Prognostic factors in soft tissue sarcoma. Tissue microarray for immunostaining, the importance of whole-tumor sections and time-dependence.

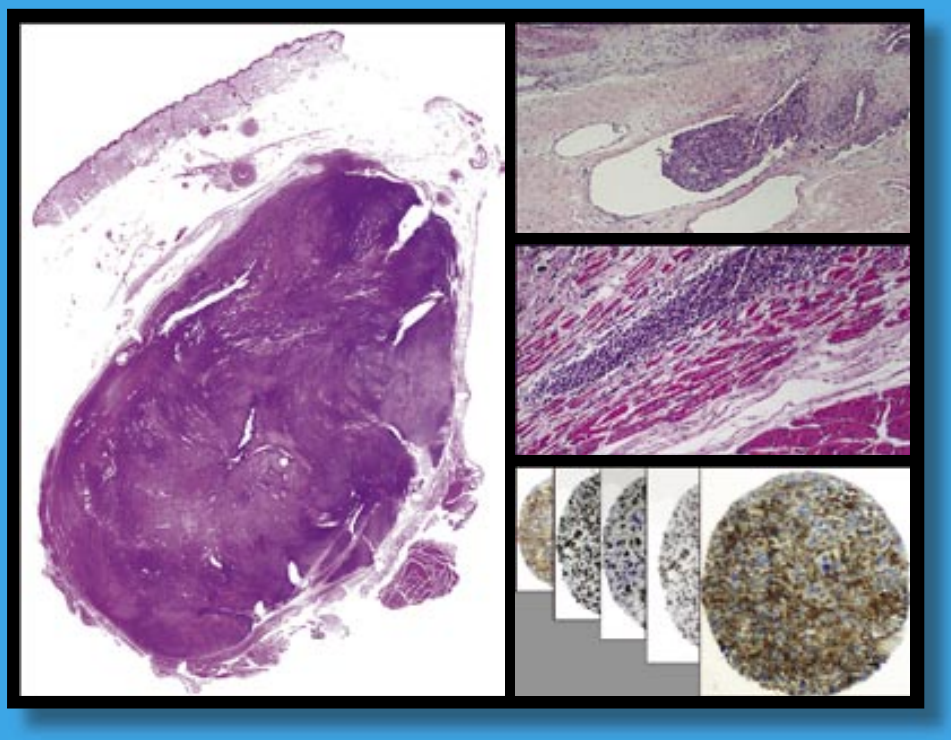
Engellau, Jacob

2004

Prognostic factors in soft tissue sarcoma

Tissue microarray for immunostaining, the importance of whole-tumor sections and time-dependence

Jacob Engellau



Acta Orthopaedica Scandinavica

Supplementum no 314 Volume 75 December 2004

Content

Studies I–IV

This work is based on the following publications:

I. Tissue microarray technique in soft tissue sarcoma: immunohistochemical Ki-67 expression in malignant fibrous histiocytoma.
J. Engellau, M. Åkerman, H. Anderson, H.A. Domanski, E. Rambech, T.A. Alvegård, M. Nilbert.
Applied Immunohistochemistry & Molecular Morphology 2001; 9(4): 358-63.

II. Expression profiling using tissue microarray in 211 malignant fibrous histiocytomas confirms the prognostic value of Ki-67.
J. Engellau, A. Persson, P-O. Bendahl, M. Åkerman, H.A. Domanski, B. Bjerkehagen, P. Lilleng, J. Weide, A. Rydholm, T.A. Alvegård, M. Nilbert.
Virchows Archiv 2004: 445: 224-30.

III. Improved prognostication using whole-tumor sections in soft tissue sarcoma: independent information from vascular invasion, necrosis, growth pattern and immunostaining.
J. Engellau, P-O. Bendahl, A. Persson, H.A. Domanski, M. Åkerman, P. Gustafson, T.A. Alvegård, M. Nilbert, A. Rydholm.
Submitted.

IV. Time dependence of prognostic factors for patients with soft tissue sarcoma: a Scandinavian Sarcoma Group study of 338 malignant fibrous histiocytomas.
J. Engellau, H. Anderson, A. Rydholm, H.C.F. Bauer, K. Sundby Hall, P. Gustafson, M. Åkerman, J. Meis-Kindblom, T.A. Alvegård, M. Nilbert.
Cancer 2004; 100: 2233-41.

Abbreviations

APC	Adenomatous polyposis coli
AUC	Area under the curve
BAX	Bcl-2-associated X protein
bcl	B-cell lymphoma/leukemia
cdk	Cyclin-dependent kinase
CGH	Comparative genome hybridization
CI	Confidence interval
CT	Chemotherapy
EORTC	European Organisation for Research and Treatment of Cancer
ER	Estrogen receptor
ESHO-RHT	European Society of Hyperthermic Oncology-Regional Hyperthermia
FISH	Flourescence in-situ hybridization
FNA	Fine needle aspiration
FNCLCC	Fédération National des Centres de Lutte Contre le Cancer
GIST	Gastrointestinal stromal tumor
Gy	Gray
H&E	Hematoxylin and erythrosine
HER2/neu	Human epidermal growth factor receptor 2. Also called c-ERBB2
HPF	High power field
HR	Hazard ratio
IHC	Immunohistochemical
Ki-67	Proliferation antigen Kiel 67
LEF	Lymphoid enhancing factor
LR	Local recurrence
LRP	Lung resistance protein
mdm2	Murine double minute 2
MDR	Multidrug resistance
MFH	Malignant fibrous histiocytoma
MMP	Matrix metalloproteinase
MPNST	Malignant peripheral nerve sheath tumor
MRP	Multidrug resistance-associated protein
NCI	National Cancer Institute
NOS	Not otherwise specified
OR	Odds ratio
p53	53 kDa protein
Pgp	P-glycoprotein
PgR	Progesterone receptor
Rb	Retinoblastoma
RMS	Rhabdomyosarcoma
ROC	Reciever operating characteristic
RR	Relative risk
RT	Radiotherapy
RT-PCR	Reverse-transcriptase plymerase chain reaction
SD	Standard deviation
SEM	Standard error of the mean
SPF	S-phase fraction
SSG	Scandinavian Sarcoma Group
STBSG	Soft Tissue and Bone Sarcoma Group
STS	Soft tissue sarcoma
TCF	T-cell factor
TMA	Tissue microarray
TNM	Tumor-Node-Metastasis
Wnt	Derived from the Drosophila gene *wingless* and oncogene *int-1*

Thesis at a glance

Study I

Aim and methods: To evaluate the high-throughput TMA technique in STS. IHC expression of the proliferation marker Ki-67 was assessed in 47 tumor blocks from 11 malignant fibrous histiocytomas (MFH) using TMA and conventional tumor sections.

Findings: TMA gave good quality staining and similar results as conventional tumor sections, with mean 9% higher staining for Ki-67 using TMA.

Conclusion: TMA can be applied to IHC studies of STS, but multiple tumor blocks should when possible be used, and optimally 3 biopsies should be obtained from each tumor block to compensate for loss and variability.

Study II

Aim and methods: To explore the prognostic value of IHC staining for Ki-67, p53, cyclin A, bcl-2, CD44, and Pgp in a series of MFH with 201 primary tumors, 44 local recurrences (LR) and 18 metastases, using the TMA technique.

Findings: Ki-67 was an independent prognostic marker for distant metastasis, whereas the other markers did not show any prognostic correlations.

Conclusion: Ki-67 should be considered for inclusion in clinical prognostication.

Study III

Aim and methods: To investigate the value of whole-tumor sections for determination of tumor-related prognostic factors in 140 primary STS of mixed histotypes. IHC staining for Ki-67, p53, cyclin A, bcl-2, ß-catenin, CD44, and Pgp was investigated in the peripheral tumor growth zone.

Findings: The whole-tumor sections facilitated identification of vascular invasion, and allowed assessment of the peripheral tumor growth pattern, which were strong prognostic factors for metastasis. Ki-67, ß-catenin, CD44 and Pgp were also strong prognostic factors for metastasis when analyzed in the peripheral tumor growth zone.

Conclusion: Whole-tumor sections enable analysis of peripheral tumor growth pattern in STS, better assessment of vascular invasion, and targeted immunostaining from the tumor periphery, all of which correlate with prognosis.

Study IV

Aim and methods: To study time-dependence of tumor-related prognostic factors in STS. Tumor size, malignancy grade, depth, necrosis, vascular invasion, mitotic rate, and LR were analyzed in 338 pleomorphic STS for the time intervals 0–2 years and >2 years.

Findings: The prognostic factors were time-dependent; their prognostic value was limited to the first 2 years of follow-up. In contrast, LR was a strong prognostic factor for metastasis regardless of when it occurred.

Conclusion: Initial prognostic factors have a value limited to the first 2 years of follow-up, whereas LR can be seen as a dynamic prognostic factor that can be monitored during follow-up.

Introduction and aims

Soft tissue sarcomas (STS) are highly malignant tumors; metastasis develops in 1/3 of the patients, most of who will die from their tumor. Multiple trials with adjuvant chemotherapy have been launched to increase survival in high-risk patients. However, there is no consensus on the criteria that should be used to define high-risk tumors for adjuvant chemotherapy and hence multiple prognostic systems are currently in use in a tumor type that accounts for barely 1% of malignancies. The overall purpose of this thesis was to identify and evaluate prognostic markers in STS of the extremities and the trunk wall, specifically:

- To evaluate the tissue microarray method for immunohistochemical studies in STS.
- To apply tissue microarray and immunostaining to identify prognostic markers for distant metastasis in a subset of pleomorphic STS; malignant fibrous histiocytoma.
- To investigate the use of whole-tumor sections to determine histopathological prognostic factors for local recurrence and distant metastasis, including peripheral tumor growth pattern in a series of mixed STS, and to study the immunohistochemical expression of molecular markers in targeted areas in the peripheral growth zone.
- To assess time-dependence of prognostic factors for distant metastasis in STS.

Epidemiology

STS may occur anywhere in the body, but 3/4 are located in the extremities, most commonly in the thigh, and 1/10 are located in the trunk wall. There is a slight male predominance, and there are no data to indicate that the incidence is changing over time. The etiology of STS is generally unknown, although an increased incidence has been reported after exposure to phenoxyacetic herbicides, chlorophenols and their contaminants (dioxin), following radiation therapy, and use of androgen-anabolic steroids [41, 74, 86, 177, 194]. Also infections with oncogenic viruses, such as human herpes virus 8 and Epstein-Barr virus, may induce a STS [177]. A number of genetic diseases such as retinoblastoma, Li-Fraumeni syndrome, Gardner's syndrome, Werner's syndrome, Beckwith-Wiedemann syndrome, nevoid basal cell carcinoma syndrome, and neurofibromatosis type I and type II, carry an increased risk of STS, but these account for only a minor part of the tumors [48, 104].

Histopathology

More than 50 histological types of STS have been identified. The histological classification is based on similarity to the mesenchymal tissue type from which the tumor supposedly stems (Table 1). In several types of STS, notably in many of the common pleomorphic sarcomas, and in synovial sarcoma, alveolar soft part sarcoma, clear cell sarcoma, extraskeletal myxoid chondrosarcoma, and epithelioid sarcoma, no similarity suggestive of tissue origin can be discerned. The age-related incidence varies among the histologic types; embryonal rhabdomyosarcoma and extraskeletal Ewing tumor/primitive neuroectodermal tumor are typical childhood tumors, and many of the synovial sarcomas occur in adolescents and young adults, whereas the median age of patients with liposarcoma and leiomyosarcoma is about 60 years, and for malignant fibrous histiocytoma

Table 1. Principal histotype classes of soft tissue tumors

WHO classification of soft tissue tumors
1. Adipocytic tumors
2. Fibroblastic/Myofibroblastic tumors
3. So-called fibrohistiocytic tumors
4. Smooth muscle tumors
5. Pericytic (perivascular) tumors
6. Skeletal muscle tumors
7. Vascular tumors
8. Chondro-osseous tumors
9. Tumors of uncertain differentiation

(MFH) about 70 years [55, 177]. Because of the different treatment strategies, STS in children are considered separately from those in adults. In adult STS, the surgical possibilities and the natural history have led to a commonly used separation of tumors located in the extremities and trunk wall from those located in the retroperitoneum, the abdomen, the thorax, and the head and neck.

During the last decades there have been considerable changes in the classification of STS. Following an increased use of ancillary techniques, such as electron microscopy, immunostaining, cytogenetics, and molecular genetics, a line of differentiation can be determined in an increasing number of STS. The lineage of differentiation is of clinical importance; in pleomorphic STS myogenic differentiation is an adverse prognostic factor [47]. This development has had particular consequence for MFH, which was introduced as a specific entity during the 1960's and quickly became the most common STS subtype [128, 132]. MFH has fibroblastic appearance but was proposed to be of histiocytic origin and was subclassified into 5 histopathological types: pleomorphic-storiform, myxoid, inflammatory, giant cell, and angiomatoid, of which the pleomorphic-storiform and the myxoid types were the most common [55, 177]. The angiomatoid type was later shown to rarely disseminate, and was reclassified as a separate entity (angiomatoid fibrous histiocytoma) [48]. During the last decade the histogenesis or line of differentiation of MFH has been debated among sarcoma pathologists. Based on thorough re-examination, including the clinical presentation and extensive immunostaining, sometimes also with the use of electron microscopy and genetic analyses, a line of differentiation can be demonstrated in many of the tumors previously classified as MFH [33, 46, 47, 69, 109]. Currently, MFH is diagnosed by ruling out other pleomorphic and myxoid sarcomas of specific lineage, foremost liposarcoma and leimyosarcoma, as well as carcinomas, melanomas and lymphomas [48, 90, 93, 112]. The sarcomas diagnosed as MFH all are considered variants of essentially fibroblastic/myofibroblastic tumors of which the storiform-pleomorphic variant is the most common and the giant-cell and inflammatory subtypes are rare. The term myxofibrosarcoma is proposed to be used synonymous with the myxoid variant of MFH [48]. This change in nomenclature has, however, not been universally accepted and at present myxofibrosarcoma and myxoid MFH are both used [112, 177].

Tumor genetics

The genetic basis of STS has been studied by cytogenetics, comparative genome hybridization (CGH), fluorescence *in situ* hybridization (FISH), reverse-transcriptase polymerase chain reaction (RT-PCR), and most recently gene expression profiling [35, 66, 105, 114, 124, 126, 134, 158, 163]. Based on the genetic aberrations STS can be divided in two main groups; one with recurrent, often simple reciprocal chromosomal translocations, and one with complex karyotypes [16]. For example, synovial sarcoma, myxoid liposarcoma, alveolar rhabdomyosarcoma, and clear-cell sarcoma are characterized by type-specific translocations (Table 2). The fusion genes that result from these translocations often involve growth factor receptors and these novel proteins may be of clinical value for diagnosis, and possibly also for early detection of recurrences [16, 35, 134]. However, fusion genes that can not be discerned due to karyotypic complexity may be present also in other sarcoma types [115]. Some fusion types, such as the *SS18/SSX1* and the *SS18/SSX2* in synovial sarcoma have been suggested to correlate with prognosis, but are not yet used in clinical prognostication [16, 89, 101]. STS containing such fusion genes are especially suitable for development of targeted therapies. The gastrointestinal stromal tumor (GIST) is a prime example of how the increased understanding of genetic alterations at a molecular level has had significant therapeutic consequences. In GIST, tyrosine kinase activation, usually KIT, is a prerequisite for the GIST diagnosis, and for the tumor phenotype. Immunohistochemical (IHC) staining for KIT expression is thus used as a diagnostic marker, and KIT also represents the target for tyrosine kinase inhibitor treatment [82].

Recent studies demonstrate that distinct gene expression profiles can be found in many STS types, including GIST, synovial sarcoma, and malignant peripheral nerve sheath tumors

Table 2. Examples of fusion genes in soft tissue tumors

Histotype	Chromosomal translocation	Fusion gene
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14)	*PAX3/FOXO1A*
	t(1;13)(p36;q14)	*PAX7/FOXO1A*
Alveolar soft part sarcoma	t(X;17)(p11;q25)	*ASPSCR1/TFE3*
Angiomatoid fibrous histiocytoma	t(12;16)(q13;p11)	*FUS/ATF1*
Clear cell sarcoma	t(12;22)(q13;q12)	*EWS/ATF1*
Congenital fibrosarcoma	t(12;15)(p13;q25)	*ETV6/NTRK3*
Dermatofibrosarcoma protuberans	t(17;22)(q22;q13)	*COL1A1/PDGFB*
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	*EWSR1/WT1*
Extraskeletal Ewing sarcoma	t(11;22)(q24;q12)	*EWSR1/FLI1*
	t(21;22)(q22;q12)	*EWSR1/ERG*
Extraskeletal myxoid chondrosarcoma	t(9;22)(q22;q12)	*EWSR1/NR4A3*
	t(9;17)(q22;q11)	*TAF15/NR4A3*
Low-grade fibromyxoid sarcoma	t(7;16)(q33;p11)	*FUS/CREB3L2*
Myxoid liposarcoma	t(12;16)(q13;p11)	*FUS/DDIT3*
Pericytoma with t(7;12)	t(7;12)(p21-22:q13-15)	*ACTB/GLI*
Synovial sarcoma	t(X;18)(p11;q11)	*SS18/SSX1* or *SSX2*

(MPNST), whereas other STS types, such as leiomyosarcoma and pleomorphic liposarcoma, show a larger variability [4, 91, 125, 158, 163]. However, even among the pleomorphic tumors, gene expression profiles reveal that some tumors form distinct subclusters, which is encouraging from a clinicopathological point of view since this may allow identification of diagnostic and prognostic distinct entities that have hitherto not been recognized.

- Type-specific chromosome translocations that result in novel fusion proteins are detected in some STS, whereas other entities display complex aberrations.
- Distinct gene expression profiles can be found in some types of STS and correlate with the specific gene fusions, but may also reveal new clinico-pathological subtypes.

Treatment

In adult STS of the extremities and the trunk wall, surgery is the mainstay of treatment. The surgical principles for STS were described by Enneking, who defined 4 types of excision [40]. An excision with an intralesional margin opens the tumor and leaves tumor tissue. A marginal excision entails *en-bloc* removal of the tumor, but surrounding tissue with possibly microscopic tumor extension is not excised. A wide excision is performed with *en-bloc* removal of the tumor and surrounding tissue. Radical excision entails an *en-bloc* resection of the tumor with the entire compartment within which the tumor has developed. An amputation can, depending on the location of the tumor, result in any of the surgical margins. The risk of a local recurrence (LR) is almost 100% with an intralesional margin, 60–80% with a marginal margin, 20–30% with a wide margin and less with a radical excision [177]. Radiotherapy (RT), administered either externally preoperatively or postoperatively, or as postoperative brachytherapy, reduces the risk of a LR [131, 166, 172, 188, 191]. The radiation dose administered is 50–60 Gy in marginal margins, but should be increased to 60–70 Gy in intralesional margins [172, 190]. Although this combined treatment reduces the rate of LR to 20–30% of patients with long follow-up, the metastasis-free survival is not affected [45, 138, 172, 193]. To reduce the number of surgical interventions and the rate of LR, centralization of STS is important for optimal diagnosis and treatment [22, 58, 145].

The role of chemotherapy in adult STS of the extremities and trunk wall is not clear. Despite the introduction of many chemotherapeutics since the beginning of the 1980's, retrospective analyzes of large series have not demonstrated any clear survival benefit or a reduction of the rate of metas-

tasis during the ensuing decades [178]. In contrast to this finding, large meta-analyses of studies on adjuvant doxorubicin-based chemotherapy have found a reduction of metastasis in STS of the extremities and trunk wall, but overall survival was only marginally improved with an absolute benefit of 7% at 10 years [1, 2]. Treatment with chemotherapy and surgical resection in patients with metastasis cures only selected patients [12, 14, 192].

- Surgery is the main treatment for STS and the surgical margin and the use of radiotherapy is important for the risk of local recurrence, but not for survival.
- Metastasis develops in 1/3 of the patients and about 1/5 develop a local tumor recurrence after long-term follow-up.
- Current chemotherapy regimens are of limited value.

Prognostic factors

Treatment failure in STS occurs as a LR, distant metastasis or both. For most cancers, the prognosis, i.e. risk of metastatic disease, is assessed by the TNM-system [53]. In STS metastases are rarely clinically present at diagnosis, and lymphatic spread is uncommon, which leaves only the primary tumor for risk assessment of metastasis. Furthermore, prognostic factors in STS may be type-specific, which complicates analyses of this rare tumor type. Prognostic factors should ideally be easily and reproducibly determined, clearly separate high-risk and low-risk groups, and preferably be present or absent in comparison to being a factor with continuously increasing risk.

In STS of the extremities and trunk wall a LR is seldom lethal, in contrast to retroperitoneal sarcomas, but causes considerable morbidity [172]. Strong prognostic factors for a LR are the surgical margin obtained, which correlates with the risk for remaining tumor tissue, and a high malignancy grade with hazard ratios (HRs) between 2 and 3 [172].

Many prognostic factors for metastasis have been proposed, such as patient age and sex, tumor depth, location, size, histotype, malignancy grade, mitotic rate, microscopic tumor necrosis, vascular invasion, DNA-ploidy, S-phase fraction (SPF), a number of molecular markers, and LR [24, 55, 139, 140]. Of these factors, patient age and sex have not been consistent, and have in population-based series not been found to be of prognostic value [55]. The independent value of tumor depth has been questioned, as there is a close correlation between tumor size and depth [151]. DNA aneuploidy has been reported to be an adverse prognostic factor in STS, and a high S-phase fraction has been reported to identify patients with a high risk of metastasis, and may also identify patients with a prolonged clinical course [5, 27, 57].

