analyses of each gender revealed that ER expression was a significant positive prognostic factor in women (P=0.017), while PgR expression was associated with a poor prognosis in men (P=0.001). Among the four possible co-expression patterns of ER and PgR, the ER-/PgR+ profile for the whole cohort, which was seen in 14% of the patients (n=34), was associated with a miserable prognosis (P<0.001). In the multivariate analysis, this ER-/PgR+ phenotype was an independent negative prognostic factor for DSS (HR=1.9, 95% CI=1.2-3.1, P=0.008) in the total patient cohort.

4.3. Paper III

The PI3K/Akt pathway is involved in cellular survival pathways by inhibiting apoptotic processes and stimulating cell growth and proliferation. Its negative prognostic value has been proven in many types of cancer. The aim of this study was to investigate the prognostic impact of Akt (Akt1) phosphorylated at threonine^{308} and serine^{473}, Akt2, Akt3, PI3K and PTEN, alone and in coexpression with ER and PgR in non-gastrointestinal stromal tumor soft tissue sarcomas (non-GIST STSs). Interobserver scoring agreement was tested for all markers. The intraclass correlation coefficients were as follows: 0.89 for p-Akt Ser^{473} (P<0.001), 0.94 for p-Akt Thr^{308} (P<0.001), 0.91 for Akt2 (P<0.001), 0.95 for Akt3 (P<0.001), 0.88 for PI3-K (P<0.001) and 0.89 for PTEN (P<0.001).

In univariate analyses of the total material, p-Akt Thr^{308} (P=0.002), Akt2 (P=0.008) and PI3K (P<0.001) were significant indicators of shorter DSS. Nuclear expression of p-Akt Thr^{308} expression showed a significantly favorable prognosis (P=0.029), compared to cytoplasmic and especially mixed cytoplasmic and nuclear expression.

Subgroup analysis based on patients' gender revealed that high expression of p-Akt Thr^{308} was a negative prognostic factor particularly for men (P=0.009 vs. P=0.064 for women). In contrast, p-Akt Ser^{473} appeared to be a negative prognosticator exclusively for female patients (P=0.023 vs. P=0.87 for men). Expression of steroid hormone receptors also showed opposite prognostic impacts depending on patient's gender. This was further proved by the co-expression of these factors. Among others, PgR-/p-Akt Ser^{473}+ phenotype tended to have an unfavorable impact in women (P=0.087) but was clearly favorable in men (P= 0.010).

In the multivariate analysis of the total material, PI3K expression by tumor cells (HR=1.5, 95% CI=1.0-2.2, P=0.042) was independent negative prognostic indicator of DSS.

5. DISCUSSION

5.1. Methods

5.1.1. Data collection and study population

As in many similar studies, the representativity of the studied population is a major issue. We have included patients from two countries, Norway and Russia, to achieve more statistical power for the statistical analyses. On the other hand, the possibility of population heterogeneity can be a potential drawback. However, the populations are both ethnically and geographically close as illustrated by a similar distribution of clinopathological variables. Despite possible differences with respect to diagnoses or treatment traditions, the revision of all tumors and the relatively limited and grossly comparable choice of curative strategies made it meaningful to study both the Norwegian and Russian patients in one cohort. Besides, the study focuses on the natural biology of the STSs and not on the treatment modalities.

5.1.1.1. Representativity of Norwegian and Russian study populations

In 23 years (1973-2006), the estimated number of STSs in Northern Norway is about 230-460 STS patients, taking into consideration the total incidence (of about 0.5 – 1% of all cancers annually) and the proportion of the population living in Northern Norway, comprising approximately 10%. There were 299 Norwegian non-GIST STSs observed in our population before excluding 132 cases due to missing clinical data or inadequate paraffin-embedded fixed tissue blocks (Figure 8). Since missing cases are at random, it can be argued that the patient population is not selection biased and therefore representative.

Though the Norwegian material is representative, one may to a larger degree question the representativity of the Russian material. The population of Arkhangelsk Oblast is about three times larger than Northern Norway. As the number of Russian population in our study (n=82) is about one third of the Norwegian material, there should definitely be more patients in the Russian material, although the time of the material collection here (1993-2004, 11 years) comprised one third (1973-2006, 33 years) of the Norwegian inclusion period. However, the number of Russian patients in our studies is about one third of the Norwegian study population. This potential selection bias should be taken into account when our results are being analyzed. We also see that subgroups of the Russian patients have significantly worse prognoses than the Norwegian cohort. However, when comparing the

clinicopathological variables, only the distribution of malignancy grade is significantly altered in the Russian versus the Norwegian material. The increased number of Russian patients with malignancy grade 3 may explain at least some of the reduced survival rate in the Russian population. This in turn can be explained by the fact that a considerable part of the Russian material was from the Arkhangelsk Regional Oncology Center, while a proportion of patients with less malignant tumors could potentially be cured locally at the local district hospitals. Hence, a selection bias of the Russian material can not be ruled out.

5.1.1.2. Patients without metastasis at the time of diagnosis and non-operated patients

Many translational studies conducted on sarcomas exclude high-risk categories of patients, namely those with inoperable status and metastasis at the time of diagnosis, from study population in terms to achieve more homogeneity. Ideally, one should analyze these groups separately, to avoid a probability of removing potential prognostic significance of individual markers, i.e. increased chance of false negative results.

We have repeated all statistical analyses based on patients who did not undergo surgery and did not have metastasis at the time of diagnosis. The exclusion reduced the total number of non-GIST STS patients from 249 to 194. Analyses have been done in these patients as a total group (n = 194) and (for article 2 and 3) separately for men and women.

Paper I

Among the markers we investigated in the first study, again TGF-β1 (P=0.035) and fascin (P=0.010) were significant indicators of shorter DSS, NF-kB p105 (P=0.050) was borderline significant, while PKC-ζ showed a tendency towards significance (P=0.096). In the Cox analysis, TGF-β1 tended to a significant value (P=0.075).

Paper II

For paper II, neither ER nor PgR expressions were significant in total group patients, but as in the published analysis PgR was a negative prognosticator in men (P=0.010) and ER a significantly favorable prognostic factor for women (P=0.041). ER-/PgR+ phenotype was an independent negative prognosticator in all patients (HR=2.8, 95% CI=1.4-5.6, P=0.007) and in the subgroup of women (HR=2.0, 95% CI=1.0-4.0, P=0.036).

Paper III

And finally, the PI3-Akt signalling proteins showed the same poor prognostic significance as in the original paper in univariate analyses of p-Akt Thr^{308} (P=0.045), Akt2 (P=0.040) and PI3K (P=0.015). The latter, however, was not longer significant in Cox

analysis. Nevertheless, after the excluding tumor grade from the multivariate analysis, PI3K appeared as an independent negative prognostic factor (HR=1.6, 95% CI=1.1-2.5, P=0.027).

5.1.1.3. Patients without gender-associated sarcomas

As our results may potentially be distorted by the inclusion of gender-related sarcomas in our study, we excluded gender-related sarcomas (44 leiomyosarcomas located in uterus) from our database and carried out both univariate and multivariate analyses for whole cohort and, for ER, PgR and PI3K-Akt signalling system proteins, separately for each gender.

In the dedifferentiation-related group of proteins, the results of this rescoring were generally unaltered with only minor changes in P values: TGF-β1 (P=0.047), fascin (P=0.013), NF-kB p105 (P=0.043) and PKC-ζ (P=0.001) were significant indicators of shorter DSS, while only TGF-β1 was significant in the multivariate analysis (HR=1.5, 95% CI=1.0-2.3, P=0.035). ER and PgR also showed comparable values, with the ER-/PgR+ phenotype as an independent negative prognosticator (HR=1.8, 95% CI=1.1-2.9, P=0.025) for the total patient cohort.

With respect to the PI3K-Akt signalling system, results corresponded to those obtained without exclusion of gender associated sarcomas, except for Akt3 evaluated in the total cohort. Unlike in previous calculation, Akt3 showed a significantly negative impact in the univariate analysis (P=0.042). PI3K alone was an independent negative prognosticator of survival among the investigated markers, though both significance and the hazard ratio were higher compared results including gender-related sarcomas (HR=1.9, 95% CI=1.2-2.9, P=0.005 versus HR=1.5, 95% CI=1.0-2.2, P=0.042).

In conclusion, the inclusion of gender-related sarcomas did not appear to alter our results.

5.1.1.4. Separate investigation of differently located sarcomas

Based on clinical and prognostic data, sarcomas located on extremities and trunk (ET), versus retroperitoneal and visceral tumors (VR) may be regarded as distinct STS entities. In the ET group, metastases are the main cause of sarcoma-related death, while local relapse is a more common cause of sarcoma-death in STSs in the VR group. We have stratified patients according to ET (n=115) versus VR (n=66) subgroups (patients with head and neck STSs (n=13) were excluded from these analyses). Significant differences and trends from the

original papers were persistent in the ET sarcomas. For the VR group, the number of patients was insufficient for reliable analyses.

5.1.1.5. Heterogeneity of histological entities in study population

Another major issue is the heterogeneity with regard to the histological entities included in the analyses. This introduces the possibility that expression of the prognostic molecules investigated could be differently expressed in some subgroups. We conducted subgroup analyses of the histological entities concerning the expression of investigated molecules, and found the same tendencies in the larger subgroups when compared to the smaller subgroups.