Malignancy grade

Malignancy grading is not uniformly performed and interpreted, but is principally based on a combination of histological type, cellularity, pleomorphism, mitotic activity, and presence of necrosis [25, 48, 92]. The two malignancy grading systems most commonly used are the National Cancer Institute (NCI) system used in North America, and the Fédération Nationale des Centres de Lutte Contre le Cancer (FNCLCC) system used in most parts of Europe [23, 31]. In Scandinavia, Sweden, Norway and Finland use a IV-tiered histological malignancy grading system based on cellularity, pleomorphism, nuclear atypia, tumor necrosis and mitotic rate [6, 107, 109], whereas a III-tiered system is used in Denmark [120]. High malignancy grade is a strong prognostic factor for metastasis [25, 55, 116, 165, 193]. However, the lack of a uniform grading system, and the uncertainty of what weights should be attributed to the various components in a grading system, the degree of subjectivity involved, and the different number of tiers used makes malignancy grade a complicated prognostic factor. In addition, in some histotypes such as the malignant peripheral nerve sheath tumor, desmoplastic small round cell tumor, extraskeletal myxoid chondrosarcoma, alveolar soft part sarcoma and epithelioid sarcoma malignancy grade lacks clinical value. Furthermore, in some STS types, such as the invariably high-grade rhabdomyosarcoma, the histological subtype is a more important factor for prediction of outcome than malignancy grade [25, 48, 92].

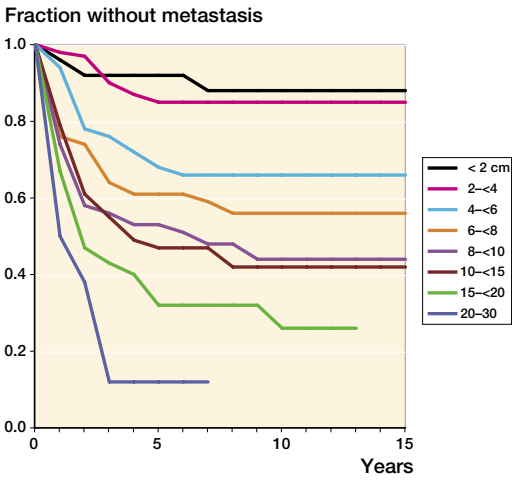


Figure 1. Continuous effect on metastasis-free survival of increasing tumor size in 338 MFH

Tumor size

Tumor size, which usually is easily measured, has repeatedly been shown to be of strong prognostic value [24, 55, 193]. As a prognostic factor, tumor size represents a continuum of increasing risk (Figure 1). Trovik et al. found that the relative risk of metastasis increased by 1.5% per 5 cm increment of tumor size [173]. The importance of size may also be histotype-specific; whereas in leiomyosarcoma, MFH and synovial sarcoma tumor size is a strong prognostic factor for metastasis this is not the case in liposarcoma [55, 177]. Furthermore, there is no consensus regarding which cut-off should be used, and dichotomization at 5 cm, 8 cm, and 10 cm have been used in different studies [24, 55, 193].

Necrosis

Tumor necrosis has in several studies been shown to be a strong prognostic factor for metastasis [32, 55, 106, 174]. Necrosis is incorporated in most malignancy grading systems, but the classification of necrosis required differs from present versus absent in the systems used in Sweden, Norway and Finland, to a cut-off at 15% of the inspected tumor areas in the NCI system, and a cut-off at 50% in the FNCLCC system [23, 31, 109].

Vascular invasion

Vascular invasion is a prognostic factor for metastasis in STS [55, 56, 106, 149, 150, 171]. Despite this, vascular invasion is not included in common grading systems [23, 31, 109]. Vascular invasion has been reported in rates between 6 and 25% in different studies of STS [55, 94, 106, 150]. To some extent this variability can be explained by different STS types being analyzed, but also in similar materials the rate of vascular invasion varies. Both false classification of vascular invasion e.g. tumor cells bulging into a vessel, or freely dispersed tumor cells within the vessel boundary due to sectioning artefacts, as well as false negative assessment due to invasion into very small vessels, or due to only small parts of the tumor being examined may also contribute to the divergent results. In addition, the size of the tumor area that should be assessed to reliably identify vascular invasion has not been defined.

Peripheral tumor growth pattern

The extent and importance of sarcoma growth into surrounding normal tissue has not been systematically studied. One previous study has suggested that infiltrating growth, compared to a pushing growth pattern, was a prognostic factor for metastasis in STS [106], but growth pattern is not incorporated into the malignancy grading systems currently used.

Local recurrence

LR is a prognostic factor for metastasis, but although a possible causal relationship between LR and metastasis has been intensely debated it has not been confirmed in population-based studies [59, 172]. Since the strong prognostic factors for a LR, i.e. high malignancy grade and tumor size, also are strong prognostic factors for metastasis, a LR could be indicative of a highly malignant tumor phenotype.

- Malignancy grade, tumor size, necrosis, and vascular invasion are strong prognostic factors in STS.
- Tumor depth is frequently used as a prognostic factor, but its independent role has been questioned.
- A local recurrence is often indicative of high malignancy, but is generally not the cause of metastasis.
- Tumor growth pattern has been suggested to represent a prognostic factor, but has not been systematically evaluated.

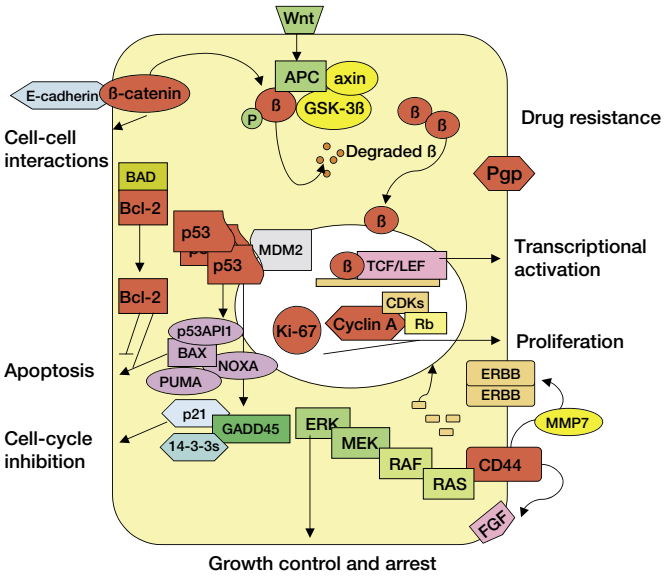


Figure 2. Molecular pathways suggested to be of prognostic importance in STS. Red symbols were examined in this thesis.

Biological markers

Several gene products, including markers of cell proliferation, cell adhesion, transcription control, apoptosis, onco-proteins and tumor suppressors have been implicated as prognostic factors in STS (Figure 2). Since sarcomas are rare, studies of the prognostic importance of biological markers have been hampered by limited sample sets, evaluation of single tumor markers, and partly contradictory results. One of our aims was to perform expression profiling using IHC and to apply the tissue-preserving TMA technology in order to study multiple tumor markers in large tumor series. The following sections review current data on the biological markers analyzed in this work with specific reference to their prognostic value.

Proliferation

Increased proliferation is one of the hallmarks of malignancy, and assays of proliferation include counting the number of mitotic figures per tumor area, determining the S-phase fraction (SPF) by flow cytometry, labeling of proliferating cells with radioactive thymidine, and IHC staining for growth-phase specific proteins such as Ki-67. In routine clinical histopathology assessment of mitotic figures in 10 microscopic high power fields (HPFs)(×40) is often used and the count is categorized as low (0–9/10 HPFs), intermediary (10–19/10 HPFs) or high (≥20/10 HPFs) [23]. The SPF has been shown to discriminate prognostic high-risk and low-risk patients for metastasis in STS but the technique is hampered by 30–40% non-informative histograms [57, 77]. Labeling of proliferating cells with radioactive thymidine is a laboratory-intense technique that is currently not commonly used, whereas IHC staining of Ki-67 protein is easy to perform and thus suitable for routine use. In STS a high Ki-67 level has been associated with poor prognosis in several studies [20, 30, 61, 62, 67, 72, 116, 175, 189]. Cut-off levels between 10% and 30% have been used, and the interpretation of the Ki-67 staining differs; should the average or the maximum labeling intensity in the tumor be used, and should proliferation be determined in the peripheral growth zone or in randomly selected areas?

Cyclins are integral proteins in the cell cycle progression during which their levels vary. Cyclin A binds to cyclin dependent kinase (cdk) 1 and cdk 2 and leads to phosphorylation of cell-cycle regulators such as the retinoblastoma (Rb) protein and p107 that are crucial to DNA replication and mitosis [119]. Increased cyclin A has been shown to correlate with low metastasis-free survival in STS [76, 130]. Furthermore, in synovial sarcoma, increased expression of cyclin A has been linked to the fusion gene *SS18/SSX1,* offering a possible explanation for the increased proliferation and poor prognosis of tumors with this fusion gene variant [187]. However, further validation of the prognostic importance of cyclin A in different histotypes of STS is needed.

ß-catenin

Dysfunctional cell-cell contact and alterations of the cell-extracellular matrix interactions are important facets of a malignant phenotype. Regulation of cell-signaling is mediated by numerous membrane-associated proteins. The transmembrane glycopro-

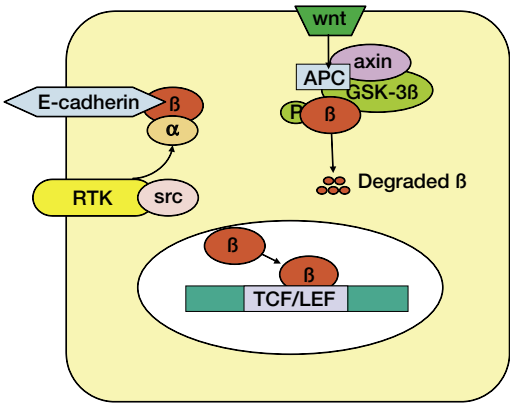


Figure 3. ß-catenin functions as part of the cadherin-catenin cell adhesion complex, and is via ß-catenin associated with the actin cytoskeleton. Phosphorylated ß-catenin is degraded by a APC-dependent complex, which is inhibited by Wnt signalling. When degradation is inhibited ß-catenin accumulates in the cytoplasm and translocates to the nucleus where transcriptional regulation through interaction with TCF/LEF takes place.

tein ß-catenin, linked to the cadherin/catenin complex, has been shown to have an adhesive function and also to play a central role in the Wnt-signalling pathway [75, 136, 159]. Loss of cadherin-mediated cell-cell adhesion occurs in the development of most epithelial malignancies. Studies of ß-catenin in STS have revealed altered expression with relocation of the ß-catenin staining to the cytoplasm and/or the nucleus in osteosarcoma, rhabdomyosarcoma, dedifferentiated liposarcoma, alveolar soft part sarcoma, and MFH [79, 153]. Translocation of ß-catenin to the cytoplasm and/or the nucleus has been detected in metastases and indeed loss of catenin-cadherin mediated cell adhesion may be of specific importance in the metastatic process [79, 87]. The prognostic importance of ß-catenin expression has been explored in two series of synovial sarcoma in which tumors with nuclear accumulation of ß-catenin were associated with a worse prognosis, although ß-catenin did not have any independent prognostic value [63, 152]. Correlations between the accumulation of ß-catenin and a high Ki-67 index has also been described in STS [99, 152]. Besides a role in cell-cell adhesion, ß-catenin also has an oncogenic function through interaction with nuclear transcription factors in the TCF/LEF families through which ß-catenin can stimulate cell proliferation [181] (Figure 3).

CD44

CD44 is a multifunctional cell surface molecule that is involved in cell proliferation, differentiation, migration, and angiogenesis. Several CD44 sequences exist because of alternative splicing and post-translational modification. Up-regulation of CD44 has been found in many cancer types, including lymphomas, colorectal cancer, renal cancer, and head and neck tumors [11, 83, 108, 123, 135, 176]. A prognostic importance of CD44 expression in STS has been suggested in two studies [83, 135]. CD44 exists in three forms; as a transmembrane receptor, an extracellular matrix associated fragment, and as a soluble fragment in the fluid phase. Depending on the extracellular cue, the cytoplasmatic domain can both promote and inhibit cell growth and metastasis. Cleavage of the transmembrane domain generates a cytosolic fragment that controls gene transcription, also of CD44 itself in a classical feedback mechanism, and an ectodomain which may accumulate in the extracellular matrix and interact with collagen, fibronectin and hyaluronan. In the fluid phase, soluble CD44 can function both as an agonist and an antagonist of the CD44 receptor itself or of matrix associated molecules [21, 141]. CD44 can through these mechanisms operate in a growth-, invasive- and metastasis promoting mode, or in a growth- and invasiveness inhibitory state, and the dominance of either of these modes can thus drive the tumor phenotype in either direction (Figure 4). Confirmatory studies of the prognostic role of CD44 in STS are needed, but phase I studies that apply anti-CD44 therapy have been suggested for tumor types in which CD44 expression has been correlated to metastasis.

p53, mdm2 and bcl-2

Controlled gene transcription is fundamental for cell control, and tumor suppressor genes are key regulators in this process. p53 alterations, either due to mutations or to defective interaction with mdm2, have been implicated in many human malignancies [180]. p53 has multiple roles including cell-cycle inhibition through activation of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$, activation of apoptotic cascades, and through interaction with tumor necrosis factor receptors [34, 36, 180]. Despite the importance of p53 in the regula-

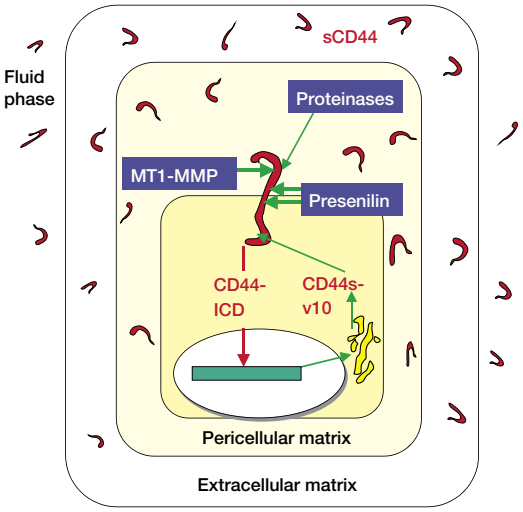


Figure 4. Transmembrane CD44 exists in a standard form and 10 isoforms. Depending on site of cleavage, a cytosolic or an extra-cellular fragment influences DNA transcription, or interacts with the pericellular, and extracellular matrix, and as a soluble fragment in a fluid phase.

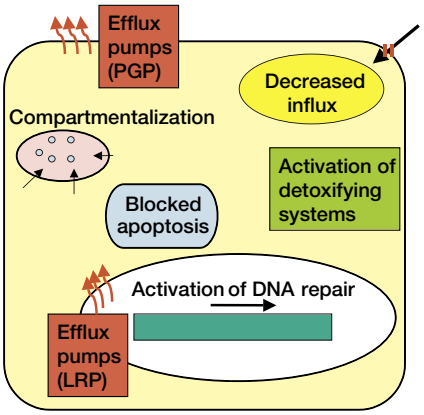


Figure 5. The cellular mechanisms of increased drug resistance to cytotoxic drugs. ATP-dependent efflux pumps actively transport drugs out of the nucleus or out of the cell.

tion of these vital cell functions, and the fact that mutated p53 has been shown to be of prognostic importance in other forms of cancer, the prognostic importance of p53 in STS is controversial [30, 67, 160]. Amplification of mdm2 is an especially important mechanism leading to p53 inactivation in STS, and some studies suggest that a possible prognostic importance of p53 may depend on co-expression of mdm2 [167, 184, 186]. However, the great number of studies that have been performed without reaching an answer as to a prognostic role of p53 in STS indicate that a possible prognostic importance of p53 immunostaining is small. The transcription regulators Rb and Bcl-2 also interact with p53 function and it is possible that combined alterations in these pathways may be of prognostic importance [36, 113, 116, 122, 185].

Multidrug resistance

Increased cellular resistance to cytotoxic drugs is mediated by several mechanisms e.g. down-regulation or loss of cell surface drug receptors, intracellular drug compartmentalization, increased resistance to apoptotic signals, increased DNA-repair, and increased drug transport from the nucleus or the cytoplasm [52] (Figure 5). The multidrug resistance (MDR) genes are believed to play a significant role in the limited efficacy of chemotherapy in most STS. The proteins that have been associated with MDR are involved in the transport of molecules from the nucleus to the cytoplasm, or from the cytoplasm to the extracellular space, whereby the intracellular drug concentration is reduced. Several MDR proteins have been identified, such as the p-glycoprotein (Pgp), the multidrug resistance-associated protein 1 (MRP1), and the lung resistance-related protein (LRP). In adult STS the levels of MDR proteins vary between histotypes with a particularly frequent expression in MFH, which explain the differences in clinical behavior between different histopathological types of STS [97]. Although MDR1 expression is regarded as important in sarcomas of both soft tissue and bone, there are no consistent data to demonstrate that the levels of Pgp influence tumor response or prognosis in STS [26, 96, 97, 182].

- Multiple biological markers have been suggested to have prognostic value in STS, but the findings in different studies are partly contradictory.
- A high proliferation, assessed by Ki-67, seem to be a consistent prognostic marker for poor outcome.
- Positive immunostaining for cyclin A, ß-catenin, CD44, and Pgp are suggested to correlate with poor prognosis, but the data are based on only a few studies.

- Immunostaining for p53 do not seem to be of independent prognostic information, but may need to be correlated with expression of mdm2, Rb, and bcl-2.

Tissue microarray

Conventional analysis of the tumor expression of biological markers uses small tumor sections for each IHC staining. These methods are quite labor intensive, and for limited tumor samples repeated sectioning may consume the paraffin-embedded tumor. Furthermore, only one tumor can be examined at a time. In 1986 Battifora [7] introduced a method in which multiple 1 mm in diameter tumor rods were wrapped in small intestine, and embedded in a paraffin block. This "sausage" block method enabled multiple tumors to be examined, and allowed multiple sections to be made for IHC staining. The method was subsequently modified from a sausage-block to a checkerboard configuration which improved the throughput [8]. The technique was, however, hampered by limitations in the identification of the individual rods, and in 1998, Kononen et al. [98] introduced the TMA technique. TMA is a tissue conserving, high-throughput technique that utilizes multiple tumor core biopsies of 0.6-1 mm in diameter that are transferred to a new paraffin block, which allows for multiple tumors to be analyzed simultaneously (Figure 6). Once the defined tumor material has been collected, TMA can be performed at a speed of 30–70 biopsies per hour, and up to 500–1000 such biopsies can be evaluated on a single slide [98, 147]. As multiple biopsies can be taken from a tumor to construct replicate TMA blocks, it has been calculated that multiple tumor biopsies from a 10 mm^2 tumor area, will allow more than 10.000 analyses [157]. However, as the TMA technique uses minimal core biopsies questions of applicability and reproducibility in large and heterogeneous tumors have been raised.

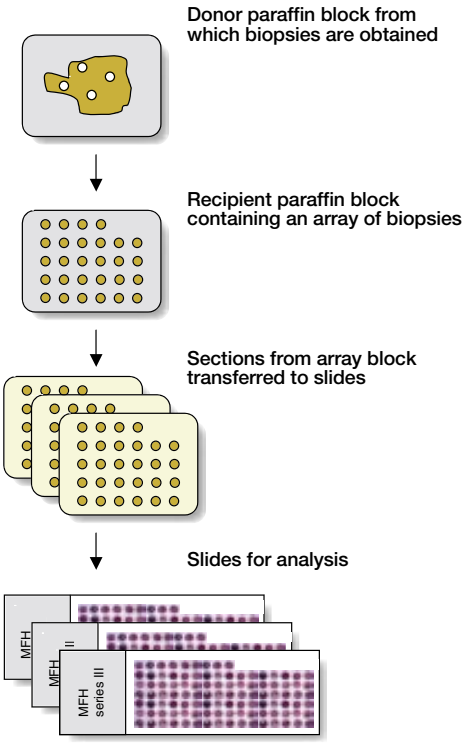


Figure 6. Schematic presentation of the tissue microarray technique.

Prognostic systems

Prognostic systems based on a few, presumably strong, factors have been developed in order to select patients for inclusion in trials of neoadjuvant or adjuvant chemotherapy. There is, however, no agreement as to which factors to include and since several prognostic systems currently are in use comparison of trial results is difficult (Table 3) [38, 42, 44, 49, 51, 78, 88, 111, 183].

- There is no consensus about optimal prognostic factors in STS and several prognostic systems are currently in use.
- Most systems include malignancy grade and tumor size, and to a varying extent include factors such as tumor depth, mitotic rate, differentiation, vascular invasion, and tumor necrosis.

Time-dependence of prognostic factors

In several types of malignancies, e.g. breast cancer, colorectal cancer, and malignant lymphomas the importance of prognostic factors changes during

Table 3. Examples of current prognostic systems for metastasis in STS

Group or Cancer Center	Protocol	High-risk criteria	Reference
EORTC STBSG	62931 Adjuvant CT	Size > 5 cm and grade II or III	Donato di Paola et al. [38]
EORTC STBSG	62771 Adjuvant CT	Size ≥ 8 cm and any grade, or Size < 8 cm and grade II, III, or LR, grade II, III, or Inadequate surgery and grade II, III	Gortzak et al. [51]
EORTC STBSG	62961/ ESHO RHT-95 Neoadjuvant CT and hyperthermia	Size ≥ 5 cm and grade II, III, and extra-compartmental	Issels et al. [78]
ISG	Adjuvant CT	Size ≥ 5 cm and deep seated, and grade III	Frustaci et al. [49]
SSG	SSG XIII Adjuvant CT	2 of the following: size > 8 cm, vascular invasion, tumor necrosis	Fernberg et al. [42]
CCOP Guidelines Initiative	Report #11-2 Adjuvant CT	Size > 5 cm and high grade and deep seated	Figueredo et al. [44]
MDA CC	Neoadjuvant CT	Size ≥ 5 cm and grade II, III or LR	Meric et al. [111]
MSK CC	Neoadjuvant and adjuvant CT	Size > 5 cm and high grade and deep seated. Nomogram	Wunder et al. [183] Kattan et al. [88].
Australia	Clinical practice CT	Diagnosed metastases	P. Choong, personal communication

Footnote: CT chemotherapy, ISG Italian Sarcoma Group, SSG Scandinavian Sarcoma Group, CCOP Cancer Center Ontario Practice, MDA MD Anderson, MSK Memorial Sloane Kettering, CC Cancer Center

follow-up [15, 29, 50, 68, 117, 142, 155]. In breast cancer, a positive steroid hormone status is initially a positive prognostic factor, but becomes a negative factor for metastasis after 3-5 years of follow-up [29, 50, 68, 155]. A similar time-dependence has been suggested in STS in a study in which the impact of strong prognostic factors, such as tumor size and malignancy grade was found to diminish over time [165]. We therefore used the Scandinavian Sarcoma Group (SSG) registry to study time-dependence of prognostic factors in a large series of STS.