5.1.1.6. Conclusion on material representativity

In conclusion, the data collection introduced problems in identifying adequate numbers of similar patients with similar tumors and with the same treatment traditions. These are all known problems when conducting STS studies. Our findings are in large hypothesis generating, and to be more conclusive future STS studies must be based on large, multi-institutional and multinational studies with possibilities to establish large enough patients STS cohorts of more homogenous tumor groups. However, all the tumors we investigated had mesenchymal derivation and belong to the same generic group. Moreover, the investigated dedifferentiation and proliferation markers probably to a large degree reflect universal and basic processes in tumorigenesis. They are described in a variety of epithelial and non-epithelial tumors of different locations and histological entities and seem not to depend on tumor type.

5.1.2. Tissue microarray

Among the obvious advantages of the TMA technique compared to whole slide assessments are the high throughput, cost benefit, possibility for large cohorts simultaneously, supreme staining standardization, reproducibility as well as relative simplicity. It is also possible to use the donor specimens for further analysis and share the material between different institutions.

Along with these apparent benefits, there are some drawbacks often discussed with regard to the use of TMAs. A common concern is whether the small core samples used in

TMA analysis give meaningful information on large tumor specimens. Instead of 0.6 mm cores, some investigators have used larger cores (2-4 mm or more) to increase the representativity [176-178]. Others suggest that punching multiple small cores from different regions better captures the heterogeneity of the tumors [169]. We chose using duplicate 0.6 mm cores which were selected to be as representative as possible, after reviewing all the original sections of the tumor and taking heterogeneity into consideration. Up to 95% correlation has been demonstrated when comparing tumour cell assessment in duplicate 0.6 mm cores versus the whole slide [169].

Another often mentioned drawback is that TMAs are not suitable to individual diagnosis of patients. In the involved institutions, all diagnostic procedures were performed using full slides, before construction of TMAs for marker studies.

5.1.3. Immunohistochemistry

IHC is only one of many techniques used to analyze tissues for expression of proteins and other molecules. More advanced techniques are available. Nevertheless, IHC continues to prove its worth. It is reliable, well developed and known, easy to interpret, widely available and routinely used in pathological laboratories. Unlike modern array techniques, IHC visualizes the final protein product, localization of the protein and not merely an up or down regulated gene etc.

5.1.3.1. Antibodies

Choosing antibodies is one of the major steps in conducting an IHC study. When available, commercial antibodies are the best choice, as they have data leaflets with rigorous specifications and are easily available for conformational studies. The next step is choosing between monoclonal and polyclonal antibodies. Monoclonal antibodies are all targeting one epitope on the antigen providing excellent specificities. In addition, they are homogenous from production lot to production lot, making conformational studies easier to conduct. The drawback of monoclonal antibodies is the chance that post processing of the tissue could lead to concealment of the targeted epitope and a type II error. Polyclonal antibodies target several epitopes on the same antigen resulting in a more robust antigen binding. The robust antigen binding happens at the cost of a risk of cross reaction with other antigens and an increased risk of a type I error.

The Sarcoma Study Group is a part of a larger Translational Cancer Research Group. All the immunomarkers we used were chosen from published literature and validated by both manufacturer and previous studies of lung cancer in our research group [119,179].

A common concern is whether improper tissue storage over years may affect the IHC results. To address this question we have divided the total material (n = 194) based on date of diagnosis, both in three categories (1973-1989, n = 48; 1990-1999, n = 97 and 2000-2006, n = 49) and in two categories (1973-1996, n = 101 and 1997-2006, n = 93). There were no significant differences (defined as $r > 0.2$, $P < 0.01$ due to multiple testing) in any of the marker expressions with regard to time-period.

5.1.3.2. Controls

Antibody specificity is ensured by a western blot showing binding of a protein of the expected size. In the case of the antibodies used in our studies, this was done by the manufacturer and presented in the data leaflets of the antibodies.

Positive and negative controls are normally conducted to control the quality of IHC experiments. Negative controls are conducted by replacing the primary antibody with a primary antibody diluent, to check for unspecific staining in the absence of the antibody. Negative controls could be made even more stringent by introducing isotype controls to check for unspecific binding. In addition, there were tissue controls with other tumor groups and normal tissue on each TMA slide, representing both positive and negative controls.

5.1.4. Statistics

There are almost as many approaches to statistical analysis of survival data as there are studies on survival and no optimal method of analysis exist. In order not to over- or under-interpret the significance of their data, investigators have to be vigilant in their choosing of different analyses. We believe that we in our analyses have found a reasonable balance between type I and type II errors. A short discussion of the statistical methods used in our studies is presented below.

5.1.4.1. Significance level

Type I errors occur when inappropriate significance levels are used. In biological studies it has become a norm to use $P < 0.05$ as the cut-off point where a difference is

considered significant. This gives that one in twenty tests for the same difference will be a type I error. When conducting a large number of tests the chance of an erroneous positive result thus increases. There have been developed several approaches for reducing the chance of type I error in the setting of multiple testing. The drawbacks of these are the increased chance of type II error. There is no consensus whether such methods should be used in prognostic studies. We chose not to conduct a correction of multiple testing as we see our studies as hypothesis generating. This gives us an increased risk of type I errors, but decreases the chance of type II errors.

5.1.4.2. Cut-off values

In prognostic biological studies the cut-off values are meant to divide the subjects under investigation into diagnostic groups based on the relative expression of proteins, mRNA etc. As biological values are continuous scales this gives a skewed view on reality and the results must be interpreted in that context. The most common approach is to dichotomize the material, but sometimes several groups give a better picture. When choosing the cut-off values the researchers must choose between using a predefined value either based on previous research, the mean or median, percentiles, standard deviations etc. or finding the cut-off value that yields the two groups with the largest possible difference in the end-point under investigation. There are drawbacks and advantages to both approaches. When using a predefined cut-off value the chances of type I errors decreases at the cost of type II errors. In many cases it is also difficult to find meaningful previous studies suggestive of a usable cut-off value. In the case of a conformational study, using a predefined value makes sense since there already is established a cut-off. In the case of a novel study, choosing the mean, median or percentiles as cut-off values makes sense in that it increases the reproducibility of the cut-offs and therefore will be easier to evaluate in a conformational study. When choosing the cut-off that yields the two groups with the largest possible difference in prognosis, the chances of type II errors decreases at the cost of type I errors. This approach makes sense in novel hypothesis generating studies where there are no predefined values to help choose the appropriate cut-offs. Such studies could be the basis of further research into novel fields and their results should be interpreted in this light. We have used the latter approach in our studies and we regard our findings as hypothesis generating. Hence, our results should be confirmed in other prognostic studies before being incorporated into clinical practice.

5.1.4.3. Survival analysis

There are several ways in which survival data can be analyzed and interpreted. One well proven method is the Kaplan-Meier (KM) analysis which tests the difference between groups in time to event data. However, the KM method does not adjust for the presence of other clinical variables. To address this point we used the Cox-proportional hazards method to adjust for clinical variables found to be significant using the KM method. This stringent method works to ensure that the variables found in our studies are in fact independent of known demographic, clinical or pathological variables and could therefore contribute when calculating the prognosis of non-GIST STS patients.

An issue is which endpoint to use. In prognostic studies there is a variety of endpoints as overall survival (OS), metastasis free survival (MFS), time to recurrence (TTR), time to progression (TTP) and as we have chosen, DSS. DSS is a well-established endpoint and, in this case, excludes patients with non-sarcoma related deaths.

5.2. Discussion of results

Sarcomas are rare tumors, and large cohorts of patients are therefore relatively rare in studies on non-GIST STS. Our study population is quite large compared to similar studies. Fully reassessed histology, scrutinized staining, visualizing and scoring processes, as well as comprehensive clinical data for each patient and rather long follow-up provides objectivity to the study performance and assessment. This was further augmented by the possibility to investigate the prognostic impact of several families of proteins which are responsible not only for tumor growth, proliferation and differentiation, but also for angiogenesis and local immunity, and estimate possible co-expressions within and between these marker families.

Although the total amount of patients in our studies is rather large, the histological subgroups are not numerous enough to conduct meaningful subgroup analyses, which is common problem in sarcoma-related research. Among other possible concerns are differences in treatment over time and between Norwegian and Russian patients and challenges regarding IHC. Nevertheless, the results of the univariate and multivariate analyses of the clinicopathological variables in the present cohort are in accordance with the published literature indicating a representative patient population and a good basis for marker analyses. An important exception is the varying malignancy grade rate between the Norwegian and Russian populations.

In summary, the results of our studies presented herein suggest the involvement of these markers in tumor growth, proliferation and (de)differentiation in development, behavior and, finally, prognosis of non-GIST STS. The exact mechanisms of such involvement are, however, yet to be elucidated.

5.2.1. Paper I

In this study we investigated the prognostic impact of a set of biomarkers in non-GIST STS patients known to participate in the process of EMT in epithelial tumors [91], but bearing other important biological functions as well. TGF-β1, fascin, NF-kB p 105 and PKC-ζ showed significant unfavorable influence on survival in the univariate analyses. Besides, high expression of TGF-β1 was a significant independent negative prognostic indicator of DSS.

TGF-β belongs to a TGF-β/BMP family of growth factors and is basically a tumor-suppressive agent whose functions include proliferation hampering and promotion of apoptosis both in normal and tumor cells. There is, however, a broad evidence of its negative influence on prognosis, described mostly in epithelial [110,111], but also in mesenchymal tumors [112-115]. The possible mechanisms of such pro-neoplastic action include receptor-inactivating mutations, selective inactivation of the tumor-inhibiting arm of this pathway [108], and TGF-β induced systemic immune suppression [109]. Other proposed modulators of TGF-β function are factors in the tumor microenvironment, particularly inflammatory cells, cancer-associated fibroblasts [109] and angiogenetic factors [117]. We found TGF-β1 to be a crucial prognostic marker. It had a significantly independent negative prognostic effect on DSS in non-GIST STS.