- Time-dependence of prognostic markers has been suggested in several cancer types.
- Tumor size and malignancy grade has been suggested to loose their prognostic value over time in STS, but time-dependence of other prognostic factors has not been assessed.

Patients and methods

Patients and tumor material

Since 1986 the SSG maintains a sarcoma registry. This registry contains approximately 90% of all STS of the extremities and trunk wall diagnosed in Sweden and Norway [9]. During the period 1986 through 1994, 1470 patients from Sweden and Norway with STS of the extremities and trunk wall were reported to the SSG registry. Tumors have been reviewed and classified by the SSG Pathology Review Group using established criteria [177]. After thorough re-evaluation of all sections available and IHC stainings performed at the primary diagnosis, new sections were cut and additional IHC was performed when considered necessary. The antibody panel included muscle specific actin, smooth muscle actin, desmin, S-100 protein, epithelial membrane antigen (EMA), cytokeratins, and the markers for melanoma and lymphoma HMB45, CD45, CD30, CD20, and CD3. Histological malignancy grading was based on a IV-tiered grading system based on cellularity, pleomorphism, nuclear atypia, tumor necrosis, and mitotic activity [6, 107, 109]. MFH were classified as storiform-pleomorphic, myxoid (at least 10% of the examined area being myxoid [112]), giant-cell, and inflammatory. The clinical follow-up included clinical examination and conventional chest radiographs every 3 months during the first 2 years and thereafter twice yearly for at least 5 years, or until metastasis occurred.

A summary of the patients in studies I–IV is presented in Figure 7 and in Table 4.

The studies were approved by the Lund University Ethics Committee.

Table 4. Summary of the tumor materials in studies I–IV

Study	Histo-type	n	Contributing center and patients (n) A	B	C	D	E	F	G	H	I
IV	MFH	338	72	80	64	23	58	14	12	10	5
II	MFH	218	74	59	62	23					
III	STS	140	140								
I	MFH	11	11								

Footnote: MFH malignant fibrous histiocytoma, STS soft tissue sarcoma, A Lund University Hospital, Lund, Sweden, B Karolinska Hospital, Stockholm, Sweden, C Det Norske Radiumhospital, Oslo, Norway, D Haukeland University Hospital, Bergen, Norway, E Sahlgrenska University Hospital, Gothenburg, Sweden, F Umeå University Hospital, Umeå, Sweden, G St Olavs Hospital, Trondheim, Norway, H Linköping University Hospital, Linköping, Sweden, I Ullevål Hospital, Oslo, Norway.

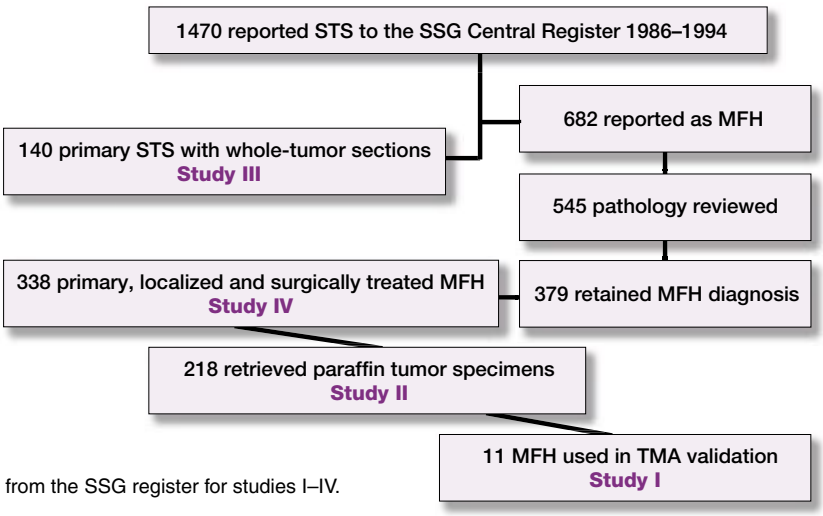


Figure 7. Patient selection from the SSG register for studies I–IV.

Study I

The study that validated the use of TMA for immunostaining in STS used tumor tissue from 11 MFHs from the department of Pathology, Lund University Hospital. Of these tumors, 10 were also included in studies II and IV, and from 9 of the patients whole-tumor sections had been established and were included in study III. 47 representative tumor blocks, median 5 (3–5) blocks from each tumor, were selected by a sarcoma pathologist. Tumor heterogeneity was assessed using the results obtained from the HPF method counting all Ki-67 staining cells in 10 HPFs that were randomly chosen from viable tumor areas in each tumor block.

Study II

From the MFH series (study IV), paraffin-embedded tumor blocks were retrieved from 218 patients, treated 1986-1994 at two centers in Sweden and two centers in Norway (Table 4). 211 tumors paraffin blocks from the primary tumor were obtained, and in addition material from the first LR in 50 patients (7 of which lacked tumor material from the primary tumor) and from the first metastasis in 20 patients. In total, 281 tumors from the 218 patients were included. Median 3 (1–7) representative tumor blocks were selected from each tumor by two sarcoma pathologists resulting in 717 tumor blocks.

From each selected tumor block a fresh section was made and stained with hematoxylin-erythrosin, and 1–4 viable and representative areas were marked by one of the pathologists. The median follow-up was 9 (4–13) years for the survivors. For the study of IHC expression, all tumors were included, but in the survival analyses, 10 patients were excluded as metastasis developed within 1 month, and the analyses were based on 201 patients (Table 5). Of the 201 MFH, 97% were high-grade; 57/201 (28%) were grade III and 138/201 (69%) were grade IV. 67/201 (33%) patients developed a LR with a median time to LR of 12 moths (45 days–11 years), and metastasis occurred in 65/201 (32%) after a median time of 12 months (66 days–7 years).

Study III

During 1988 through 2000, 298 patients with primary, non-metastatic STS of the extremities and the trunk wall were referred before any surgery to the Musculoskeletal Tumor Center in Lund. Of these, 262 underwent surgery without preoperative radiotherapy or neoadjuvant chemotherapy. From 140 patients with mixed histopathological tumor types whole-tumor sections from the primary tumor had been performed, and of these, 17 MFH were also included in studies II and IV (Table 4). The median follow-up time for the survivors was 5.8 (2.5–14) years. During the follow-up, 24 (17%) patients developed a LR after a median time of 12 (1–100) months, and 54/140 (39%) developed metastasis after a median time of 10 (1–152) months (Table 6).

Table 5. Clinicopathological data in 201 MFH (study II)

Factor	n	(%) [a]
Sex		
Men	105	(52)
Women	96	(48)
Age, median (range) years	71	(19–96)
Site		
Trunk wall	24	(12)
Upper extremity	44	(22)
Lower extremity	133	(66)
Depth		
Subcutaneous	77	(38)
Deep-seated	124	(62)
Size, median (range) cm	7	(1–30)
Malignancy grade		
I	0	
II	6	(3)
III	57	(28)
IV	138	(69)
Quality of local treatment		
Adequate	154	(77)
Inadequate	47	(23)
Local recurrence	67	(33)
Surgically treated	54	(81)
Metastasis	65	(32)
Surgically treated	25	(38)

[a] Values for age and size are median (range)

Study IV

During 1986 through 1994, 682 tumors were reported as MFH to the SSG registry, and 545/682 (80%) of these were histologically reviewed by the SSG Pathology Review Group. 166 were reclassified; 98 were classified as another type of sarcoma, 3 as malignant tumors other than sarcoma, 33 as benign lesions, 30 were excluded due to insufficient or non-representative material and 2 patients

Table 6. Clinicopathological data in 140 STS (study III)

Factor	n	(%) [a]
Sex		
Men	79	(56)
Women	61	(44)
Age, median(range) years	69	(16–94)
Site		
Trunk wall	10	(7)
Upper extremity	28	(20)
Lower extremity	102	(73)
Size, median (range) cm	8	(2–28)
Depth		
Superficial	47	(34)
Deep-seated	93	(66)
Histopathological diagnosis		
Leiomyosarcoma	46	(33)
Liposarcoma	19	(14)
Malignant fibrous histiocytoma	18	(13)
Myxofibrosarcoma	18	(13)
Soft tissue sarcoma NOS	12	(9)
MPNST	10	(7)
Synovial sarcoma	8	(6)
Extraskeletal myxoid chondrosarcoma	3	(2)
Myofibroblastic sarcoma	2	(1)
Angiosarcoma	2	(1)
Fibromyxoid sarcoma	1	(0.5)
Malignant mesenchymoma	1	(0.5)
Malignancy grade		
I	5	(4)
II	13	(9)
III	24	(17)
IV	98	(70)
Tumor necrosis	83	(59)
Vascular invasion	50	(36)
Infiltrating peripheral growth pattern	100	(71)
Quality of local treatment		
Adequate	124	(89)
Inadequate	16	(11)
Adjuvant chemotherapy	8	(6)
Local recurrence	24	(17)
Metastasis	54	(39)

[a] Values for age and size are median (range)

Table 7. Clinicopathological data in 338 MFH (study IV)

Factor	n	(%) [a]
Sex		
Men	181	(54)
Women	161	(46)
Age, median (range) years	70	(19–91)
Site		
Trunk wall	39	(12)
Upper extremity	86	(25)
Lower extremity	213	(63)
Size, median (range) cm	7	(1–30)
Depth		
Superficial	142	(42)
Deep-seated	196	(58)
Referral pattern		
Virgin	133	(39)
After FNA	73	(22)
After coarse needle	10	(3)
After incisional biopsy	31	(9)
After marginal excision	31	(9)
After wide excision	3	(1)
After intralesional excision	47	(14)
After local recurrence	9	(3)
Not referred	1	(0.3)
Malignancy grade		
I	1	(0.3)
II	8	(2)
III	99	(29)
IV	230	(68)
Tumor necrosis	187	(55)
Vascular invasion	31	(9)
Quality of local treatment		
Adequate	273	(81)
Inadequate	65	(19)
Local recurrence	98	(29)
Metastasis	110	(39)

[a] Values for age and size are median (range)

with cutaneous tumors were excluded. Another 21 patients with metastasis at the time of diagnosis, 12 patients with intralesional surgery without adjuvant RT, 4 patients who received no treatment, and 4 patients with incomplete follow-up were also excluded, leaving 338 patients for the study. The minimum follow-up time for the survivors was 6 years; the median follow-up for the entire group of patients was 7 (0.2–15) years. During the follow-up period (until June 30, 2001) 194 patients died, of whom 98 (50%) died of sarcoma, 21 (11%) died with sarcoma but of other causes, 75 (39%) died of other causes and without tumor. Overall, 98/338 (29%) patients developed a LR and metastasis occurred in 110 (33%) patients after a median time of 14 months (2 months–7 years) (Table 7).

In the series of 201 primary MFH (study II), all tumors were reviewed by an experienced sarcoma pathologist and classified as myxoid MFH if >10% of the examined area was myxoid. In this series 79/201 (39%) were myxoid tumors, of which 33/79 (0.4) were subcutaneous and 46/79 (0.6) deep-seated. 2 of the subcutaneous tumors and 3 of the deep-seated tumors were low-grade (grade II). Superficial myxoid MFH of low malignancy grade is commonly perceived to have a low-risk of metastasis, but in the series of 201 MFH, there were no significant differences in metastasis-free

survival in the myxoid tumors compared to the non-myxoid tumors (data not shown). The myxoid subset was therefore not separately analyzed in this series. Despite the large proportion of myxoid tumors, metastasis occurred in 32% in this series (Table 5), but this is explained by almost all of the myxoid tumors being high-grade. In the series of 140 mixed STS (study III), the rate of metastasis was 39% (Table 6), and in the series of 338 MFH (study IV) it was 33% (Table 7). These rates of metastasis are in agreement with previous reports [24, 137, 154, 165, 193].

Tissue microarray

For the establishment of a TMA block a fresh 5-µm section was made from each selected tumor block and stained with H&E. 1 to 4 viable and representative areas were marked, and tissue cylinders with a diameter of 0.6 mm were punched from corresponding areas in the donor tumor block and brought into the recipient paraffin block (the TMA block) using a custom-made manual tissue arrayer (Beecher Instruments, Silver Spring, MD, USA)[85, 98]. The tissue biopsies were aligned and marked for identification. In study I, 3 core biopsies from each tumor block was made, resulting in 141 core biopsies from 11 tumors. In study II, 2410 core biopsies were obtained with a median of 9 (3–10) biopsies from each tumor in order to minimize the importance of intra-tumor variability. In study III, 140 whole-tumor blocks were used, but in 1 tumor too little tissue remained in the paraffin block, and in 3 liposarcomas no surrounding tissue remained and therefore TMA was performed in 136 tumors. From these tumors, median 6 (5–7) core biopsies were made along a perpendicular line in the tumor periphery in the whole-tumor sections and brought into a recipient paraffin block, which resulted in 963 core biopsies. In infiltrating tumors the cylinders were taken from the infiltrating zone. For the IHC stainings fresh 5-µm sections from the TMA blocks were transferred to glass slides (DAKO Chem-Mate Capillary Gap Microscope slides, 57 mm, DAKO A/S, Glostrup, Denmark).

Immunohistochemistry

For immunostaining the sections were dewaxed and rehydrated. To achieve antigen retrieval, the sections were pretreated in 10 mM citrate buffer (pH 6.0) in a microwave at 900 W for 8 min followed by 15 min at 350 W. An automated immunostainer, TechMate® 500Plus immunostainer (DAKO A/S, Glostrup, Denmark) was used for all the immunostainings, with DAKO Chem-Mate Kit peroxidase/3-3' diaminobenzidine (DAKO A/S, Glostrup, Denmark). After counterstaining with hematoxylin the slides were dehydrated in ascending concentrations of ethanol to xylene and mounted. The slides were blinded and were independently evaluated by the investigators.

The selection of biological markers analyzed was based on previous studies that had suggested a prognostic importance in STS (Table 8). Commercially available antibodies were used (Table 9). Appropriate positive controls were used, as were buffers and dilutions according to the manufacturer's recommendations. All the immunostainings were scored independently by the investigators. The cut-off levels for the respective immunostains were as follows; any tumor cell nuclear expression of Ki-67, cyclin A and p53 was accepted as positive, regardless of staining intensity, and the tumor specimens were scored as 0 (no immunostaining), 1 (positive staining, but below the cut-off level), and 2 (positive staining above the cut-off level). Immunostaining for bcl-2 was scored as 0 (no tumor cells stained), 1 (weak staining of the membrane or cytoplasm in less than the cut-off level of tumor cells), and 2 (strong staining above the cut-off), and care was taken to disregard infiltrating lymphocytes in the scoring. For ß-catenin, staining was evaluated as; 0 (no tumor cells stained), 1 (predominately cytoplasmic staining), 2 (predominately nuclear staining), and frequency; + (<20% of the tumor cells), ++ (20–50% of tumor cells), and +++ (>50% of tumor cells). Practically all positively stained tumor cells showed staining of the cytoplasm. Isolated nuclear staining was rare and difficult to differentiate from cytoplasm staining, and as positive tumor samples were few, the tumors were subsequently classified as negative or positive. CD44 was classified as aberrant if clear-cut staining was present in the cytoplasm, and/or

Table 8. Published studies (≥40 tumors) of prognostic importance of biological markers in STS

Marker	Histotype	Patients (n)	Prognostic importance	Reference
Ki-67	mixed	355	M	Moller Nielsen M., Thesis, Odense University, Odense, Denmark, 2001
	mixed	216	U	Jensen V. et al., Histopathology 32: 536-46, 1998
	mixed	193	NS	Hasegawa T. et al., Cancer 95: 843-521, 2002
	mixed	182	M	Choong PF. et al., Apmis 102: 915-24, 1995
	mixed	174	U	Drobnjak M. et al., J Natl Cancer Inst 86: 549-54, 1994
	mixed	126	M	Rudolph P. et al., Am J Pathol 150: 1997-2007, 1997
	mixed	123	M	Huutanen RL. et al., Cancer Res 59: 2885-90, 1999
	mixed	121	M	Heslin MJ. et al., Cancer 83: 490-7, 1998
	mixed	104	U	Rohr UP. et al., Verh Dtsch Ges Pathol 82: 45-50, 1998
	MFH	65	NS	Åhlen J. et al., Oncol Rep 10: 1641-5, 2003
	mixed	65	M	Levine EA. et al., J Clin Oncol 15:3249-57, 1997
	mixed	60	NS	Golouh R. et al., Mod Pathol 9: 919-24, 1996
	mixed	57	M	Niezabitowski A. et al., Gen Diagn Pathol 142: 327-33, 1997
	MFH	54	U	Yang P. et al., Cancer 76: 618-25, 1995
	mixed	47	U	Hoos A. et al., Cancer 92: 869-74, 2001
	MPNST	49	M	Watanabe T. et al., Histopathology 39: 187-97, 2001
	mixed	40	U	Stefanou DG. et al., Anticancer Res 18: 4673-81, 1998
p53	mixed	355	NS	Moller Nielsen M., Thesis, Odense University, Odense, Denmark, 2001
	mixed	216	U	Jensen V. et al., Histopathology 32: 536-46, 1998
	mixed	207	U	Cordon-Cardo C. et al., Cancer Res 54: 794-9, 1994
	mixed	198	M	Wurl P. et al., Diagn Mol Pathol 6: 249-54, 1997
	mixed	174	U	Drobnjak M. et al., J Natl Cancer Inst 86: 549-54, 1994
	mixed	145	M	Taubert H. et al., Cancer Res 18: 4134-6, 1996
	mixed	121	NS	Heslin MJ. et al., Cancer 83: 490-7, 1998
	mixed	96	U	Kawai A. et al., Cancer 73: 2499-505, 1994
	mixed	86	U	Wurl P. et al., Oncogene 16: 1183-5, 1998
	mixed	70	NS	Nakanishi H. et al., Oncology 54: 238-44, 1997
	MFH	65	NS	Åhlen J. et al., Oncol Rep 10: 1641-5, 2003
	mixed	60	NS	Golouh R. et al., Mod Pathol 9: 919-24, 1996
	MFH	54	NS	Yang P. et al., Cancer 76: 618-25, 1995
	MFH	52	U	Reid AH. et al., Diagn Mol Pathol 5: 65-73, 1996
	mixed	40	NS	Stefanou DG. et al., Anticancer Res 18: 4673-81, 1998
mdm2/p53	mixed	121	NS	Heslin MJ. et al., Cancer 83: 490-7, 1998
	mixed	86	U	Wurl P. et al., Oncogene 16: 1183-5, 1998
	MPNST	49	NS	Watanabe T. et al., Histopathology 39: 187-97, 2001
cyclin A	mixed	126	U	Huutanen RL. et al., Cancer Res 59: 2885-90, 1999
	mixed	55	M	Nogushi T. et al., Am J Pathol 156: 2135-47, 2000
Pgp	mixed	65	M	Levine EA. et al., J Clin Oncol 15:3249-57, 1997
	mixed	55	M	Nakanishi H. et al., J Cancer Res Clin Oncol 123: 352-6, 1997
	mixed	44	NS	Coley HM. et al., Eur J Cancer 36: 881-8, 2000
CD44	mixed	133	U	Maula S. et al., Br J Cancer 84: 244-52, 2001
	mixed	62	M	Peiper M. et al., Anticancer Res 24: 1053-6, 2004
	mixed	47	U	Kahara N. et al., Virchows Arch 436: 574-8, 2000
bcl-2	mixed	216	NS	Jensen V. et al., Histopathology 32: 536-46, 1998
	mixed	102	NS	Dan´ura T. et al., Cancer Lett 178: 167-74, 2002
	mixed	70	M	Nakanishi H. et al., Oncology 54: 238-44, 1997
	MFH	65	U	Åhlen J. et al., Oncol Rep 10: 1641-5, 2003
ß-catenin	SS	62	U	Saito T. et al., J Pathol 192: 432-50, 2000
	Mixed	56	NS	Kuhnen C. et al., Mod Pathol 13: 1005-13, 2000
	SS	44	U	Hasegawa T. et al., Hum Pathol 32: 257-63, 2001

MFH malignant fibrous histiocytoma, LMS leiomyosarcoma,
SS synovial sarcoma
U prognostic importance in univariate analysis,
M prognostic importance in multivariate analysis,
NS no significant prognostic importance

Table 9. Immunohistochemical markers and evaluation criteria (studies I–III)

Marker	Clone	Company	Dilution	Normal control	IHC cut-off
Ki-67	MIB-1	DAKO	1:1000	Colon	>20%
Cyclin A	6E6	Novocastra	1:100	Colon	>5%
P53	DO-7	DAKO	1:300	Colon	>20%
Bcl-2	M0887	DAKO	1:50	Colon	>10%
ß-catenin	14	Transduction Laboratories	1:5000	Colon	Any + vs –
CD44	F1044-2	Novocastra	1:500	Tonsil	>25%
Pgp	JSB-1	Novocastra	1:10	Cerebrum	>10%

Footnote: Citrate buffer was used in all the immunostainings.

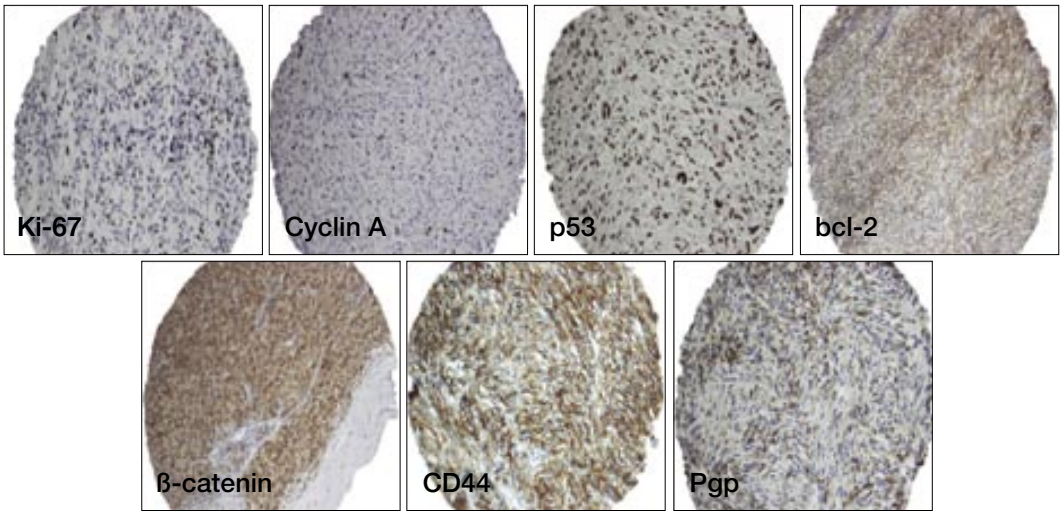


Figure 8. TMA sections (×20) with positive staining for the markers Ki-67, cyclin A, p53, bcl-2, ß-catenin, CD44, and Pgp.

if strong membrane staining with concomitant staining in the cytoplasm was found. CD44 was scored as 0 (no tumor cells stained), 1 (<10%), 2 (10–25%), and 3 (>25%). Pgp, when present, stained the cytoplasm and was scored as 0 (no immunostaining), 1 (≤10%), and 2 (>10%), (Table 9 and Figure 8).

In study I, the Ki-67 immunostaining was evaluated in 4 ways. In the tumor block sections, 400 cells on or in contact with a line, and all tumor cells in 10 randomly chosen HPFs were counted and the fraction of positively stained cells calculated. In the TMA-slide all cells in a HPF (×40) which almost covered the entire area of the cylinder section, were counted and scored in 20% classes independently by 3 of the investigators. In study II, 2 authors independently evaluated the array slides, whereafter a pathologist reviewed 14% of the array sections without access to the previous scores. In study III, the TMA slides were evaluated independently by 2 authors and in cases with discrepant findings, the TMA sections were jointly reevaluated and a consensus was reached.

Whole-tumor sections

In study III, whole-tumor sections were used. Following fixation of the tumor in neutral buffered 10% formaldehyde, at least a 1 cm transverse whole-tumor section was made from the largest diameter between the proximal and distal tumor poles and including the surrounding tissue. For dehydration a dehydrator with protocols tested for different types of tissues and sample thicknesses was used. After the dehydration, which took up to

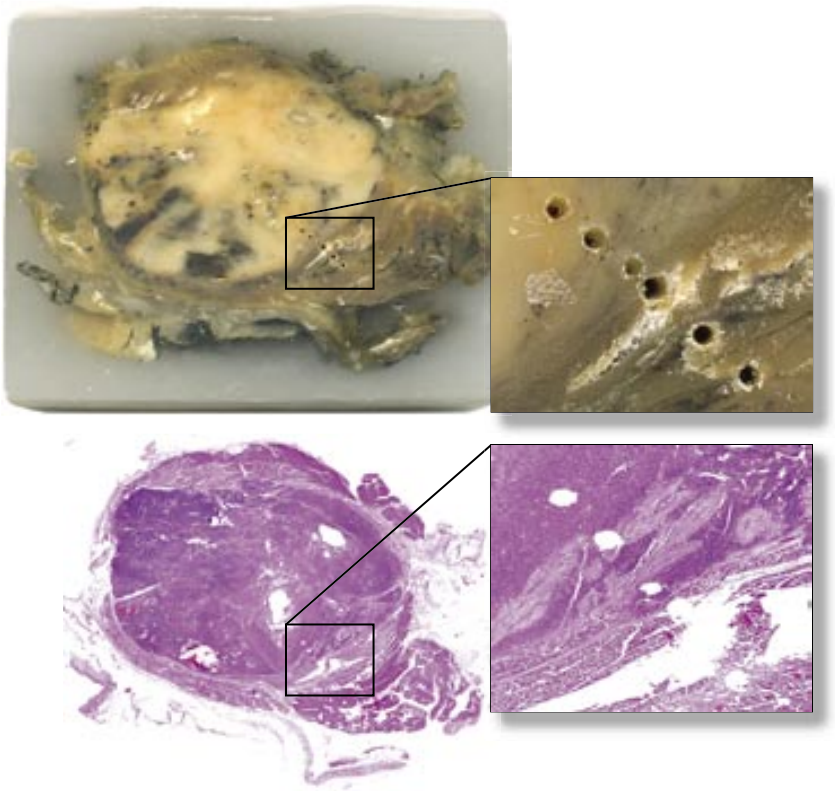


Figure 9. Whole-tumor paraffin block from a grade IV, deep-seated leiomyosarcoma, 7 cm in diameter with peripheral TMA punches (top). Corresponding section with minimal tissue loss following TMA (bottom).

72 h, the sections were paraffin-embedded. Using a whole-tumor microtome, 5 µm sections were made, mounted on large glass slides and stained with hematoxylin-erythrosin B for evaluation (Figure 9).

Tumor necrosis was defined as the presence of amorphous cellular debris, usually associated with a neutrophil polymorphonuclear cell response, or clustering of dead cells, and apoptotic bodies or cell ghosts. We employed no lower limit of a necrotic area. Hyalinosis, edema, fibrin exudates lacking tumor cells, or acellular areas of fibrosis were not defined as necrosis.

Vascular invasion was defined as the presence of tumor cells within any space having an obvious endothelial lining, whether within the tumor or in the tumor rim. The tumor cells had to be adherent to the vessel wall, or associated with adherent fibrin, red blood cells, or leucocytes. Bulging of tumor into a vessel with intact endothelial lining was not accepted as intravascular tumor growth.

Peripheral tumor growth pattern was microscopically assessed and classified as pushing if no sign of infiltrative growth could be seen, as focally infiltrating if ≤25% of the tumor rim showed signs of infiltration, and as extensively infiltrating if >25% of the tumor border showed infiltration into the surrounding tissues. As there were no prognostic differences in the subsequent analyses between the 2 subsets of infiltrative growth pattern, they were combined, and in the further analyses the growth pattern was classified as infiltrating *versus* pushing (Figure 10).

Statistics

In study I, in which the use of TMA in MFH was validated for Ki-67 staining, the intratumor heterogeneity was assessed using the variance component model,

$$y_{ij}=m+\delta_i+\varepsilon_{ij},$$

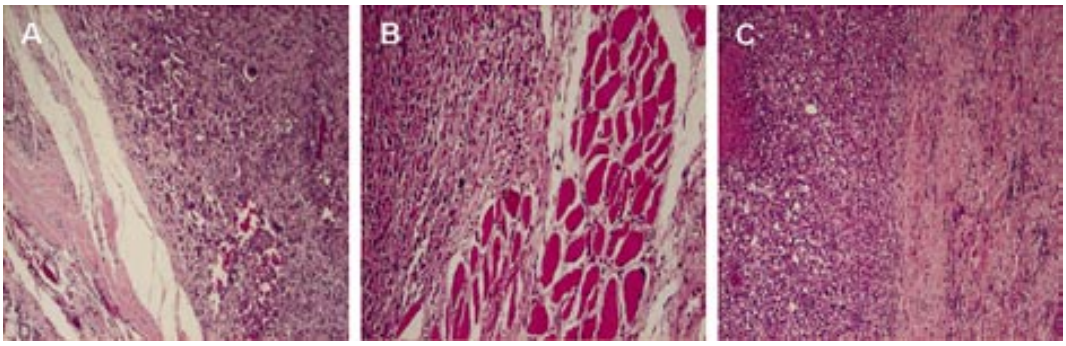


Figure 10. Peripheral tumor growth patterns in STS. Pushing growth (A), focally infiltrating growth in surrounding muscle (B), and widely infiltrating growth (C).

where y_{ij} is the proportion of positive cells in sample j within block i, m is the true proportion in the tumor, δ_i is the deviation of block i from the average of the tumor and ε_{ij} is the deviation of sample j from the average of block i. The standard deviations σ_δ and σ_ε of δ_i and ε_{ij} measure the variation between blocks and within blocks, respectively, and they are estimated by analysis of variance. Given k tumor blocks and n samples per block, the standard error of the mean (SEM) of all y_{ij} in the whole tumor is

$$SEM = \sqrt{(\frac{\sigma_\delta^2}{k} + \frac{\sigma_\varepsilon^2}{kn})}$$

In the special case where the standard deviations between and within blocks are equally large ($\sigma_\delta = \sigma_\varepsilon = \sigma$) the formula simplifies to

$$SEM = \sigma\sqrt{(\frac{1}{k} + \frac{1}{kn})}$$

which allows comparison of how the number of blocks (k) and the number of samples within the blocks (n) affects the SEM. The results of Ki-67 staining with the line count method and the HPF method for each tumor block were compared by plotting the difference between the two methods against the mean of the methods [13]. A reference value for each tumor block was constructed by combining the values obtained with the line count method and the HPF method, and subsequently compared with the values using the tissue microarray method by plotting these against each other for each tumor block.

In study II correlations between the immunohistochemical staining patterns were assessed using Pearson correlations. The immunohistochemical expression of the factors analyzed in the primary tumors, the local recurrences and the metastases were tested for trend using the nonparametric Wilcoxon matched pairs signed-rank test. Exact McNemar tests were used for sparse matched 2-by-2-tables. Univariate and multivariate analysis with time to metastasis as endpoint was done using Cox proportional hazards model.

In study III the Cox proportional hazards model was used for estimating hazard ratios and proportional hazard assumptions were checked using Schoenfields test [156]. Areas under ROC curves were compared using an algorithm suggested by DeLong [37]. The performance of prognostic indices with optimal weights, derived from Cox-models, was similar to the performance with equal weights for the 3 factors. Therefore, all factors were assigned a weight of 1.0 (data not shown).

The Cox proportional hazards model was used also in univariate and multivariate analysis for the prognostic factors using the for the time intervals 0–10 moths and >10 months. Necrosis was, however, dropped from the models for the time interval 0-10 months, as necrosis predicted metastasis in this interval with 100% sensitivity. All the estimates for this interval were therefore restricted to tumors with necrosis in order to assess the independent prognostic information of the other factors in tumors with necrosis. The estimates for the remaining factors correspond to separate models with one factor added at a time in a series of 3-factor (0–10 months), or 4-factor analysis, one for each time interval.

In study IV pair-wise associations between the dichotomized prognostic factors were analyzed using odds-ratios. The effect of the prognostic factors on the endpoint time to metastasis was illustrated by means of cumulative incidence curves with death as a competing event [84]. The relative risk of metastasis was analyzed univariately and multivariately for the prognostic factors using the Cox proportional hazards model for the entire follow-up, and also for the time intervals 0–2 years and >2 years. To study how the absolute effect of the prognostic factors varied with time, the probability of metastasis the following year was calculated by prognostic group yearly for the first 5 years using life-table methods.

For studies II–IV p-values correspond to two-sided tests and values below 0.05 were considered significant. For the statistical analyses Stata 8.0 and 8.1 (STATA Corp., College Station, TX, U.S.A.) were used.

Results and discussion

Application of tissue microarray (study I)

In the TMA validation study, we addressed the heterogeneity of IHC expression of Ki-67 in 47 tumor blocks from 11 MFH. The results obtained with conventional methods of assessment, the line count method and assessment of 10 HPFs, were compared with TMA-based assessment. The mean number of cells in each HPF was 280 (70–800) cells.

The two methods used on conventional sections, the line count method, and the HPF method showed a high degree of agreement with a mean difference per tumor block of 1.7 (95% CI 0.7–2.6)% (Figure 11). When the results using TMA were compared to a reference value composed of the combined Ki-67 count of both the line method and the HPF method, we found a good accordance, with mean 8.6 (3.5–28)% higher values for TMA than the reference value (Figure 12). This difference was interpreted as a result of TMA biopsies having been taken from selected areas of the tumors, whereas in the conventional methods the areas counted were randomly selected. The staining quality on TMA was perceived as good, and we reviewed the areas on the Ki-67 stained conventional sections from where the biopsies had been taken but found no difference in the quality of staining that could explain these differences (unpublished data).

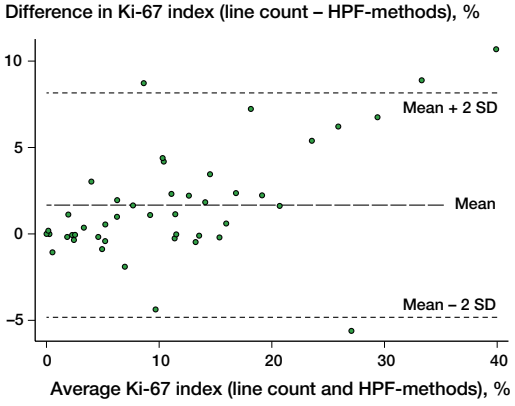


Figure 11. The degree of agreement between the line count and HPF method in assessment of Ki-67 staining in 45 tumor blocks from 11 MFH. The difference in percentage between the two methods is plotted against the mean of the methods for each tumor block (study I).

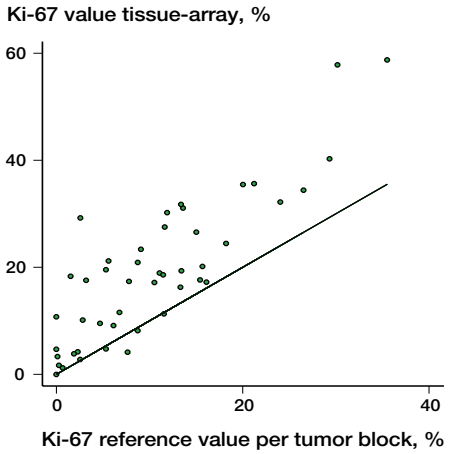


Figure 12. Agreement between TMA and reference value of Ki-67 staining count in 45 tumor blocks from 11 MFH (study I).

The median interblock SD for Ki-67 was 2.3 (0.9–9.5)% and the median intrablock SD was 2.5 (1.0–5.0)% (Table 10, Figure 13). As the TMA biopsies obtained from each tumor block were almost covered by a HPF (×40) with an area of 0.16 mm^2, the impact of tumor heterogeneity

Table 10. Interblock and intrablock standard deviation (SD) of Ki-67 staining in 11 MFH (study I)

Tumor no.	Tumor blocks (n)	SD (%) interblock	SD (%) intrablock
1	3	2.2	2.3
2	3	2.3	5.0
3	4	1.6	2.3
4	5	2.2	2.5
5	5	9.5	3.4
6	3	5.0	2.5
7	5	5.8	3.5
8	5	6.9	4.4
9	4	3.1	2.9
10	5	1.3	1.0
11	5	0.9	2.2

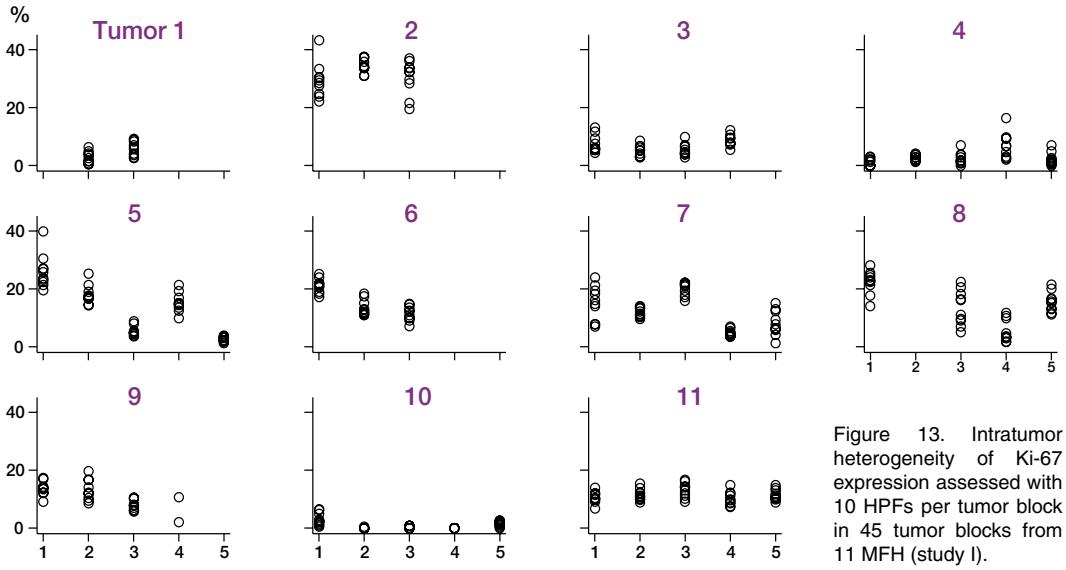


Figure 13. Intratumor heterogeneity of Ki-67 expression assessed with 10 HPFs per tumor block in 45 tumor blocks from 11 MFH (study I).

Table 11. Relative influence of the number of core biopsies (n) and the number of tumor blocks (k) on the SEM (study I)

	$n = 1$	2	3	4	5
$k = 1$	1.41	1.22	1.15	1.12	1.10
2	1.00	0.87	0.82	0.79	0.77
3	0.82	0.71	0.67	0.65	0.63
4	0.71	0.61	0.58	0.56	0.54
5	0.63	0.55	0.52	0.50	0.49

on the TMA sampling could be estimated. From each tumor 3–5 tumor blocks were used, and from each tumor block 10 HPFs were counted. As the median interblock and intrablock SD were found to be almost identical ($\sigma_\delta = \sigma_\varepsilon = \sigma$) a comparison of how the number of blocks (k) and the number of samples within the blocks (n) affects the SEM could be made (Table 11). These data show that taking more than 3 core biopsies from each tumor block only has a marginal effect on the SEM values. Therefore, we concluded that several, ideally 3, tumor blocks should be sampled from each tumor to account for tumor heterogeneity, whereas the number of samples from each tumor block primarily should affirm that each tumor block could be assessed.

The results in the TMA validation study are in accordance with other studies in which TMA has been found to reproduce clinicopathological correlations utilizing conventional sections for IHC staining, or using DNA isolated from entire tumor pieces or from paraffin sections. These studies have included different tumor types, such as colorectal cancer, breast cancer, urinary bladder cancer, prostate cancer, and bone and STS [18, 19, 43, 71, 73, 98, 118, 127, 146, 157, 169]. In 59 fibroblastic tumors comparisons between TMA and conventional sections were made for the markers Ki-67, p53 and pRB by Hoos et al. [73], who found reliable readings from triplicate TMA cores in 96% for Ki-67, 98% for p53 and 91% for pRB, and both methods showed a prognostic correlation between Ki-67 expression and metastasis. Nielsen et al. [124] performed cDNA microarray profiling of 77 STS, including 44 synovial sarcomas, and found that the characteristic gene expression patterns in synovial sarcoma could be verified at the protein level by IHC staining TMA samples. They also evaluated antibodies for their specificity in synovial sarcoma using TMA-based immunostaining, and found that the expression levels were in agreement with previous studies based on conventional staining. In a hierarchical cluster analysis of the immunostaining results the histological subtypes were distinctly discriminated.

The ability to identify clinicopathological correlations using TMA is perhaps more important than comparison between the absolute results using TMA or conventional sections. Furthermore, there

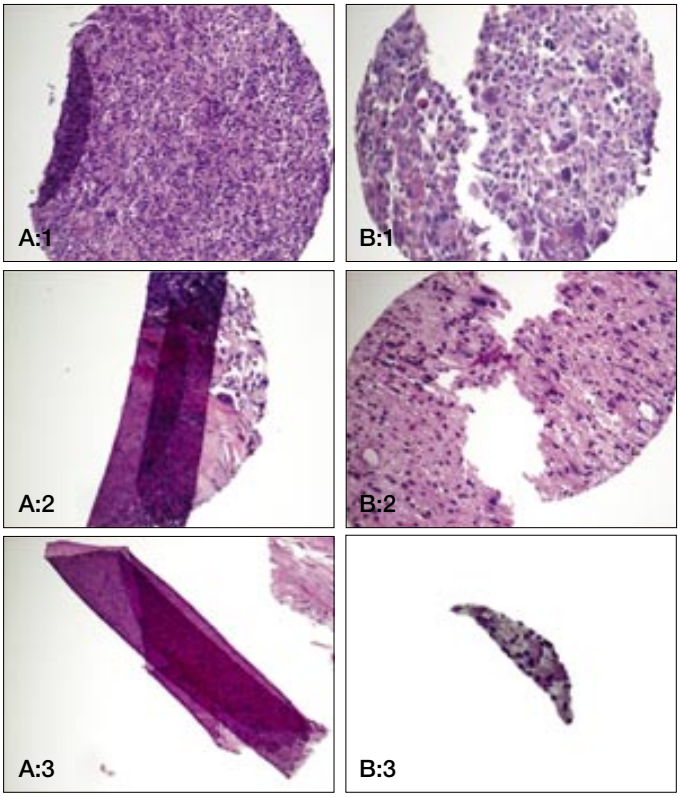


Figure 14. Patterns of damage in TMA, examples of folded (A:1–3) and torn sections (B:1–3).

is no general agreement as to how IHC expression patterns should be interpreted. Should foci with the highest staining intensity be used? How large an area of tumor should be examined? How many tumor blocks from a large tumor should be stained and evaluated? Studies applying TMA to breast cancer have confirmed the clinicopathological correlation between amplification of *ERBB2* and *MYC* in steroid receptor negative, p53 expressing tumors [98, 169]. Camp et al. [19] obtained an accuracy rate of >95% if 2–3 TMA core biopsies were used to study the expression of ER, PgR and HER2/neu in breast cancer. In urinary bladder cancer, Nocito et al. [127] reliably assessed histological grade and Ki-67 labelling index using 4 replicate TMA sections.