Fascin is an actin-bundling protein that is found in membrane ruffles, microspikes, and stress fibers [180]. In tumorigenesis, it augments cell motility and is therefore an important factor in the progression and metastasis of cancers [123]. Fascin is reported to be overexpressed in sarcomatoid, in contrast to conventional, non-small cell lung carcinoma [126]. In leiomyomatous tumors of the uterus it was associated with a higher malignancy grade [127]. Our data are in accordance with these findings, we found fascin expression to be associated with a shorter STS survival in univariate analyses.

The majority of studies devoted to NF-kB agree that this marker enhances tumor invasiveness and metastasis resulting in shorter DSS. This marker is demonstrated to be constitutively activated in various tumor cells where it promotes tumor cell proliferation and survival, as well as metastasis, inflammation, invasion, and angiogenesis [118]. On the other

hand, there are some studies which show tumor inhibiting role of NF-kB. Thus, in a recent work by Al-Saad et al., NF-kB p 105 was reported to have a favorable impact on DSS in operable non-small cell lung carcinoma patients [119]. We found NF-kB p 105 expression in STS to indicate a poor prognosis.

The polarity complex proteins Par-6 and PKC-ζ have been identified as EMT-associated factors [129], increasing proliferation, migration and invasiveness in cell cultures [130,131]. Cornford et al. reported that PKC-ζ expression was significantly higher in prostatic carcinomas than in non-neoplastic prostate tissue [132]. We were unable to find studies on Par-6 expression in human sarcomas through PubMed searches. In our study, we observed PKC-ζ expression to be a significant indicator of shorter DSS, while Par-6 did not show any prognostic significance.

E-cadherin, being responsible for epithelial cell junction, is rarely expressed in STS, except for synovial and epithelioid sarcomas, as well as mesothelioma, which naturally express both epithelial and mesenchymal markers. As expected, E-cadherin was in this study expressed aberrantly in a minority of STS and failed to demonstrate any association with survival.

Vimentin is a recognized marker of advanced aggressivity in epithelial tumors. Its negative influence on patient survival has been demonstrated in several human cancers including breast [181], gastric [182] and oral carcinoma [183]. The STSs which by definition express vimentin are not generally investigated for the prognostic importance of its grade of expression. In our material, all tumor cells were positive for vimentin, but at varying degrees. All STSs were dichotomized as strongly positive tumors or not, but there was no difference in survival between these two groups.

The results indicate that the factors known as EMT markers in epithelial malignancies have similar roles in the progression of STSs. This, in turn, since STSs are already mesenchymal neoplasms, means that EMT, as a term, is either misnomer or just particular "epithelial" example of a broader idiom, such as dedifferentiation or anaplasia.

5.2.2. Paper II

In this paper we investigated distribution of female steroid hormone receptors, ER and PgR, and found that at least one of them was expressed in 53% of non-GIST STS cases when 1% is considered a positivity threshold. Further, we found PgR to be a negative prognostic factor for DSS in non-GIST STS male patients, while ER expression by STS was a positive

prognosticator for female patients. Additionally, we investigated all possible ER/PgR co-expression profiles and found the ER-/PgR+ phenotype to be an independent negative prognostic factor for DSS in the whole cohort of non-GIST STS patients.

Steroid hormones, and therefore their receptors too, are known to stimulate the progression of breast cancer as well as other gynecological tumors. ER served for decades as a predictor of success of hormone-ablation therapy for ER-positive in contrast to ER-negative breast cancers [137,138]. A diversity of soft tissue tumors expresses both ER and PgR [139,184-186], but there is much uncertainty concerning the steroid hormone receptor expression value in the mesenchymal tumors. This is probably due to vagueness of positivity cut-off for non-gynecological tumors which is as high as 10% in most of studies. We have modified the Allred score [187] for STSs and used 1% positivity as cut-off value. The strong and moderate (score 3 and 2, respectively) hormone receptor expression occurred mostly in sarcomas of uterus, pelvis and breast, while the weak (score 1) expression of both ER and PgR was surprisingly evenly distributed among location, gender and age. Generally, 36% of the tumors expressed ER and 30% expressed PgR in our material.

The rate of ER and PgR expression in leiomyomatous tumors of uterus was frequently demonstrated to rise with the grade of differentiation of malignant tumors from benign leiomyoma to high grade malignant leiomyosarcoma [139,140]. However, the information concerning steroid hormone receptor expression in soft tissue tumors outside the gynaecological area is scarce and controversial. In our study, ER expression (using positivity threshold at 1%) had a positive impact on survival in women (univariate analysis), but failed to show any significant value in the Cox proportional hazards analysis. PgR expression showed a clearly negative impact on DSS in men and slightly positive, but not significant influence on survival in women.