Loss of TMA sections during the different steps in the TMA process, principally during the IHC staining, is the main reason for taking multiple TMA biopsies. Most TMA series report loss of about 10–15% of the sections, either due to empty spots on the slide, folded sections or by sections being torn by the microtome [73, 118]. In the TMA validation study, all the biopsies were lost from 1 tumor block, but 6 biopsies from the remaining 3 tumor blocks were successfully retrieved. We experienced a loss of 24% of the TMA samples; 4% during the biopsy retrieval and mounting in the recipient paraffin block, and a further 20% during the IHC staining. In the series of 201 primary MFH (study II), 2 tumors lost all the sections in the staining for Pgp, but also in this study median 3 (1–7) tumor blocks had been selected from each tumor and no other tumors were lost in any of the stainings performed. We found median 7 (5–19)% of the sections damaged, but only 4 (3–4)% were lost. In the series of 140 mixed STS (study III), 1 tumor was lost in all the IHC stainings, and a median of 19 (2–23)% of the TMA sections were damaged, but only 4 (3–6)% lost. The higher rate of loss found in the TMA validation study probably reflects a learning curve as our experience in performing TMAs increased. In the series of 140 mixed STS (study III), a somewhat higher rate of damaged sections (19%) were seen compared to 7% in the series of 201 primary MFH (study II), and this was probably caused by differences in tissue textures in the peripheral zones of the tumors, which were targeted in the former series. When the microtome encounters different tissue textures shearing forces tend to damage the sections (Figure 14). The recommendation of using triplicate TMA sections for analysis is in accordance with the results of several studies in different tumor types, and will minimize the loss of data, thereby providing a reliable IHC expression profile. In the TMA validation study, we also demonstrated how examining additional areas from a tumor block have a minor impact on the results, and that the number of TMA biopsies from each tumor block primarily should ensure material from every block.

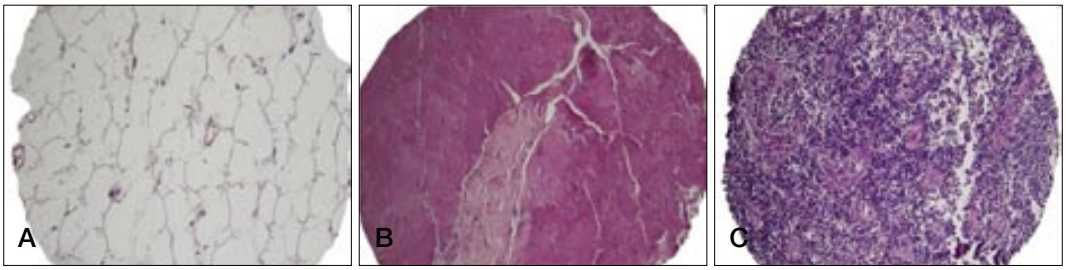


Figure 15. Non-representative TMA sections containing adipose tissue (A), muscle (B) and an inflammatory nodule (C).

In TMA, the areas targeted are important. Inadequate alignment of the biopsy needle can result in retrieval of normal tissue, or hemorrhagic or necrotic foci (Figure 15). In order to maximize the number of evaluable TMA sections and to provide a true estimate of the marker studied, heterogeneity in both tumor tissue and staining patterns needs to be taken into account. Nuclear staining and staining patterns evaluated based on a presence or an absence of staining can often be determined from one TMA section containing representative tumor tissue. In contrast, for immunostains that are predominately cytoplasmic, and for markers with heterogeneous staining patterns, or when staining is evaluated in 3 or more categories a higher number of non-assessable samples may be seen due to discordant readings. In these instances, generally 2 or more TMA sections are required to obtain an acceptable level of reproducibility [73]. However, the fraction of tumors with heterogeneous findings is probably also dependent on the tumor type studied. Markers with a high degree of intratumor heterogeneity and with different staining patterns in the periphery and in the center of the tumor are likely to be more vulnerable to TMA interpretations. Thus, for a correct immunohistochemical characterization of certain markers, core biopsies from the periphery as well as the center of the tumor may be required [19].

In the series of 140 mixed STS (study III), in which the peripheral growth zones of the tumors were specifically targeted for TMA, the prognostic value of the biological markers used differed compared to the results obtained in the series of 201 primary MFH (study II), from which the tumor blocks were selected for tumor viability and representativity but did not include the adjacent normal tissue. Consequently, the TMA biopsies may have been obtained from more central tumor regions in these latter series. In univariate analysis with time to metastasis as endpoint there was only concordance of the prognostic value of a high Ki-67 expression, with had a hazard ratio (HR) of 1.9 in the series of 201 primary MFH, and HR 2.4 in the series of 140 mixed STS (Table 12). This observation suggests that targeting the peripheral tumor growth zone may be important.

Table 12. Univariate analysis of prognostic value of TMA-based IHC expression patterns for metastasis in 201 MFH (study II) and 140 mixed STS (study III)

Factor [a]	Univariate analysis for metastasis-free survival					
	201 MFH			140 STS		
	HR	95% CI	p-value	HR	95% CI	p-value
Ki-67	1.9	1.2–3.2	0.009	2.4	1.3–4.1	0.003
cyclin A	1.3	0.8–2.0	0.4	2.9	1.5–5.6	0.002
p53	1.1	0.6–1.9	0.9	0.7	0.32-1.6	0.4
bcl-2	1.5	0.7–3.2	0.3	0.5	0.20–1.2	0.1
ß-catenin	–	–	–	2.5	1.4–4.4	0.001
CD44	1.0	0.6–1.6	1.0	2.4	1.4–4.2	0.001
Pgp	0.9	0.5–1.4	0.6	2.1	1.1–3.8	0.02

[a] Refers to cut-off levels in Table 9.

- TMA is comparable to using conventional sections for determining IHC staining of Ki-67 in MFH.
- More than 1 tumor block should be used for TMA due to intratumoral heterogeneity, and optimally 3 TMA biopsies per tumor block should be obtained to compensate for loss of core sections.
- The localization of TMA sampling may influence the results; our data suggest that evaluation in viable tumor regions in the peripheral growth zones may be important.

Table 13. Immunhistochemical positive staining patterns in 201 MFH (study II) and 140 mixed STS (study III)

Factor [a]	201 MFH		140 STS	
	Total n	%	Total n	%
Ki-67	61	30	37	27
cyclin A	108	54	81	60
p53	49	24	27	20
bcl-2	17	8	21	16
ß-catenin	-	-	23	17
CD44	121	60	42	31
Pgp	81	40	25	19

[a] Refers to cut-off levels in Table 9.

Table 14. Univariate analysis of prognostic factors for local recurrence in 124 STS with adequate local treatment (study III)

			Local recurrence-free survival Univariate analysis		
Factor	n	(%)	HR	95% CI	p-value
Tumor size >8 cm	53	43	2.3	0.94–5.8	0.07
Deep location	78	63	1.8	0.63–4.9	0.7
Tumor necrosis	73	59	2.8	1.0–7.7	0.05
Vascular invasion	45	36	2.4	0.97–6.0	0.06
Grade IV	91	73	3.9	0.91–17	0.07
Infiltrating growth	92	74	∞	–	0.001 [b]
Ki-67 [a]	37	27	2.1	0.81–6.0	0.1
cyclin A [a]	81	60	3.6	1.2–11	0.03
p53 [a]	27	20	1.4	0.52–4.0	0.5
bcl-2 [a]	21	16	1.4	0.45–4.1	0.6
ß-catenin [a]	23	17	2.4	0.84–6.0	0.1
CD44 [a]	42	31	1.1	0.42–2.9	0.8
Pgp [a]	25	19	1.4	0.46–4.3	0.6

[a] Refers to cut-off levels in Table 9. [b] log-rank test.

Prognostic value of immunohistochemistry (studies II–III)

Immunostaining for prediction of metastasis

In STS the prognostic value of IHC staining for tumor-related markers has been evaluated in about 100 articles, of which 37 larger studies are summarized in Table 8, with Ki-67 and p53 being the most extensively studied markers. Few studies, however, have analyzed multiple biological markers both univariately and multivariately, and compared the value of IHC with clinico-pathological data (Table 8) [67, 116]. We evaluated multiple IHC staining patterns in 201 primary MFH and using whole-tumor sections in a series of 140 mixed STS (Table 13). The frequencies of positive staining patterns for the different IHC markers were similar between the 2 studies (II and III) regarding the markers Ki-67, p53 and cyclin A, whereas bcl-2, CD44 and Pgp showed a lower frequency of positive staining in the series with mixed STS than in the MFH series (Table 13). 17 MFH were included in both studies, and the stainings did not differ in these tumors between the 2 studies (data not shown). Thus, the differences detected may be attributed to histotype-specific characteristics, but may also reflect the use of whole-tumor sections with evaluation of the peripheral tumor growth zone in study III.

Ki-67

We detected Ki-67 staining in >20% of the tumor nuclei in 30% of MFH and in 27% of the whole-tumor sections from the mixed STS series (Table 13). These results are similar to the frequencies

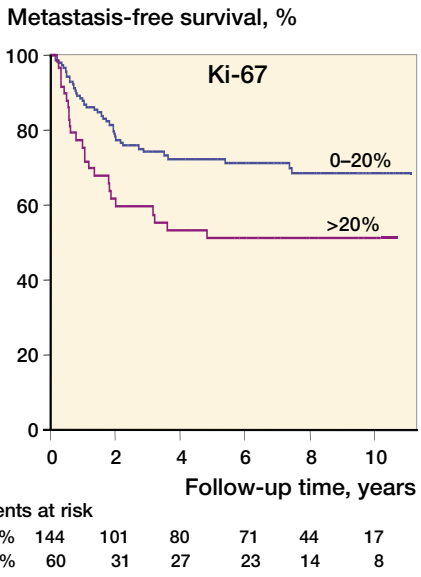


Figure 16. Metastasis-free survival stratified by Ki-67 expression in 201 MFH (study II).

reported in other series [20, 39, 61, 62, 67, 72, 116, 189]. In both our studies, a high (>20%) Ki-67 expression was a strong prognostic factor for metastasis (Figure 16, Table 15). Ki-67 is the most extensively studied biological marker in STS, and high expression has in several studies been shown to be an independent prognostic factor (Table 8). The prognostic value of Ki-67 has been questioned

Table 15. Multivariate analysis of prognostic factors for metastasis in 201 MFH (study II) and in 140 mixed STS (study III)

	Metastasis–free survival									
	201 MFH					140 STS				
Factor	n	(%)	HR	95% CI	p–value	n	(%)	HR[b]	95% CI	p–value
Tumor size >8 cm	85	42	3.0	1.6–5.7	0.001	66	47	1.2	0.65–2.1	0.6
Deep location	124	62	1.4	0.7–2.8	0.3	93	66	1.1	0.61–2.2	0.7
Tumor necrosis	113	58	1.2	0.6–2.4	0.5	83	59	2.8	1.2–6.9	0.02
Vascular invasion	21	11	1.0	0.5–2.0	0.9	50	36	3.5	1.9–6.6	<0.001
Grade IV	138	69	–	–	–	98	70	0.88	0.33–2.2	0.7
Infiltrating growth	–	–	–	–	–	100	71	3.2	1.3–7.9	0.01
Ki-67 [a]	61	30	1.8	1.1–3.2	0.03	37	27	1.9	1.0–3.5	0.04
cyclin A [a]	108	54	–	–	–	81	60	1.9	0.94–3.8	0.07
p53 [a]	49	24	–	–	–	27	20	0.46	0.20–1.0	0.06
bcl-2 [a]	17	8	–	–	–	21	16	0.77	0.30–2.0	0.6
ß-catenin [a]	–	–	–	–	–	23	17	2.7	1.5–4.8	0.001
CD44 [a]	121	60	–	–	–	42	31	2.1	1.2–3.7	0.008
Pgp [a]	81	40	–	–	–	25	19	2.4	1.3–4.5	0.007

[a] refers to cut–off levels in Table 9. HR Hazard Ratio, CI Confidence Interval.
[b] Hazard ratio adjusted for grade IV, deep location, size >8 cm, tumor necrosis and vascular invasion in multivariate analysis.

for MFH by Jensen et al. [80] who found a prognostic value in other histotypes of STS but not in MFH. Our results support the prognostic value of Ki-67 expression also in MFH. Hence, enough evidence for Ki-67 as a prognostic marker should have accumulated from retrospective studies, and we suggest that Ki-67 should be evaluated prospectively and considered as a routine IHC marker in pleomorphic STS.

Cyclin A

In the largest series of cyclin A expression in STS to date, Huuhtanen et al. (1999) reported a median cyclin A level of 11% in 126 mixed STS, and found a significant prognostic value for cyclin A expression using a cut-off level of 6% [76]. In MFH (study II), and in mixed STS (study III), we found a high (>5%) cyclin A expression in 54% and 60% of the tumors, respectively (Table 13). In MFH we could not demonstrate any prognostic value of cyclin A expression, whereas in the series of mixed STS (study III), high cyclin A expression was prognostic for metastasis in univariate analysis, with HR 2.9 (Table 17), but not in multivariate analysis (HR 1.9) (Table 15). Oda et al. used a cut-off of 10% in a series of 43 myxofibrosarcomas, and did not show an independent value of a high cyclin A expression in multivariate analysis, although univariate analysis suggested one [130]. In their series, the relatively limited number of tumors could account for the lack of prognostic value found for a cyclin A expression using a higher cut-off level.

Table 16. Immunohistochemical expression patterns in disease progression in 201 MFH (study II)

Factor[a]	Primary tumor (n=201) n (fraction)	Local recurrence (n=44) n (fraction)	Metastasis (n=18) n (fraction)
Ki-67	61 (0.3)	21 (0.5)	9 (0.5)
CyclinA	108 (0.5)	29 (0.7)	13 (0.7)
p53	49 (0.2)	13 (0.3)	6 (0.3)
bcl-2	17 (0.1)	5 (0.1)	2 (0.1)
CD44	121 (0.6)	30 (0.7)	11 (0.6)
Pgp	81 (0.4)	24 (0.5)	9 (0.5)

[a] Refers to cut-off levels in Table 9.

p53

p53 staining was found in 24% of the MFH and in 20% of mixed STS series(Table 13), which was similar to the rates found by other investigators [36, 39, 62, 116]. We did not find any prognostic value of p53 staining for metastasis or LR (Tables 12, 14 and 15). The data reported on the prognostic value of p53 expression in STS are partly conflicting, and although several studies have reported a prognostic

Table 17. Univariate analysis in 201 MFH (study II) and in 140 mixed STS (study III)

	Metastasis–free survival									
	201 MFH					140 STS				
Factor	n	(%)	HR	95% CI	p–value	n	(%)	HR	95% CI	p–value
Tumor size >8 cm	85	42	3.8	2.3–6.4	<0.001	66	47	2.0	1.2–3.5	0.01
Deep location	124	62	2.6	1.4–4.7	0.003	93	66	1.6	0.8–2.9	0.2
Tumor necrosis	113	58	2.5	1.4–4.5	0.001	83	59	4.2	2.1–8.4	<0.001
Vascular invasion	21	11	1.8	0.9–3.6	0.1	50	36	4.9	2.8–8.7	<0.001
Grade IV	138	69	1.7	0.9–2.9	0.08	98	70	2.6	1.3–5.4	0.008
Infiltrating growth	–	–	–	–		100	71	4.6	1.9–11	0.001
Ki-67 [a]	61	30	1.9	1.2–3.2	0.009	37	27	2.4	1.3–4.1	0.003
cyclin A [a]	108	54	1.3	0.8–2.0	0.4	81	60	2.9	1.5–5.6	0.002
p53 [a]	49	24	1.1	0.6–1.9	0.9	27	20	0.7	0.3–1.6	0.4
bcl-2 [a]	17	8	1.5	0.7–3.2	0.3	21	16	2.5	0.2–1.2	0.1
ß-catenin [a]	–	–	–	–		23	17	2.5	1.4–4.4	0.001
CD44 [a]	121	60	1.0	0.6–1.6	1.0	42	31	2.4	1.4–4.2	0.001
Pgp [a]	81	40	0.9	0.5–1.4	0.6	25	19	2.1	1.1–3.8	0.02

[a] refers to cut–off levels in Table 9. HR Hazard Ratio, CI Confidence Interval.

value in univariate analysis, the independent value of p53 expression in multivariate analysis has only been shown in a small number of studies (Table 8). There are several possible reasons for this. Many antibodies used for detecting p53, including DO-7 used in our studies, recognize both mutated and wild-type p53. There are more than 10.000 *TP53* mutations reported in human tumors; the majority being missense mutations located in the DNA binding domain of *TP53* [60]. As a consequence of the amino acid substitution, configuration alterations may lead to prolonged half-life of p53, and nuclear accumulation, but also varying functional consequences of p53 as a multifunctional transcription factor [70]. Since the p53 protein participates in cell-cycle control, apoptosis, senescence, differentiation, genomic stability, and in DNA replication and repair, detection of p53 accumulation is probably not a sufficient assay for understanding the functional effects on the many pathways p53 is involved in [70, 180]. Consequently, when p53 expression was analyzed in conjunction with the cell-cycle co-regulator Rb, Würl et al. [185] showed that concomitant high expression of both p53 and Rb correlated with worse outcome, and that absence of over-expression of either, or none of the proteins, correlated with a better prognosis in 198 STS. The mdm2 protein, involved in the ubiquitin-mediated degradation of p53, could also be considered when analyzing p53 expression, and tumors with over-expression of both p53 and mdm2 may have a worse outcome [186].

bcl-2

We detected bcl-2 staining in 53% of the MFH and in 24% of the mixed STS, and a high staining (>10% of the cells) in 8% of the MFH, and in 16% of the mixed STS (Table 13). Dan'ura et al. [36] and Miettinen et al. [113] have reported bcl-2 staining in about 1/3 of STS with the highest frequencies observed in MFH and synovial sarcomas. Only 8 synovial sarcomas were included in the whole-tumor section series (study III), but indeed 6 of these showed a high staining pattern. We did not detect any prognostic significance from bcl-2 staining, which is in agreement with Jensen et al. [80]. Our results stand in contrast to the prognostic value of bcl-2 found in 2 previous studies in STS; Åhlen et al. [3] found a prognostic value of bcl-2 in 65 MFH in which 20% of the tumors showed bcl-2 accumulation, and this was also reported in a series of 70 mixed STS with 43% bcl-2 positivity by Nakanishi et al. [122]. The prognostic value of bcl-2 in STS is probably limited as the results in several large studies appear to be contradictory (Table 8). The questionable prognostic value of bcl-2 in STS is also suggested in the findings by Dan'ura et al. [36] who showed that although expression of bcl-2, and Bax correlated to an apoptotic index on microscopy, and that there were histotype spe-

cific differences of these expression patterns, they lacked prognostic value in 102 STS.

ß-catenin

Cytoplasmic accumulation of ß-catenin has been studied in 3 larger (>50 tumors) series of STS that have reported staining in 21–57% of the tumors with the highest frequency reported in synovial sarcomas [63, 99, 152]. In our series of mixed STS, 17% of the tumors showed cytoplasmic staining for ß-catenin, which was found to be a strong prognostic factor for metastasis in both univariate and multivariate analysis (Tables 13, 15 and 17). In addition to a structural role in the cadherin-catenin complex, ß-catenin can act as a transcriptional factor in the nucleus by serving as a co-activator of the TCF/LEF family of DNA-binding proteins [28]. Increased intracellular levels of ß-catenin may thereby influence transcription of genes involved in proliferation and transcriptional activation such as *cyclin D1* and *c-myc*, whose promoters contain TCF/LEF binding sites [65, 161, 168].

CD44

We found CD44 staining in >25% of the tumor cells in 60% of MFH and in 31% of the mixed STS using whole-tumor sections (Table 13). These levels were similar to those previously reported, although comparisons are difficult, since the methods of assessment, and the cut-off levels differ [83, 108, 135]. A high CD44 expression was also a strong prognostic factor for metastasis in our series of 140 mixed STS with a HR of 2.4, but not in the series of 201 MFH (Table 17). Although up-regulation of CD44 has been shown in several tumor types, such as cancers, lymphomas and mesenchymal tumors, the prognostic value of CD44 is seemingly conflicting as both improved and impaired survival has been found [11, 83, 108, 123, 135, 176]. CD44 is encoded by a single gene, but multiple isoforms of CD44 are generated by alternative splicing. Assessment can be made of the standard (CD44s) variant, and any of its 10 isoforms, CD44v1-10. In STS there are only a few reports of CD44 expression and consequences of up- or down-regulation. Peiper et al. [135] found improved survival in tumors with high (>20%) CD44s expression in a series of mixed STS. In contrast, in an experiment with CD44 knockout mice, Weber et al. [176] showed that formation of metastasis was virtually aborted in mice lacking the CD44 gene products. In a series of 47 STS, Kahara et al. [83] demonstrated that most sarcomas expressed CD44s, but that the isoform CD44v6 was more commonly expressed in high-grade tumors, and this isoform was also correlated to a shorter metastasis-free survival. Also Maula et al. [108] found that high CD44s expression, and particularly the isoform CD44v6 was associated with an increased risk of LR, but not with survival. The results from the 140 mixed STS (study III) are in agreement with the latter 3 studies that implicated CD44s as a prognostic factor for metastasis in STS. A possible explanation for the contrasting results could be found in the multiple functions of CD44s and its isoforms.