The value of ER/PgR coexpression profiles is well studied in breast carcinoma. In a few words, any hormone receptor positivity gives better prognosis for success of antihormonal therapy [188,189]. In our study, the ER-/PgR+ profile (14% of the tumors) was a significantly unfavourable factor for the whole patient cohort both in univariate and in multivariate analyses.

This study is, to our knowledge, the first to elucidate the distribution and prognostic value of steroid hormone receptors in non-GIST STSs. Both ER and PgR were surprisingly frequently expressed in sarcomas irrelatively to patient's gender and location of the tumor.

Their prognostic significance is not much of a surprise, since both of them in essence are growth factors.

5.2.3. Paper III

In this paper we investigated the expression of a set of biomarkers belonging to the Akt-PI3K signaling pathway in non-GIST STS patients. In univariate analyses, p-Akt Thr^{308}, Akt2 and PI3K showed significant unfavorable influence on survival of the whole cohort of patients. In addition, PI3K was found to be an independent negative prognostic factor of DSS in these patients. We have also elucidated different prognostic effects of Akt phosphorylation site, alone and with regard to patient's gender. Further, the co-expressions of these markers and female steroid hormone receptors suggested additive effects or even possible synergisms between these different pathways in non-GIST STSs.

PI3K is, via PDK1 and mTORC2 dependent activation, an upstream regulator of all Akt isoforms, and plays an important role in the PI3K/Akt pathway. Its high expression has been implicated as an adverse prognostic factor in many types of cancer [190-192]. In STS, we observed that PI3K expression was a significant independent indicator of shorter DSS.

Being a promoter of cell survival, Akt1 has been regarded as a major factor in many types of cancer [145-147]. The majority of studies agree on negative prognostic impact of Akt expression [149-151], while there are several reports demonstrated an opposite action of Akt [152,153]. The latter studies utilized antibodies against p-Akt Ser^{473}, while the former were based on p-Akt Thr^{308} expression [150,193]. Al-Saad et al. [179] have recently compared the prognostic impact of Akt phosphorylated at both sites and demonstrated that expression of p-Akt Thr^{308}, but not p-Akt Ser^{473}, negatively influenced prognosis in patients with non-small cell lung cancer. For the whole cohort, we have also found that p-Akt Thr^{308} expression was associated with a shorter STS survival in the univariate analyses, while p-Akt Ser^{473} expression had no significance. However, calculated separately for each gender, high expression of p-Akt Thr^{308} was a negative prognostic factor particularly for men, in contrast to p-Akt Ser^{473}, which appeared to be a negative prognosticator exclusively for female patients. Such gender diversity prompted us to investigate coexpressions of these markers with ER and PgR, which were recently shown to activate PI3K/Akt signalling pathway [194,195]. In our study, the prognostic diversity of these factors in men and women was enhanced in the co-expression profiles: male patients with STSs expressing simultaneously p-Akt Thr^{308} and PgR had statistically significantly reduced survival. For women, the ER-/ p-Akt Ser^{473}+ expression

profile was the most unfavorable phenotype. The co-expression of PI3K with both ER and PgR showed multiple independent negative impacts on survival in STS patients with the phenotypes ER-/PI3K+ in women and PgR+/PI3K+ in men being the least favorable.

Akt2 has been described mostly as a contributor of the insulin signaling pathway, but in Akt1 deficient mice it is also proved to substitute, at least partly, the role of Akt1 in growth and proliferation [196]. We found Akt2 expression to be associated with significantly shorter DSS in univariate analysis. This might be explained by the extra-endocrine function of Akt2 [196].

Le Page et al. reported that nuclear Akt1 and Akt2 expression significantly correlated with favorable outcome in 63 prostate cancer patients, while cytoplasmic Akt1 expression correlated with a higher risk of postoperative prostate-specific antigen (PSA) recurrence and shorter PSA recurrence interval [197]. In the present study, we were able to find such dependence only for nuclear p-Akt Thr^{308} expression, which proved to be prognostically favorable compared to cytoplasmic and especially mixed cytoplasmic and nuclear location.

The exact mechanisms by which Akt phosphorylation site and combined Akt/PI3K and steroid hormone receptors coexpression influence on intracellular signaling cannot be elucidated by translational studies. The findings may, however, indicate that these factors are in play in the progression process of STSs'.

6. CONCLUSIONS AND IMPLICATIONS FOR FURTHER RESEARCH

We have investigated three sets of markers augmenting tumor growth, proliferation and dedifferentiation in non-GIST STS patients. Several markers and also interesting co-expressions proved to be independent prognostic factors. Although the precise molecular interactions resulting in STS tumor cell dedifferentiation and proliferation are still unclear, our findings may help to identify a subgroup of patients with aggressive tumors which require adjuvant therapy.