Pgp

We found PgP staining (>25%) in 40% of MFH and in 19% in mixed STS (with 6/18 MFH in the latter series having high Pgp expression) (Table 13), which is in line with reports from other authors who have reported Pgp expression in >50% of MFH and in varying frequencies in other STS entities [97, 102, 121]. We could not detect any prognostic importance of Pgp in the MFH series, whereas it was a strong prognostic factor for metastasis in the mixed STS series (Table 15). In a meta-analysis of 27 studies including 631 patients with osteosarcoma, Pakos and Ioannidis [133] demonstrated that Pgp was not associated with histological response to chemotherapy, but was a strong prognostic factor for disease relapse and progression. In STS, Pgp expression has been suggested as a marker for poor sensitivity to chemotherapy and worse survival [81, 102]. In rhabdomyosarcoma (RMS), Komdeur et al. [95] showed that pediatric tumors differ from adult tumors in the expression of multidrug resistance associated proteins. The expression of one of the MDR proteins, LRP was more pronounced in adult tumors and with age at diagnosis, and was also clearly less expressed in alveolar RMS. This offers an explanation for the increased sensitivity to therapy and better survival seen in pediatric RMS, and with the better outcome of alveolar form of RMS compared to embryonal and pleomorphic forms. In the same series, the expression of Pgp or the third MDR protein investigated, MRP1, did not

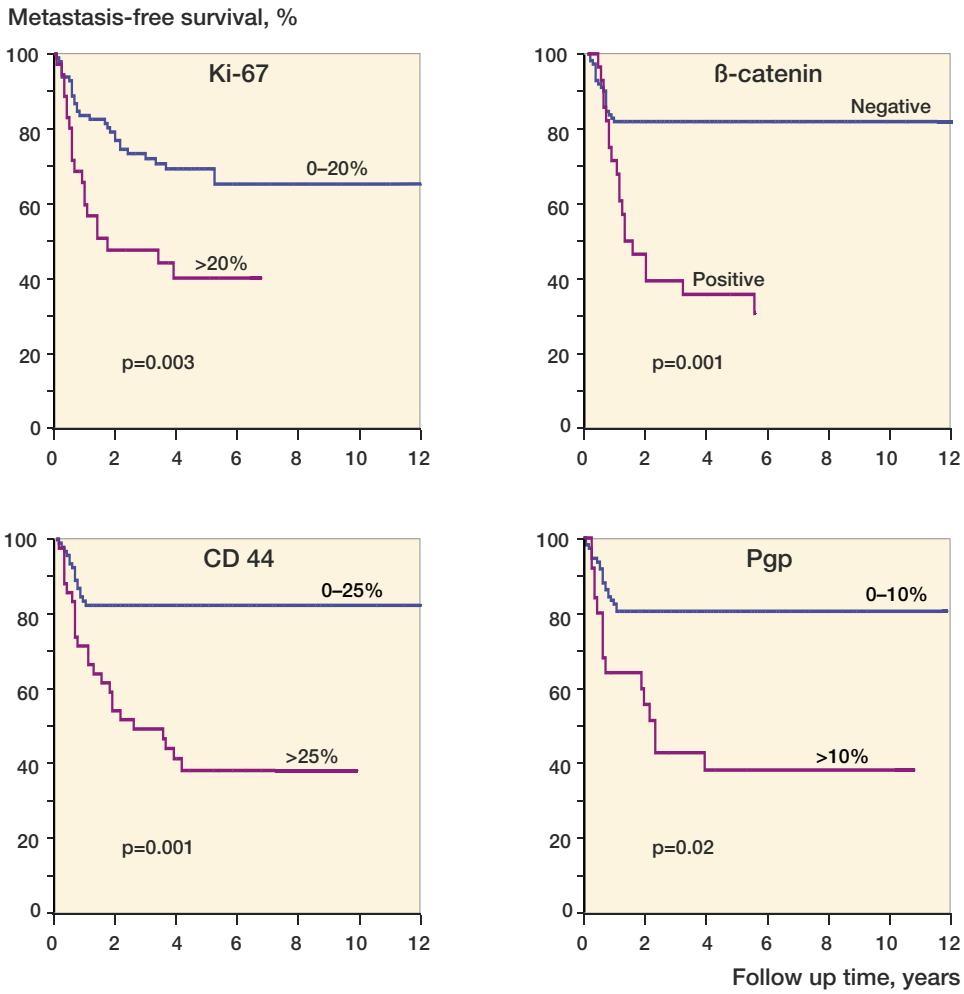


Figure 17. Metastasis-free survival according to IHC staining for Ki-67, ß-catenin, CD44, and Pgp in 140 mixed STS with whole-tumor section and peripheral TMA sampling (study III).

correlate with the expression of LRP, and although both were commonly expressed in RMS, Pgp and MRP1 were suggested to be independently regulated. Since chemotherapy was rarely used in our series, chemosensitivity was not addressed, but our findings concur with the suggested prognostic value of Pgp in STS.

In summary, only Ki-67 gave independent prognostic information in our series of 201 MFH (study II). In this series representative tumor areas from regular tumor blocks were selected by a sarcoma pathologist, whereas the peripheral tumor growth zone as identified in whole-tumor sections were specifically targeted for analysis in study III. In the latter series immunostaining for ß-catenin, CD44 and Pgp were in addition to Ki-67 found to be strong and independent factors for metastasis (Figure 17).

Immunostaining for prediction of local recurrence

The rates of LR in the MFH-based series of 338 patients and 201 patients were 29% and 33%, respectively. This was higher than in the series of 140 mixed STS, in which the rate of LR was 17%, and also higher than in previously reported, large series of MFH [137, 154]. This higher rate of LR can be explained by almost all the tumors in the MFH-based series being high-grade tumors, the long follow-up (more than 6 years for the survi-

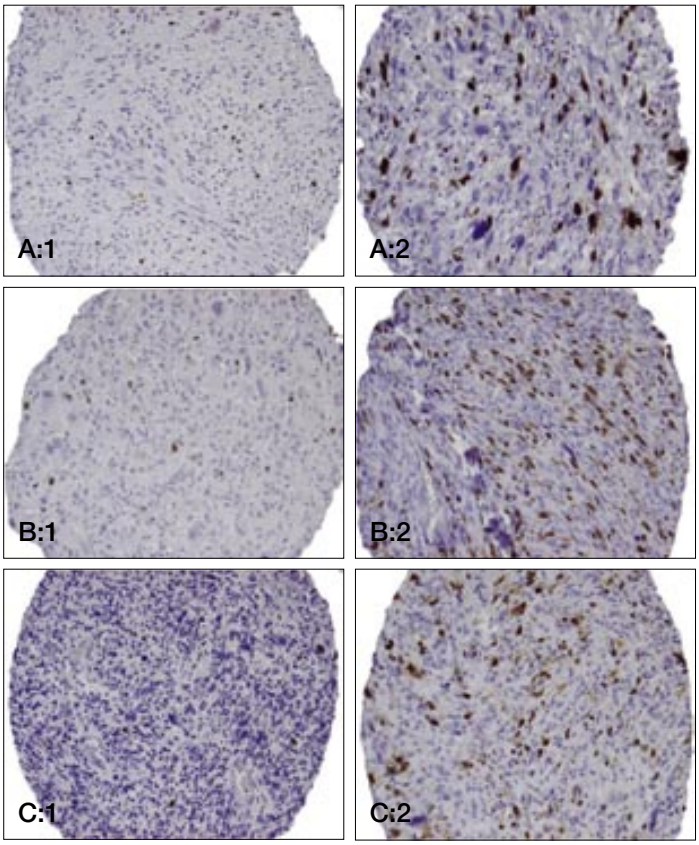


Figure 18. Up-regulation of cyclin A expression from primary tumors to metastases in 3 primary MFH (A:1-C:1) and the corresponding metastases (A:2-C:2).

vors), and the large proportion of myxoid tumors (38%), which are known to have a higher risk of LR [110].

In study II, none of the biological markers studied correlated with an increased risk of LR in MFH (data not shown), but in study III, using whole-tumor sections from 140 mixed primary STS, we found a correlation between expression of cyclin A and development of LR. The univariate analysis, performed in a subset of 124 tumors and with adequate local treatment, showed that tumors with cyclin A expression had a HR of 3.6 for development of LR (Table 14). Data on IHC expression in relation to LR are scarce, but Maula et al. [108] demonstrated an increased risk of LR for the isovariant CD44v6, but not for overall CD44 immunoreactivity in a series of 133 mixed STS and Heslin et al. [67] studied the markers Ki-67, p53, mdm2 and Rb in 121 mixed STS without detecting any significant correlation to the risk of LR.

IHC staining in primary tumors, local recurrences and metastases

In study II, primary tumor samples were available from 211 MFH, 50 LRs, and 20 metastases. The IHC expression increased from primary tumors to LRs and further in the metastases for Ki-67, cyclin A, p53 and Pgp, but not for bcl-2 and CD44 (Table 16). However, when the IHC expression levels were compared in matched tumor samples, the LRs did not significantly differ compared to the primary tumors for any of the markers (data not shown). When metastases were compared to the matched primary tumors, only cyclin A was consistently upregulated in all 18 metastases (p=0.03) (Figure 18).

These findings support the concept of a LR being a consequence of residual disease after surgical treatment of the primary tumor, whereas metastasis rather represents a clonal evolution of highly malignant cells with a metastatic phenotype. The results are also in accordance with a previous study using matched tumor samples from primary tumors and metastases in osteosarcoma. Oda et al. [129] demonstrated that the Ki-67 labelling index was higher in the metastases than in the primary tumor in a series of 25 osteosarcomas, whereas no difference in the expression of p53 was detected.

- IHC can add independent prognostic information in STS.
- Proliferation, assessed by Ki-67, should be included in studies of STS as a prospective prognostic marker.
- ß-catenin, CD44 and Pgp are candidate IHC prognostic factors for further studies.
- Based on the IHC findings, a local recurrence seems to represent regrowth of residual tumor.

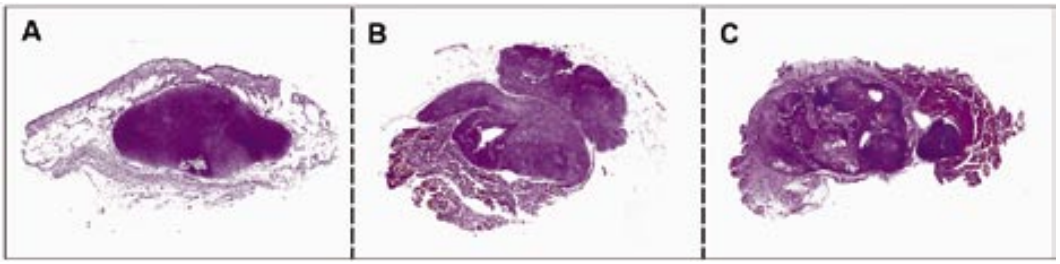


Figure 19. Whole tumor sections of a 4 cm s.c. leiomyosarcoma, grade IV (A), a 6 cm deep-seated MFH, grade IV (B), and an 8 cm deep-seated MFH, grade IV (C) (study III).

Value of whole-tumor sections (study III)

Whole-tumor sections are used in several tumor types, e.g. prostate cancer, rectal cancer and laryngeal cancer for better histopathological evaluation of e.g. tumor depth, invasion into adjacent tissues, and perineural growth [143, 164, 179]. At the Musculoskeletal Tumor Centre in Lund, we have increasingly used whole-tumor sections in addition to conventional sections for histopathologic examination in STS (Figure 19). The purpose was to evaluate the prognostic information obtained using whole-tumor sections with regard to previously established prognostic factors, peripheral tumor growth pattern, and immunostainings for markers associated with prognosis in STS.

Necrosis

Tumor cell necrosis is an important prognostic factor for metastasis in STS [6, 10, 32, 55, 64, 100, 120, 148, 171]. Necrosis is incorporated in the American NCI grading system, the French FNCLCC- grading system, and in the two grading systems used in Scandinavia [23, 31, 109, 120]. Good reproducibility in determining microscopic tumor necrosis has been demonstrated; Guillou et al. [54] reported concordance in 81% of tumors among 15 pathologists in determining necrosis, and Gustafson et al. [56] found concordance in 77% of 200 STS evaluated independently by sarcoma pathologists at 3 centers.

Despite the importance attributed to microscopic tumor necrosis there is no agreement regarding the amount of necrosis relevant for prognostication. In the NCI grading system a 15% cut off is used, whereas in the FNCLCC-grading system and in the Danish grading system by Myhre-Jensen a 50% cut-off is used [23, 31, 120]. In a study of 282 patients van Unnik et al. [174] found that necrosis, when determined as present or absent on the reviewed histopathology slides, was a discriminator for metastasis, and Gustafson et al. [56] found in 200 STS that necrosis had a prognostic value irrespective of how small the necrotic area was. As necrosis is included in histological malignancy grading, it follows that the proportion of tumors with necrosis increases with grade.

In our series (studies II and III), necrosis was assessed as present or absent, and was found in 60% of the tumors (Table 15). In the 201 MFH (study II), necrosis was not present in any of the 6 low-grade (grades I and II) tumors, but found in 15/57 (0.3) grade III, and in 98/138 (0.7) grade IV tumors. In the mixed STS series (study III) 1/18 low-grade tumors had foci of necrosis on whole-tumor sections, and necrosis was found in 6/24 grade III tumors, and in 76/98 grade IV tumors. The different frequencies observed in these 2 studies indicate that there is a small benefit in using whole-tumor sections for determining the presence of necrosis. For quantification of necrosis the use of whole-tumor sections also offer an obvious advantage in that a whole tumor plane can be examined.

Necrosis was a prognostic factor in univariate analysis for metastasis in both the MFH series, and in the mixed STS series, with HRs of 2.5 and 4.2, respectively (Table 17).

In a multivariate analysis, necrosis lost its prognostic value in the series of 201 MFH, whereas it remained an independent, strong factor for metastasis in the series of mixed STS with a HR of 2.8 (Table 15). One reason for this discrepancy could be that using whole-tumor sections improved the detection of necrosis in the grade IV tumors, and also foci of necrosis in 1 of the low-grade tumors, with an impact on the survival analysis. It

is unlikely that histotype-specific differences had an influence, as it has previously been shown that necrosis is a strong prognostic factor for metastasis in all the major histotypes included in studies II and III; MFH, leiomyosarcoma, and liposarcoma [55, 64, 170].

In the subset of 124 mixed STS analyzed for LR necrosis was in univariate analysis also found to be of prognostic importance for LR (Table 14). In this set the low rate of LR (19/124) yielded a low statistical power, and the impact of probable confounding factors, such as malignancy grade and vascular invasion could not be multivariately analyzed, which makes the interpretation of the independent value of necrosis difficult.

Vascular invasion

Vascular invasion was found at a considerably higher frequency using whole-tumor sections; only 21/201 (10%) MFH (study II) had demonstrable vascular invasion, compared to 50/140 (36%) in the 140 mixed STS (study III) in which whole-tumor section were assessed (Table 15). In the MFH, vascular invasion did not have any prognostic value for metastasis. This contrasts sharply to the strong prognostic importance that vascular invasion had in the mixed series of STS, in which the commonly accepted strong factor tumor size lost its independent prognostic value (Table 15). The importance of vascular invasion was evident when comparing the rate of metastasis in tumors with and without vascular invasion; 56/180 (0.3) MFH without demonstrated vascular invasion metastasized, compared to 19/90 (0.2) in the mixed STS. By determining vascular invasion patients with a very high probability of metastasis could be identified; in the series of mixed STS 35/50 (0.7) tumors with vascular invasion metastasized (Table 18).

Vascular invasion has previously been reported to be a prognostic factor for metastasis in STS [5, 55, 106, 144, 150, 171]. The 36% vascular invasion observed in the present series of mixed STS is considerably higher than what has been reported in previous studies [24, 55, 94, 106, 139]. In these studies vascular invasion was usually included in the entity "bone or neurovascular invasion", which varied between 6% and 20%.

Our results indicate that whole-tumor sections facilitate the identification of vascular invasion. In the subset of 17 MFH utilized both in study II and in study III, there were 1/17 tumors with vascular invasion in the series of 201 MFH (study II), but an additional 3 tumors showed vascular invasion when whole-tumor sections were analyzed in study III.

Table 18. Development of metastasis in relation to vascular invasion and grade in 201 MFH (study II) and in 140 mixed STS (study III)

Grade	201 MFH		140 mixed STS	
	Met/Vi+	Met/Vi–	Met/Vi+	Met/Vi–
I	0	0	0	0
II	0	1/6	1/1	1/12
III	0/1	15/56	5/6	2/18
IV	9/20	40/118	29/43	16/55
Total	9/21	56/180	35/50	19/90

Footnote: Met metastasis, Vi vascular invasion, +/– present/absent.

Determination of vascular invasion could possibly have been further augmented by the use of immunostaining against endothelial antigens, such as CD31, CD34 or factor VIII, which facilitate assessment of small vessels. In the pathology review of the tumors subsequently included in the series of 201 MFH, and in the series of mixed STS, only microscopy of H&E stained sections was employed. The different rates of vascular invasion in these two series indicate a risk of false negative assessment using conventional tumor sections. Careful microscopy of the tumor areas determines vascular invasion in larger veins and venules, where tumor cells within the endothelial lining are adherent to the vessel wall and associated with fibrin and blood corpuscles (Figure 20). Many reasons for false negative assessment regarding vascular invasion exist, for example too small a tumor area investigated, and invasion in very small vascular structures. There are also several findings that erroneously could be interpreted as vascular invasion. Examples of such false positive vascular invasion are tumor cells in extended contact with a venous vessel with an intact endothelium, tumor bulging into a vessel space without disruption of the endothelium, small hemorrhagic foci with fibrin, red blood cells and leucocytes intermingled with tumor cells, but lacking an endothelial vascular structure can mimic vascular invasion. Also,

sectioning artifacts with tumor cells within a vascular structure, but without adherence to the vessel wall and not associated with fibrin, red blood cells or leucocytes can resemble vascular invasion (Figure 20). In the study of 200 STS by Gustafson et al. [56], experienced sarcoma pathologists independently assessed the same histopathologic material with good agreement for vascular invasion (Kappa value 0.77).

Peripheral tumor growth pattern

Peripheral tumor growth pattern has been suggested to be of prognostic importance for both LR and metastasis in a previous study of STS [106]. However, to our knowledge growth pattern has not been systematically investigated, and is not included in any of the grading systems, or prognostic systems in use for STS [23, 92, 183].

In the mixed STS series, the peripheral growth pattern was a strong factor not only for LR but also for metastasis (Tables 14 and 15). In the 124 tumors with adequate local treatment, tumor growth pattern distinguished between tumors that developed a LR and those that did not. Of the 32 tumors with a pushing growth pattern 16 were superficial (13/16 grades III or IV) and 16 deep-seated (10/16 grades III or IV). RT was given after marginal resection in 6/32 tumors. None of these 32 tumors recurred locally.

92/124 tumors had an infiltrating growth pattern, 30 were superficial tumors (all of which were grades III or IV) and 62 were deep-seated tumors (57 of which were grades III or IV). Among the superficial tumors, 5 LRs occurred; 4/22 recurred after a wide resection, 1/6 after a marginal resection with RT, and none of 2 tumors with a wide resection and RT. In the 62 infiltrating deep-seated

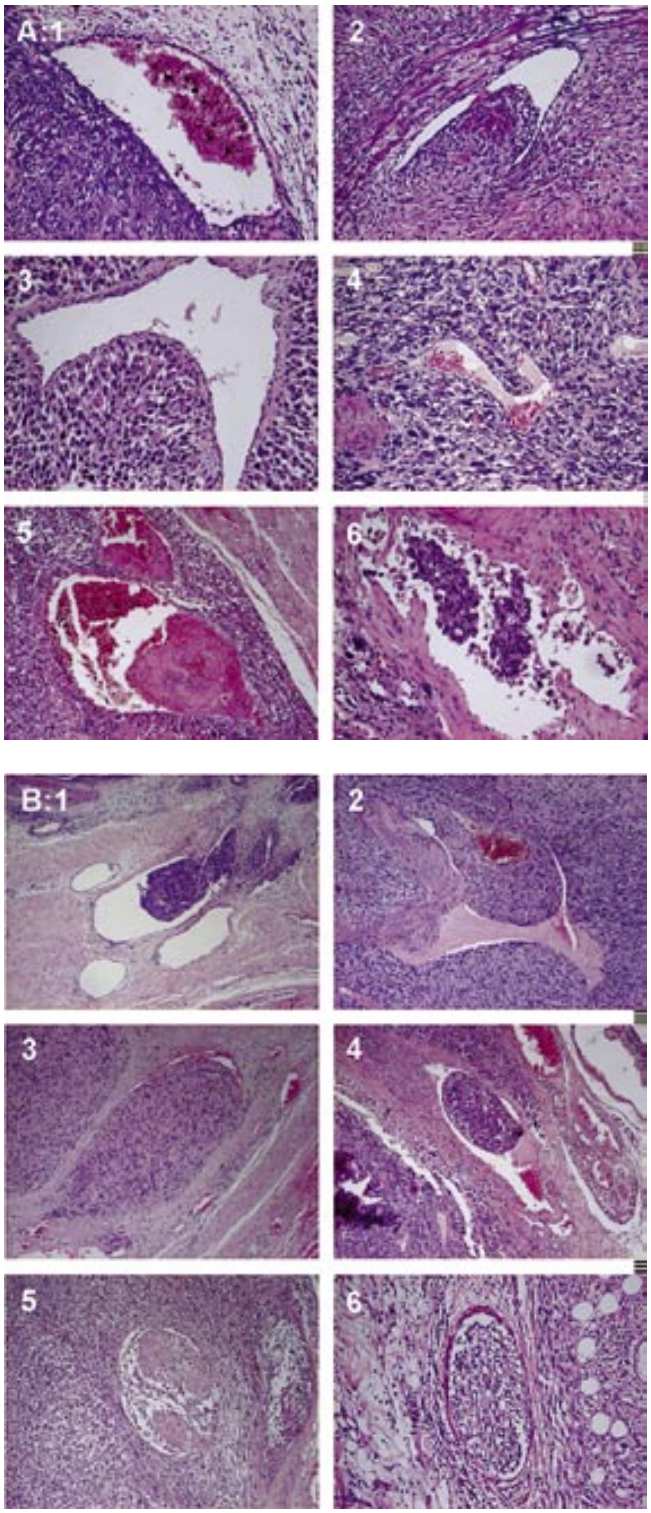


Figure 20. Illustration of false (A:1-6) and true (B:1-6) vascular invasion in STS.

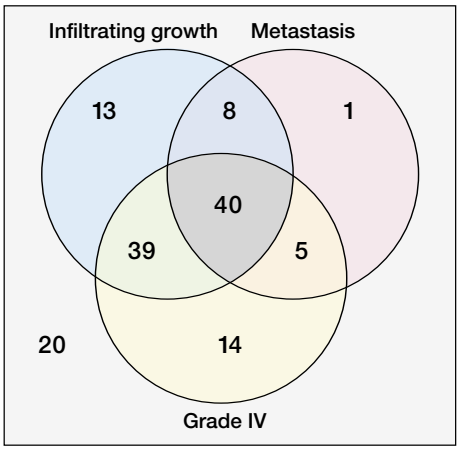


Figure 21. Venn diagram illustrating the distribution of growth pattern, malignancy grade and metastasis in 140 STS (study III).

tumors there were 14 LRs, all in the 57 grade III or IV tumors. Of these, 9/34 occurred after a wide resection without RT, and 5/20 following marginal resection with RT. After a wide resection with RT in 3 patients, there were no LRs. These findings indicate that when a wide surgical margin has been obtained in tumors with a pushing growth pattern, also in deep-seated and high-grade tumors, the need of adjuvant RT can be questioned. In contrast, tumors with an infiltrating growth pattern have a high risk of LR, and RT should probably be administered also after a wide margin, for both superficial and deep-seated tumors.

Infiltrative growth pattern was a strong prognostic factor for metastasis in univariate analysis (HR 4.6) (Table 17) as well as in multivariate analysis (HR 3.2) (Table 15). Among the 40 pushing tumors, 5/19 grade IV tumors metastasized, compared with 40/79 of the grade IV tumors with infiltrating growth pattern. Of the 42 grade III-I tumors, 1/21 of the tumors with a pushing growth metastasized, compared to 8/21 tumors with an infiltrating growth pattern (Figure 21).

Mandard et al. [106] demonstrated that infiltrative growth pattern was of prognostic importance for metastasis in STS, and proposed a prognostic system based on size, local treatment, necrosis and growth pattern, but not including malignancy grade. With this system an improved discrimination of high-risk patients, compared to NCI and FNCLCC systems, was shown. Our findings indicate that growth pattern is of prognostic value for both LR and metastasis in STS.

In the malignancy grading system outlined by Broders et al. [17] the principles of cellular differentiation used in grading of carcinomas were applied in fibrosarcoma. In this system infiltrative or pushing tumor growth was one of the histologic features assessed, the other factors being the degree of cellularity, cellular pleomorphism or anaplasia, mitotic activity (frequency and abnormal mitotic figures), and degree of necrosis. In mesenchymal tumors, also tumors perceived as benign in the sense that metastasis in principle never occurs, such as desmoids or dermatofibrosarcoma protuberans can grow extensively infiltrating. This may partly explain why the tumor growth pattern was attributed less importance in the subsequent malignancy grading systems. Assessment of the amount of necrosis and mitotic activity, in conjunction with differentiation and histological diagnosis, has to a greater extent formed the basis for current histological malignancy grading [92].

It is a common view that all high-grade STS grow infiltratively [40, 48, 90, 177]. Our findings suggest that a substantial proportion of high-grade STS have pushing borders, and that these may represent a less malignant subset of otherwise histologically similar high-grade tumors.

Prognostic indices

A prognostic index was based on the commonly used prognostic factors in STS of the extremities and trunk wall i.e. tumor size with a cut-off at 5 cm as used in most prognostic systems, depth (superficial *versus* deep) and malignancy grade (IV *versus* III–I). This index was compared with an index generated from the strongest factors in the series of 140 mixed STS, i.e. vascular invasion, growth pattern and tumor necrosis (Table 15). The index based on size, depth, and malignancy grade provided an AUC-value of 0.68, and the index based on vascular invasion, growth pattern and necrosis resulted in an improved AUC-value of 0.80 (p=0.005) (Figure 22).

The value of IHC for prognostication in STS was assessed by adding the prognostic information of the biological markers to the prognostic index. If also the two strongest IHC-factors, ß-catenin and Pgp, were added, the AUC increased to 0.85 com-

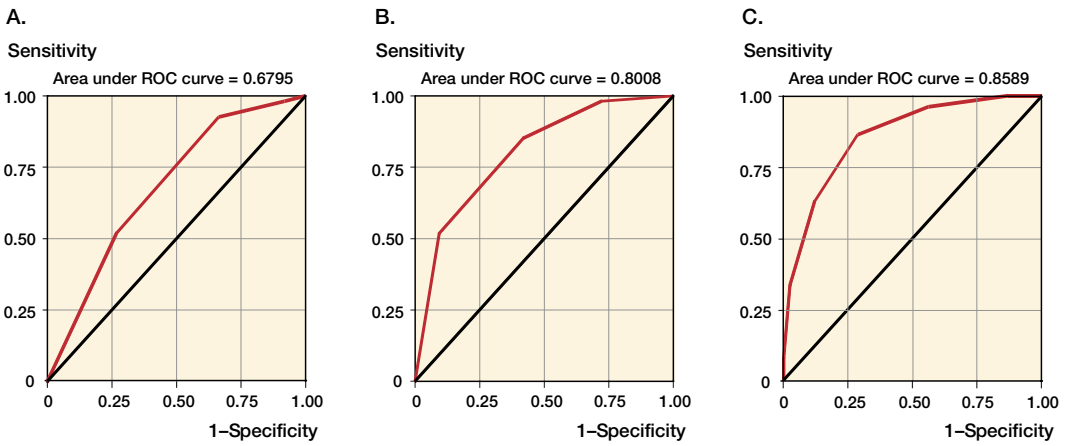


Figure 22. ROC-curves depicting the predictive accuracy of three prognostic systems for metastasis (study III).
A. Malignancy grade (IV), tumor size (>5cm) and depth (deep-seated tumor).
B. Vascular invasion, necrosis and infiltrative peripheral tumor growth pattern.
C. Vascular invasion, necrosis, infiltrative growth pattern, Ki-67, ß-catenin, CD44 and Pgp.

pared with the index based on vascular invasion, growth pattern and necrosis (p=0.01). Although not clinically feasible, an index can be constructed with all the independent prognostic factors that was found in the series of mixed STS; i.e. vascular invasion, infiltrating growth necrosis, Ki-67 >20%, ß-catenin positivity, CD44 >25% and Pgp >10%. This index had an AUC of 0.86 (Figure 22). The benefit of adding biological makers suggests that IHC probably should be prospectively investigated and used to improve prognostication of STS.

- Whole-tumor sections may improve determination of vascular invasion and necrosis and allow assessment of peripheral tumor growth pattern, all of which are of strong prognostic importance for metastasis in STS.
- Prediction of metastasis in STS may be improved by considering the factors vascular invasion, necrosis, and peripheral tumor growth pattern.

Time-dependence of prognostic factors

Time-dependence of prognostic factors in STS was specifically addressed in a series of 338 MFH for the factors tumor size, depth, necrosis, vascular invasion, and mitotic rate. The cumulative incidence of metastasis was at 5 years approximately 0.4–0.5 among patients with the respective adverse prognostic factors (>8 cm, deep-seated, necrosis, vascular invasion and high mitotic rate) at diagnosis compared to 0.2–0.3 among patients without the factor (Figure 23).

The rate of metastasis was highest during the first 2 years of follow-up, when 78/110 (0.7) metastases occurred, and 32/110 (0.3) occurred later than 2 years, of which only 4 occurred later than 5 years from diagnosis. The fraction of patients that metastasized within the categories of the prognostic factors is shown in Table 19. All prognostic factors were found to correlate, with ORs of 1.3–13 yielded by pair-wise comparisons (data not shown).

Univariate analysis showed that all factors possessed a strong prognostic value over the entire follow-up period and also in the time interval 0–2 years. Malignancy grade yielded no additional prognostic information for any of the time intervals multivariately analyzed (data not shown). Multivariate analysis using the factors size, depth, necrosis, vascular invasion, mitotic rate and a LR, revealed that only tumor size, tumor necrosis and LR were of prognostic importance over the first 2 years of follow-up, and over the entire follow-up period. Beyond 2 years, only tumor depth and LR correlated with time to metastasis (Table 20). In multivariate analysis without LR, the same pattern was seen (data not shown).

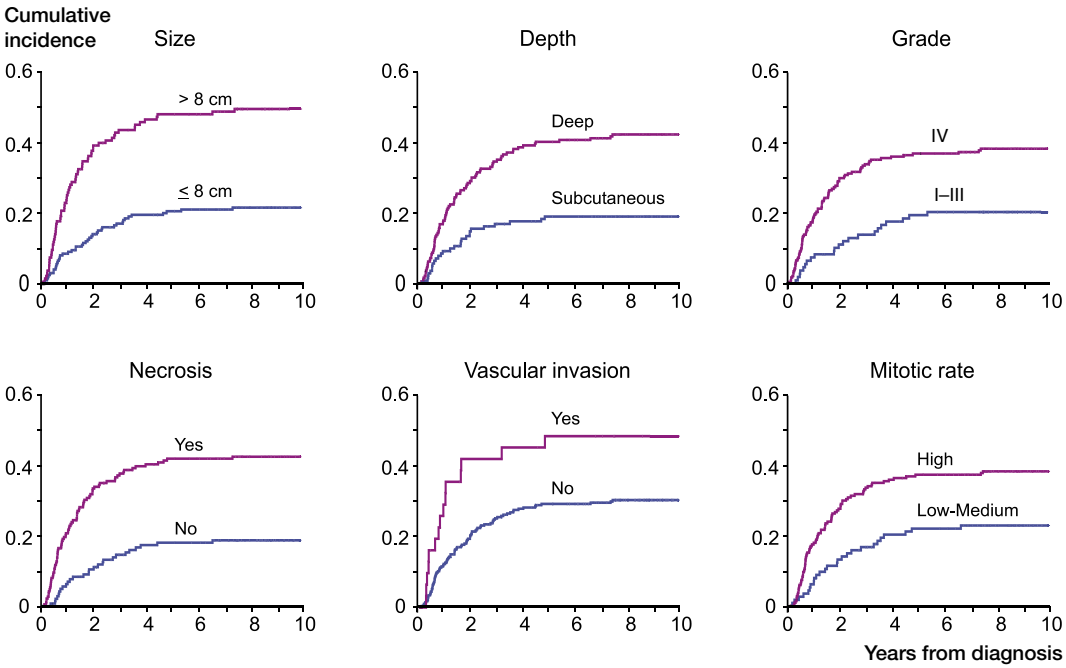


Figure 23. Cumulative incidences of metastases in 338 patients with MFH by prognostic factors; size, depth, malignancy grade, necrosis, vascular invasion and mitotic rate (study IV).

In the series of 140 mixed STS, a possible time-dependence of the prognostic factors was also investigated. In this series, 43/54 metastases occurred during the first 2 years. The statistical power with only 11 metastases diagnosed later than 2 years did not allow a separate analysis for the time frame >2 years. Therefore, the time intervals were dichotomized at the median time to metastasis, i.e. 10 months. In univariate analysis of tumor related prognostic factors and biological markers, microscopic tumor necrosis was the factor with the most striking time-dependence. Metastasis occurred during the period 0–10 months only in the tumors with necrosis, which made necrosis a strong predictor of early metastasis. After 10 months, necrosis lost its prognostic value. Several other strong prognostic factors, such as tumor size and malignancy grade, were of importance in the early time-period in univariate analysis, and thereafter lost their prognostic value. Vascular invasion, and invasive growth pattern were of prognostic value for the period 0–10 months as well as for the period >10 months. The biological markers, Ki-67, and Pgp were the only IHC markers of prognostic value for early metastasis. During the later time-period (>10 months) Ki-67 remained of prognostic value, whereas Pgp lost its prognostic importance, and cyclin A, ß-catenin, cyclin A and CD44 emerged as strong prognostic factors for metastasis occurring >10 months (Table 21). In multivariate analysis, necrosis, vascular invasion and Pgp-expression were the only factors with independent prognostic value for early metastasis. For the time period >10 months, vascular invasion remained of prognostic value, and infiltrative growth pattern, ß-catenin and CD44 provided independent prognostic information, whereas necrosis and the other biologic markers did not yield prognostic information (Table 21).

In the series of 338 MFH, the probability of metastasis the following year decreased over time, and for patients in the high-risk and low-risk groups converged already after 2–3 years. For patients with tumors >8 cm the probability of metastasis decreased from 0.24 at diagnosis, to 0.10 after 2 years, and 0.02 after 5 years. The same pattern was observed also for tumor depth, malignancy grade, necrosis, vascular invasion, and mitotic rate (Figure 24). There may be several reasons for this finding. The biological importance of an initial factor may

Table 19. Initial prognostic factors and metastasis data in 338 MFH (study IV)

Factor	Patients	Fraction with metastasis	Patients with metastasis during FU	
			0–2	>2 years
Tumor size (cm)				
≤8	199	0.2	27	16
>8	135	0.5	51	16
Undetermined	4	0	0	0
Tumor depth				
Subcutaneous	142	0.2	21	6
Deep seated	196	0.4	57	26
Tumor grade				
I+II	1+8	0.2	1	1
III	99	0.2	10	10
IV	230	0.4	67	21
Tumor necrosis				
No	143	0.2	15	12
Yes	187	0.4	63	17
Undetermined	8	0.4	0	3
Vascular invasion				
No	294	0.3	60	29
Yes	31	0.5	13	2
Undetermined	13	0.5	5	1
Mitotic rate/10 HPF [a]				
Low-medium (0–9)	114	0.2	15	11
High (≥ 10)	218	0.4	63	20
Undetermined	6	0.2	0	1
Patients at risk at start of interval			338	237

[a] HPF high power field (x40)

diminish during follow-up, provided the patients survive metastasis-free, and the results in the series of 140 mixed STS indicate that this is the case for e.g. tumor necrosis. Heterogeneity within prognostic categories as a result of errors of measurements and distribution of unknown biological factors with bearing on metastasis represent another possible explanation. In the 2 series (studies III and IV), the difference in the frequency of vascular invasion in the series of 338 MFH (10%), and the series of mixed STS (36%) suggest that false negative findings could contribute to the convergence of probabilities between the prognostic categories. It could be expected that the rate of metastasis would remain higher among patients in the high-risk categories. At diagnosis the proportion of true high-risk patients is higher in the high-risk category than in the low-risk category, which is reflected in the higher relative risk of metastasis during the first few years of follow-up. Later during follow-up most true high-risk patients in both risk categories have metastasized and thus leave the study population, which explains both the decrease in overall risk and the convergence of risk of metastasis in the high-risk and the low-risk categories.

A LR was a strong and independent prognostic factor for metastasis in both the series of 338 MFH (study IV) and in the mixed STS series (study III),

Table 20. Univariate and multivariate analyses of time to metastasis in 338 MFH for initial tumor-associated prognostic factors and local recurrence (study IV)

Factor	Whole follow–up			0–2 years			> 2 years		
	RR	95% CI	p–value	RR	95% CI	p–value	RR	95% CI	p–value
Univariate analysis									
Size >8 cm	3.0	2.0–4.4	<0.001	3.3	2.1–5.3	<0.001	2.3	1.2–4.6	0.02
Deep seated	2.5	1.6–3.8	<0.001	2.1	1.3–3.4	0.004	3.9	1.6–9.5	0.003
Grade IV	2.3	1.4–3.6	0.001	3.3	1.7–6.2	<0.001	1.2	0.6–2.6	0.6
Necrosis present	2.8	1.8–4.4	<0.001	3.8	2.2–6.7	<0.001	1.6	0.7–3.3	0.2
Vascular invasion	2.1	1.2–3.6	0.008	2.6	1.4–4.7	0.002	1.0	0.2–4.1	1
High mitotic rate	1.9	1.2–3.0	0.004	2.5	1.4–4.3	0.002	1.1	0.5–2.4	0.7
Local recurrence	2.5	1.6–3.9	<0.001	2.4	1.3–4.2	0.004	2.7	1.3–5.6	0.008
Multivariate analysis									
Size >8 cm	1.9	1.2–3.0	0.005	2.2	1.3–3.7	0.005	1.4	0.6–3.1	0.5
Deep seated	1.5	0.9–2.5	0.1	1.2	0.7–2.2	0.4	2.7	1.0–7.1	0.04
Necrosis present	1.9	1.2–3.0	0.01	2.2	1.2–4.2	0.009	1.4	0.6–3.1	0.5
Vascular invasion	1.5	0.8–2.7	0.2	1.6	0.9–3.1	0.1	1.0	0.2–4.3	0.6
High mitotic rate	1.2	0.8–2.0	0.4	1.6	0.9–2.9	0.1	0.8	0.3–1.7	0.5
Local recurrence	3.0	1.9–5.0	<0.001	2.9	1.5–5.4	0.001	3.2	1.4–7.0	0.004

RR Relative Risk, CI Confidence Interval.

Table 21. Univariate and multivariate analysis of time to metastasis for the time period 0–10 months and >10 months in 140 STS (study III)

Factor	0–10 months			> 10 months		
	HR	95% CI	p–value	HR	95% CI	p–value
Univariate analysis						
Size >8 cm	2.1	0.98–4.7	0.06	1.9	0.87–4.1	0.1
Deep seated	1.5	0.65–3.7	0.3	1.6	0.67–3.8	0.3
Grade IV	5.8	1.4–24	0.02	1.7	0.73–4.1	0.2
Necrosis present	∞	–	<0.001 [c]	1.9	0.87–4.1	0.1
Vascular invasion	5.9	2.5–14	<0.001	4.3	2.0–9.2	<0.001
Infiltrative growth	5.6	1.3–24	0.02	4.1	1.4–12	0.01
Ki-67 [a]	2.2	1.0–4.9	0.04	2.5	1.1–5.7	0.03
p53 [a]	0.71	0.24–2.1	0.5	0.70	0.21–2.3	0.6
Cyclin A [a]	2.3	0.92–5.7	0.08	3.6	1.4–9.2	0.007
ß-catenin [a]	1.7	0.73–3.8	0.2	3.6	1.7–8.1	0.001
CD44 [a]	2.0	0.94–4.4	0.07	2.9	1.3–6.5	0.007
Pgp [a]	2.5	1.1–5.7	0.02	1.6	0.64–4.2	0.3
Local recurrence [b]	2.7	0.4–20	0.3	7.8	2.5–24	<0.001
Multivariate analysis						
Necrosis present	∞	–	–	1.1	0.51–2.6	0.7
Vascular invasion	3.1	1.3–7.3	0.01	3.5	1.6–7.7	0.002
Infiltrative growth	3.0	0.70–12	0.1	3.2	1.0–9.8	0.04
Ki-67 [a]	1.4	0.63–3.0	0.4	2.4	0.99–5.9	0.05
Cyclin A [a]	1.1	0.42–2.7	0.9	2.5	0.95–6.6	0.06
ß-catenin [a]	1.4	0.62–3.3	0.4	6.6	2.8–15	<0.001
CD44 [a]	1.4	0.64–3.2	0.4	1.6	1.0–2.5	0.03
Pgp [a]	2.4	1.1–5.5	0.04	2.0	0.76–5.0	0.2
Local recurrence [b]	1.8	0.23–14	0.6	4.0	1.4–14	0.01

HR Hazard ratio, CI Confidence Interval.
[a] Refers to cut–off levels in Table 9.
[b] Based on 124 patients with adequate local treatment.
[c] log–rank test.

with a HR of 3.0 and 5.6, in univariate analysis for the whole follow-up period. In comparison to most of the initial tumor-related prognostic factors, the prognostic value of LR was not time-dependent, and remained a strong prognostic factor for metastasis whenever it occurred (Tables 20 and 21). In the mixed STS series (study III) only 3 LRs occurred during the early time-interval of 0–10 months (HR 2.7, p=0.3). In the later time-interval LR was a strong prognostic factor, with a HR of 7.8 (Table 21). In the larger series of MFH (study II), LR was a prognostic factor during both the early and later time-intervals with a HR of 2.5 and 2.7, respectively (Table 20). LR was an independent prognostic factor for metastasis in both series, and was in fact the most consistent strong prognostic factor during the time intervals analyzed (Tables 20 and 21). The implications of a LR after wide surgical margins was suggested by Simon and Enneking [162] who already in 1976 reported that "if a patient had a local recurrence after our definitive procedure, the prognosis was poor". Although some grade I STS, notably myxofibrosarcoma, have a great propensity for LR and yet rarely metastasize, in most STS a LR signifies an aggressive tumor phenotype whenever it occurs, regardless of the primary tumor characteristics. Hence, occurrence of a LR should, for most types of STS, raise the question of additional treatment with adjuvant chemotherapy.