Moreover, the biomarkers indicating such aggressiveness can represent molecular targets with the future development of small-molecule targeted therapy. PI3/Akt pathway components belong to the family of serine-threonine kinases, which are comprehended as "drugable" [148], and it was already shown effect of such Akt targeted agents on several subtypes of sarcomas *in vitro* [198,199]. ER and PgR positivity, found to be surprisingly common in STSs could possibly identify patients who may have benefit from endocrine therapy.

TMA and IHC have proven to be reliable and feasible methods for biomarker studies on tissues. While these methods might not be the most novel, they are well-proven and highly reliable when one takes into account their limitations. Our group will continue to conduct TMA and IHC studies on non-GIST STSs. We would particularly like to explore factors responsible for TGF-β modulation, such as, matrix metalloproteinases, integrins, angiogenic and inflammatory agents as well the isoforms and specific receptor of this enigmatic growth factor. This also concerns ER and PgR isotypes.

In addition we have started to measure proliferation-related micro-RNAs by *in situ* hybridization in paraffinized tissue from non-GIST STS patients. We hope to further elucidate prognostic factors in non-GIST STS patients as well as seek further knowledge on the impact of proliferation and (de)differentiation in this patient group.

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APPENDIX

HISTOLOGIC CLASSIFICATION OF SOFT TISSUE TUMORS

Fibroblastic/myofibroblastic tumors
- **Benign**
  - Nodular fasciitis (including intravascular/cranial)
  - Proliferative fasciitis/myositis
  - Organ-associated pseudosarcomatous myofibroblastic proliferations
  - Ischemic fasciitis
  - Fibroma of tendon sheath
  - Pleomorphic fibroma of skin
  - Nuchal-type fibroma/Gardner-associated fibroma
  - Elastofibroma
  - Nasopharyngeal angiofibroma
  - Keloid
  - Collagenous fibroma (desmoplastic fibroblastoma)
  - Fibrous hamartoma of infancy
  - Infantile digital fibromatosis
  - Myofibroma/myofibromatosis
  - Juvenile hyaline fibromatosis
  - Gingival fibromatosis
  - Fibromatosis colli
  - Infantile fibromatosis
  - Calcifying aponeurotic fibroma
  - Calcifying fibrous pseudotumor
- **Intermediate**
  - Adult-type fibromatosis
    - Superficial (palmar, plantar, penile, knuckle pads)
    - Deep (extra-abdominal, abdominal, intra-abdominal)
- **Malignant**
  - Pleomorphic undifferentiated sarcoma/malignant fibrous histiocytoma
    - Storiform-pleomorphic type
    - Myxoid type
    - Giant cell type
    - Inflammatory type

Lipomatous tumors
- **Benign**
  - Lipoma
  - Angiolipoma
  - Myolipoma
  - Chondroid lipoma
  - Spindle cell/pleomorphic lipoma
  - Lipoblastoma/lipoblastomatosis
  - Myelolipoma
  - Hibernoma
  - Lipomatosis
- **Intermediate**
  - Atypical lipoma (superficial well-differentiated liposarcoma)
- **Malignant**
  - Atypical lipomatous tumor/well-differentiated liposarcoma
    - Lipoma-like
    - Sclerosing
    - Spindled
    - Inflammatory
  - Myxoid/round cell liposarcoma
  - Pleomorphic liposarcoma
  - Dedifferentiated liposarcoma

Smooth muscle tumors and related lesions
- **Benign**
  - Leiomyoma
  - Angiomyoma
  - Intranodal palisaded myofibroblastoma
  - Mammary myofibroblastoma
  - Benign genital stromal tumors
    - Angiomyofibroblastoma
    - Cellular angiofibroma/angiomyofibroblastoma of male genital tract
    - Aggressive angiomyxoma
    - Superficial cervicovaginal myofibroblastoma
  - Intravenous leiomyomatosis
  - Leiomyomatosis peritonealis disseminata
- **Malignant**
  - Leiomyosarcoma

Extragastrointestinal stromal tumors
- **Benign**
- **Malignant**

Skeletal muscle tumors
- **Benign**
  - Cardiac rhabdomyoma
  - Adult rhabdomyoma
  - Fetal rhabdomyoma
    - Myxoid (classic)
    - Intermediate (cellular, juvenile)
  - Genital rhabdomyoma
- **Malignant**
  - Embryonal rhabdomyosarcoma
    - Usual type
    - Botryoid type
    - Spindle cell type
  - Alveolar rhabdomyosarcoma
  - Pleomorphic rhabdomyosarcoma
  - Sclerosing rhabdomyosarcoma
  - Other (rhabdoid features, anaplastic features)
  - Rhabdomyosarcoma with ganglion cells (ectomesenchymoma)

Tumors of blood and lymph vessels
- **Benign**
  - Papillary endothelial hyperplasia
  - Hemangioma
    - Capillary hemangioma
    - Cavernous hemangioma
    - Venous hemangioma
    - Arteriovenous hemangioma
    - Pyogenic granuloma
    - Acquired tufted hemangioma
    - Hobnail hemangioma
    - Spindle cell hemangioma
  - Lymphangioma
  - Lymphiomyoma/lymphangiomyomatosis
  - Angiomatosis
  - Lymphangiomatosis
- **Intermediate**
  - Epithelioid hemangioendothelioma
  - Hobnail hemangioendothelioma (retiform, Dabska-type)
  - Epithelioid sarcoma-like hemangioendothelioma
  - Kaposiform hemangioendothelioma
  - Polymorphous hemangioendothelioma
- **Malignant**
  - Angiosarcoma
  - Kaposi sarcoma

Perivascular tumors
- **Benign**
  - Glomus tumor
    - Usual type
    - Glomangioma (glomuvenous malformation)
    - Glomangiomyoma
    - Glomangiomatosis
  - Myopericytoma
  - Hemangiopericytoma-like tumor of nasal passages

Continued

Malignant
- Malignant glomus tumor

Synovial tumors

Benign
- Tenosynovial giant cell tumor
  - Localized type
  - Diffuse type

Malignant
- Malignant tenosynovial giant cell tumor

Mesothelial tumors

Benign
- Adenomatoid tumor

Intermediate
- Multicystic mesothelioma
- Well-differentiated papillary mesothelioma

Malignant
- Diffuse mesothelioma
  - Epithelial type
  - Sarcomatoid type
  - Biphasic type

Peripheral nerve sheath tumors and related lesions

Benign
- Traumatic neuroma
- Mucosal neuroma
- Pacinian neuroma
- Palisaded encapsulated neuroma
- Morton's interdigital neuroma
- Nerve sheath ganglion
- Neuromuscular hamartoma
- Neurofibroma
  - Usual type (localized)
  - Diffuse
  - Plexiform
  - Epithelioid
  - Pigmented
- Schwannoma
  - Usual type
  - Cellular
  - Plexiform
  - Degenerated (ancient)
  - Epithelioid
  - Neuroblastoma-like
- Melanotic schwannoma
- Perineurioma
  - Intraneural
  - Extraneural
- Granular cell tumor
- Neurothekeoma
  - Myxoid type
  - Cellular type
- Ectopic meningioma
- Glial heterotopia

Malignant
- Malignant peripheral nerve sheath tumor (MPNST)
  - Usual type
  - MPNST with rhabdomyoblastic differentiation (malignant Triton tumor)
  - Glandular MPNST
  - Epithelioid MPNST
- Malignant granular cell tumor
- Clear cell sarcoma of tendon and aponeurosis
- Malignant melanotic schwannoma
- Extraspinal ependymoma

Primitive neuroectodermal tumors and related lesions

Benign
- Ganglioneuroma
- Pigmented neuroectodermal tumor of infancy (retinal anlage tumor)

Malignant
- Neuroblastoma
- Ganglioneuroblastoma
- Ewing sarcoma/primitive neuroectodermal tumor
- Malignant pigmented neuroectodermal tumor of infancy

Paraganglionic tumors (paraganglioma)

Benign

Malignant

Extraskeletal osseous and cartilaginous tumors

Benign
- Myositis ossificans
- Fibro-osseous pseudotumor of digits
- Fibrodysplasia ossificans progressiva
- Extraskeletal chondroma/osteochondroma
- Extraskeletal osteoma

Malignant
- Extraskeletal chondrosarcoma
  - Well-differentiated chondrosarcoma
  - Myxoid chondrosarcoma
  - Mesenchymal chondrosarcoma
- Extraskeletal osteosarcoma

Miscellaneous tumors

Benign
- Tumoral calcinosis
- Congenital granular cell tumor
- Myxoma
  - Cutaneous
  - Intramuscular
- Juxta-articular myxoma
- Ganglion
- Amyloid tumor

Intermediate
- Ossifying fibromyxoid tumor
- Inflammatory myxohyaline tumor
- Mixed tumor/myoepithelioma/parachordoma
- Pleomorphic hyalinizing angiectatic tumor
- Hemangiopericytoma/solitary fibrous tumor/giant cell angiofibroma
- Perivascular epithelioid cell family of tumors (PEComa)

Malignant
- Synovial sarcoma
- Alveolar soft part sarcoma
- Epithelioid sarcoma
- Desmoplastic small round cell tumor
- Malignant extrarenal rhabdoid tumor

Figure 1E. Histologic classification of soft tissue tumors. *From Weiss SW, Goldblum R: Enzinger & Weis's Soft Tissue Tumors, 5th edn. Philadelphia: Mosby, Elseiver Inc; 2008. Permission obtained from Elseiver Inc.*

Paper 1

Paper 2

Paper 3

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