Recent studies in breast cancer [29, 50, 68, 155], lymphomas [117] and colorectal cancer [15, 142] have suggested that the relevance of prognostic factors varies during follow-up. In breast cancer, a positive tumor steroid hormone receptor status is a favorable prognostic factor at diagnosis, but after 3–5 years is a relative risk factor for metastasis [29, 50, 68, 155]. In aggressive lymphomas, Mounier et al. [117] showed that performance status, lactate dehydrogenase level and extranodal involvement,

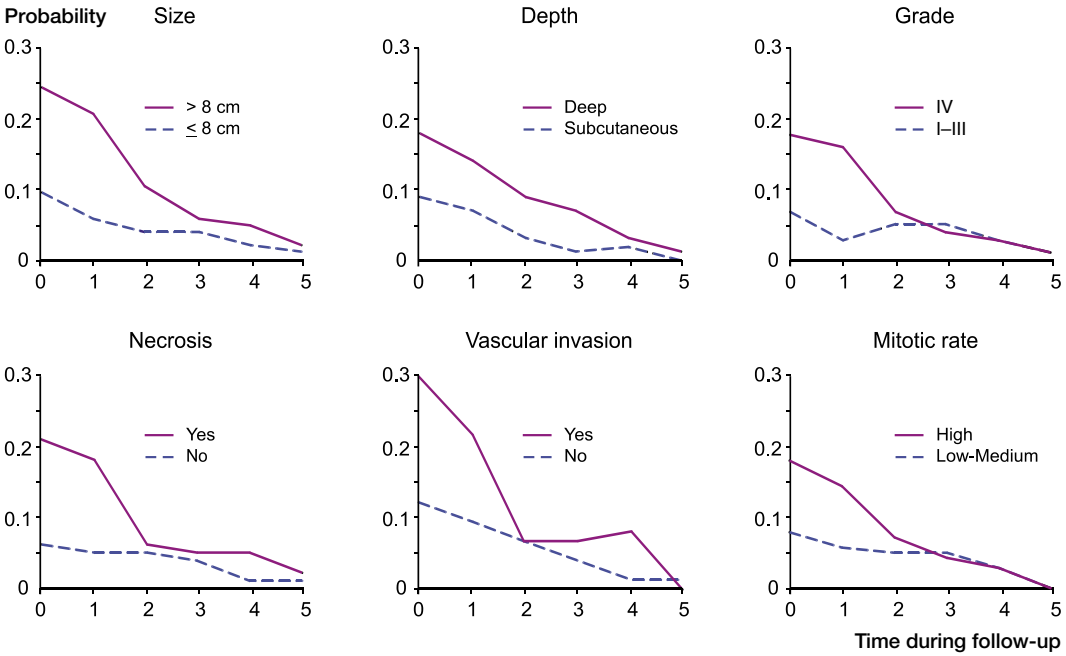


Figure 24. Annual probability of metastasis for 338 MFH by prognostic factors; size, depth, malignancy grade, necrosis, vascular invasion and mitotic rate.

all of which are included in the International Prognostic Index, are time-dependent. Few studies have addressed this question in STS. Lewis [103] demonstrated that high malignancy grade (high *versus* low) had no prognostic importance for metastasis occurring >5 years in 262 patients with STS of the extremities who were disease-free after 5 years. Stojadinovic et al. [165] reported that the relative risk associated with malignancy grade (high *versus* low) for STS of the extremities and trunk wall was 8 for the first 3 years and decreased to 2 thereafter. Similarly, they found that the relative risk associated with tumor size (>5 cm *versus* ≤5 cm) was 3 for the first year and 1.5 thereafter.

- Tumor-related prognostic factors in STS are time-dependent and most factors lose their value after 2 years of follow-up.
- Tumor necrosis is a strong predictor for early metastasis, and local recurrence signifies an increased risk of metastasis whenever it occurs.

Conclusions

- Tissue microarray is applicable for assessment of staining patterns of biological markers in STS, and yields similar results as conventional tumor sections.

- Prognostication in STS can be improved by immunohistochemical staining for Ki-67, which should be considered for routine use in pleomorphic STS. ß-catenin, CD44 and Pgp represent candidate markers for further prognostic evaluation in STS

- Immunohistochemical staining may yield a more accurate estimate for prognostic applications if performed in the peripheral tumor growth zone.

- Whole-tumor sections are of value for assessment of prognostic factors in STS. Vascular invasion, necrosis, and peripheral tumor growth pattern are strong prognostic factors for metastasis, and growth pattern also for local recurrence.

- Tumor-related prognostic factors as determined at diagnosis are time-dependent, and have lost their value after 2 years follow-up. In contrast, local recurrence after wide surgical margins can be perceived as a dynamic prognostic factor during follow-up, which when diagnosed indicates a malignant tumor phenotype with an increased risk of metastasis.

Acknowledgements

My supervisor, *Associate Professor Mef Nilbert*, Department of Oncology, Lund University, who works harder than most, yet always is available for advice and discussions, and never leaves an e-mail unanswered for longer than a few hours, regardless of the hour it was sent. With her relentless enthusiasm and kindness she is a role-model to emulate, if only possible in small parts.

My co-supervisor, *Associate Professor Thor Alvegård*, Department of Cancer Epidemiology, Lund University, for generously providing research-time for this work, and for his assistance with contacts throughout the SSG.

Professor Anders Rydholm, Department of Orthopedics, Lund University, for raising the issue of the importance of whole-tumor sections and peripheral tumor growth pattern in STS, and for generously sharing his vast knowledge of STS.

Associate Professor Måns Åkerman and *Henryk Domanski MD*, Department of Pathology and Cytology, Lund University, for reviewing and selecting the histopathological material at the contributing centers in Sweden and Norway and for being such good teachers in the art of histomorphology.

Harald Anderson Associate professor, Department of Cancer Epidemiology and *Pär-Ola Bendahl PhD*, Department of Oncology, Lund University, for their commitment and application of their great knowledge in biostatistics to the issues raised in the projects.

Annette Persson BSc, Department of Pathology and Cytology, Lund University, and *Eva Rambech BSc,* Department of Oncology, Lund University, for help with the tissue microarrays, whole-tumor sections, and for performing distinct immunohistochemical stainings.

Bodil Bjerkehagen MD, PhD, Department of Pathology, The Norwegian Radium Hospital, Oslo, *Peer Kåre Lilleng MD, PhD*, Department of Pathology, Haukeland University Hospital, Bergen, and *Johan Wejde MD, PhD*, Department of Pathology and Cytology, Karolinska Hospital, Stockholm, for assisting in the selection and compiling of the histopathological material at their centers and for constructive advice.

Kirsten Sundby Hall MD, PhD, Department of Oncology, The Norwegian Radium Hospital, Oslo, *Professor Henrik Bauer*, Department of Orthopedic Surgery, Karolinska Hospital, Stockholm, *Professor Jeanne Meis-Kindblom*, Department of Pathology, Sahlgrenska University Hospital, Gothenburg and *Associate Professor Pelle Gustafson*, Department of Orthopedics, Lund University, for contributing with follow-up data and for constructive advice.

Evy Nilsson, at the SSG Central register, for invaluable and efficient help with the patient data.

Mona Martinsson, at the Southern Swedish Tumor Registry for help with the graphics.

Martin Sjöbeck MD, at the Department of Pathology and Cytology, Lund for assistance with photography.

Professor Håkan Olson, Department of Oncology Lund University, for generously contributing to the research time made available.

Professor Dick Killander, Department of Oncology, Lund University, for supporting the work in this thesis.

My colleagues and friends at the Department of Oncology, Lund University Hospital, for creating an amicable and dynamic work-atmosphere in which research is inherently linked to the clinical responsibilities.

My fellow PhD-students and lab mates at the Kamprad lab, for the helpful and friendly environment in which the laboratory work was conducted.

The SSG Pathology Review Group, for the important work in reviewing the histopathological material in Sweden, Norway and Finland.

The SSG affiliated centers and doctors, for reporting original and follow-up data on sarcoma patients.

My wife Lena, whose love I am proud to deserve.

The Swedish Cancer Society, the Swedish Childrens Cancer Society, the John and Augusta Persson Foundation, the B. Kamprad Cancer Foundation, the G. Nilsson Cancer Foundation, the Gustaf V Fund, and the Lund University Hospital Fund for financial support. Research time was granted from Region Skåne and from the Medical Faculty at Lund University.

References

1. Sarcoma Meta-analysis Collaboration: Adjuvant chemotherapy for localised resectable soft-tissue sarcoma of adults: meta-analysis of individual data. Lancet 350: 1647-54, 1997.
2. Adjuvant chemotherapy for localised resectable soft tissue sarcoma in adults. Sarcoma Meta-analysis Collaboration (SMAC). Cochrane Database Syst Rev: CD001419, 2000.
3. Ahlen J, Weng WW, Brosjo O, et al. Evaluation of immunohistochemical parameters as prognostic markers in malignant fibrous histiocytoma. Oncol Rep 10: 1641-5, 2003.
4. Allander SV, Illei PB, Chen Y, et al. Expression profiling of synovial sarcoma by cDNA microarrays: association of ERBB2, IGFBP2, and ELF3 with epithelial differentiation. Am J Pathol 161: 1587-95, 2002.
5. Alvegard TA, Berg NO, Baldetorp B, et al. Cellular DNA content and prognosis of high-grade soft tissue sarcoma: the Scandinavian Sarcoma Group experience. J Clin Oncol 8: 538-47, 1990.
6. Angervall L, Kindblom LG, Rydholm A, et al. The diagnosis and prognosis of soft tissue tumors. Semin Diagn Pathol 3: 240-58, 1986.
7. Battifora H. The multitumor (sausage) tissue block: novel method for immunohistochemical antibody testing. Lab Invest 55: 244-8, 1986.
8. Battifora H, Mehta P. The checkerboard tissue block. An improved multitissue control block. Lab Invest 63: 722-4, 1990.
9. Bauer HC, Trovik CS, Alvegard TA, et al. Monitoring referral and treatment in soft tissue sarcoma: study based on 1,851 patients from the Scandinavian Sarcoma Group Register. Acta Orthop Scand 72: 150-9, 2001.
10. Becker RL, Jr., Venzon D, Lack EE, et al. Cytometry and morphometry of malignant fibrous histiocytoma of the extremities. Prediction of metastasis and mortality. Am J Surg Pathol 15: 957-64, 1991.
11. Benassi MS, Ragazzini P, Gamberi G, et al. Adhesion molecules in high-grade soft tissue sarcomas: correlation to clinical outcome. Eur J Cancer 34: 496-502, 1998.
12. Billingsley KG, Lewis JJ, Leung DH, et al. Multifactorial analysis of the survival of patients with distant metastasis arising from primary extremity sarcoma. Cancer 85: 389-95, 1999.
13. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1: 307-10, 1986.
14. Blay JY, van Glabbeke M, Verweij J, et al. Advanced soft-tissue sarcoma: a disease that is potentially curable for a subset of patients treated with chemotherapy. Eur J Cancer 39: 64-9, 2003.
15. Bolard P, Quantin C, Esteve J, et al. Modelling time-dependent hazard ratios in relative survival: application to colon cancer. J Clin Epidemiol 54: 986-96, 2001.
16. Borden EC, Baker LH, Bell RS, et al. Soft tissue sarcomas of adults: state of the translational science. Clin Cancer Res 9: 1941-56, 2003.
17. Broders AC HR, Meyerding HW. Pathological features of soft tissue sarcoma. Surg Gynecol Obstet: 267-80, 1939.
18. Bubendorf L, Kononen J, Koivisto P, et al. Survey of gene amplifications during prostate cancer progression by high-throughout fluorescence in situ hybridization on tissue microarrays. Cancer Res 59: 803-6, 1999.
19. Camp RL, Charette LA, Rimm DL. Validation of tissue microarray technology in breast carcinoma. Lab Invest 80: 1943-9, 2000.
20. Choong PF, Akerman M, Willen H, et al. Prognostic value of Ki-67 expression in 182 soft tissue sarcomas. Proliferation – a marker of metastasis? Apmis 102: 915-24, 1994.
21. Cichy J, Pure E. The liberation of CD44. J Cell Biol 161: 839-43, 2003.
22. Clasby R, Tilling K, Smith MA, et al. Variable management of soft tissue sarcoma: regional audit with implications for specialist care. Br J Surg 84: 1692-6, 1997.
23. Coindre JM. Symposium 14: Controversial topics in soft tissue pathology. Histopathology 41: 227-248, 2002.
24. Coindre JM, Terrier P, Bui NB, et al. Prognostic factors in adult patients with locally controlled soft tissue sarcoma. A study of 546 patients from the French Federation of Cancer Centers Sarcoma Group. J Clin Oncol 14: 869-77, 1996.
25. Coindre JM, Terrier P, Guillou L, et al. Predictive value of grade for metastasis development in the main histologic types of adult soft tissue sarcomas: a study of 1240 patients from the French Federation of Cancer Centers Sarcoma Group. Cancer 91: 1914-26, 2001.
26. Coley HM, Verrill MW, Gregson SE, et al. Incidence of P-glycoprotein overexpression and multidrug resistance (MDR) reversal in adult soft tissue sarcoma. Eur J Cancer 36: 881-8, 2000.
27. Collin F, Chassevent A, Bonichon F, et al. Flow cytometric DNA content analysis of 185 soft tissue neoplasms indicates that S-phase fraction is a prognostic factor for sarcomas. French Federation of Cancer Centers (FNCLCC) Sarcoma Group. Cancer 79: 2371-9, 1997.
28. Conacci-Sorrell M, Zhurinsky J, Ben-Ze'ev A. The cadherin-catenin adhesion system in signaling and cancer. J Clin Invest 109: 987-91, 2002.
29. Coradini D, Daidone MG, Boracchi P, et al. Time-dependent relevance of steroid receptors in breast cancer. J Clin Oncol 18: 2702-9, 2000.

30. Cordon-Cardo C, Latres E, Drobnjak M, et al. Molecular abnormalities of mdm2 and p53 genes in adult soft tissue sarcomas. Cancer Res 54: 794-9, 1994.
31. Costa J (1990) The grading and staging of soft tissue sarcomas. Pathobiology of Soft Tissue Tumors, ed. C. Fletcher and P. McKee Edinburgh, UK: Churchill Livingstone.
32. Costa J, Wesley RA, Glatstein E, et al. The grading of soft tissue sarcomas. Results of a clinicohistopathologic correlation in a series of 163 cases. Cancer 53: 530-41, 1984.
33. Costa MJ, Weiss SW. Angiomatoid malignant fibrous histiocytoma. A follow-up study of 108 cases with evaluation of possible histologic predictors of outcome. Am J Surg Pathol 14: 1126-32, 1990.
34. Coultas L, Strasser A. The role of the Bcl-2 protein family in cancer. Semin Cancer Biol 13: 115-23, 2003.
35. Dahlen A, Fletcher CD, Mertens F, et al. Activation of the GLI oncogene through fusion with the beta-actin gene (ACTB) in a group of distinctive pericytic neoplasms: pericytoma with t(7;12). Am J Pathol 164: 1645-53, 2004.
36. Dan'ura T, Kawai A, Morimoto Y, et al. Apoptosis and expression of its regulatory proteins in soft tissue sarcomas. Cancer Lett 178: 167-74, 2002.
37. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated reciever operating curves: A nonparametric approach. Biometrics 44: 837-845, 1988.
38. Donato Di Paola E, Nielsen OS. The EORTC soft tissue and bone sarcoma group. European Organisation for Research and Treatment of Cancer. Eur J Cancer 38 Suppl 4: S138-41, 2002.
39. Drobnjak M, Latres E, Pollack D, et al. Prognostic implications of p53 nuclear overexpression and high proliferation index of Ki-67 in adult soft-tissue sarcomas. J Natl Cancer Inst 86: 549-54, 1994.
40. Enneking WF, Spanier SS, Goodman MA. A system for the surgical staging of musculoskeletal sarcoma. Clin Orthop (153): 106-20, 1980.
41. Eriksson M, Hardell L, Adami HO. Exposure to dioxins as a risk factor for soft tissue sarcoma: a population-based case-control study. J Natl Cancer Inst 82: 486-90, 1990.
42. Fernberg JO, Hall KS. Chemotherapy in soft tissue sarcoma. The Scandinavian Sarcoma Group experience. Acta Orthop Scand (Suppl 311) 75: 77-86, 2004.
43. Fernebro E, Dictor M, Bendahl PO, et al. Evaluation of the tissue microarray technique for immunohistochemical analysis in rectal cancer. Arch Pathol Lab Med 126: 702-5, 2002.
44. Figueredo A, Bramwell V, Bell RS, et al. 2002 Oct.: Toronto (ON): Cancer Care Ontario (CCO). p. 27.
45. Fleming JB, Berman RS, Cheng SC, et al. Long-term outcome of patients with American Joint Committee on Cancer stage IIB extremity soft tissue sarcomas. J Clin Oncol 17: 2772-80, 1999.
46. Fletcher CD. Pleomorphic malignant fibrous histiocytoma: fact or fiction? A critical reappraisal based on 159 tumors diagnosed as pleomorphic sarcoma. Am J Surg Pathol 16: 213-28, 1992.
47. Fletcher CD, Gustafson P, Rydholm A, et al. Clinicopathologic re-evaluation of 100 malignant fibrous histiocytomas: prognostic relevance of subclassification. J Clin Oncol 19: 3045-50, 2001.
48. Fletcher CDM, Unni KK, Mertens FE, (2002) Pathology and Genetics of Tumours of Soft Tissue and Bone. World Health Organisation Classification of Tumours, ed. C.D.M. Fletcher, K.K. Unni, and F. Mertens. Lyon: IARC Press.
49. Frustaci S, Gherlinzoni F, De Paoli A, et al. Adjuvant chemotherapy for adult soft tissue sarcomas of the extremities and girdles: results of the Italian randomized cooperative trial. J Clin Oncol 19: 1238-47, 2001.
50. Gebauer G, Fehm T, Lang N, et al. Tumor size, axillary lymph node status and steroid receptor expression in breast cancer: prognostic relevance 5 years after surgery. Breast Cancer Res Treat 75: 167-73, 2002.
51. Gortzak E, Azzarelli A, Buesa J, et al. A randomised phase II study on neo-adjuvant chemotherapy for ‚high-risk' adult soft-tissue sarcoma. Eur J Cancer 37: 1096-103, 2001.
52. Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer 2: 48-58, 2002.
53. Greene FL, Page DL, Fleming ID, et al., eds. (2002) AJCC Cancer Staging Handbook / American Joint Committee on Cancer. 6th. edition, ed. F.L. Greene, Springer-Verlag: New York, Berlin, Heidelberg.
54. Guillou L, Coindre JM, Bonichon F, et al. Comparative study of the National Cancer Institute and French Federation of Cancer Centers Sarcoma Group grading systems in a population of 410 adult patients with soft tissue sarcoma. J Clin Oncol 15: 350-62, 1997.
55. Gustafson P. Soft tissue sarcoma. Epidemiology and prognosis in 508 patients. Acta Orthop Scand Suppl 259: 1-31, 1994.
56. Gustafson P, Akerman M, Alvegard TA, et al. Prognostic information in soft tissue sarcoma using tumour size, vascular invasion and microscopic tumour necrosis-the SIN-system. Eur J Cancer 39: 1568-76, 2003.
57. Gustafson P, Baldetorp B, Ferno M, et al. Prognostic implications of various models for calculation of S-phase fraction in 259 patients with soft tissue sarcoma. Br J Cancer 79: 1205-9, 1999.
58. Gustafson P, Dreinhofer KE, Rydholm A. Soft tissue sarcoma should be treated at a tumor center. A comparison of quality of surgery in 375 patients. Acta Orthop Scand 65: 47-50, 1994.
59. Gustafson P, Rooser B, Rydholm A. Is local recurrence of minor importance for metastases in soft tissue sarcoma? Cancer 67: 2083-6, 1991.
60. Hainaut P, Hollstein M. p53 and human cancer: the first ten thousand mutations. Adv Cancer Res 77: 81-137, 2000.

61. Hasegawa T, Yamamoto S, Yokoyama R, et al. Prognostic significance of grading and staging systems using MIB-1 score in adult patients with soft tissue sarcoma of the extremities and trunk. Cancer 95: 843-51, 2002.
62. Hasegawa T, Yokoyama R, Lee YH, et al. Prognostic relevance of a histological grading system using MIB-1 for adult soft-tissue sarcoma. Oncology 58: 66-74, 2000.
63. Hasegawa T, Yokoyama R, Matsuno Y, et al. Prognostic significance of histologic grade and nuclear expression of beta-catenin in synovial sarcoma. Hum Pathol 32: 257-63, 2001.
64. Hashimoto H, Daimaru Y, Takeshita S, et al. Prognostic significance of histologic parameters of soft tissue sarcomas. Cancer 70: 2816-22, 1992.
65. He TC, Sparks AB, Rago C, et al. Identification of c-MYC as a target of the APC pathway. Science 281: 1509-12, 1998.
66. Helman LJ, Meltzer P. Mechanisms of sarcoma development. Nat Rev Cancer 3: 685-94, 2003.
67. Heslin MJ, Cordon-Cardo C, Lewis JJ, et al. Ki-67 detected by MIB-1 predicts distant metastasis and tumor mortality in primary, high grade extremity soft tissue sarcoma. Cancer 83: 490-7, 1998.
68. Hilsenbeck SG, Ravdin PM, de Moor CA, et al. Time-dependence of hazard ratios for prognostic factors in primary breast cancer. Breast Cancer Res Treat 52: 227-37, 1998.
69. Hollowood K, Fletcher CD. Malignant fibrous histiocytoma: morphologic pattern or pathologic entity? Semin Diagn Pathol 12: 210-20, 1995.
70. Hollstein M, Hergenhahn M, Yang Q, et al. New approaches to understanding p53 gene tumor mutation spectra. Mutat Res 431: 199-209, 1999.
71. Hoos A, Nissan A, Stojadinovic A, et al. Tissue microarray molecular profiling of early, node-negative adenocarcinoma of the rectum: a comprehensive analysis. Clin Cancer Res 8: 3841-9, 2002.
72. Hoos A, Stojadinovic A, Mastorides S, et al. High Ki-67 proliferative index predicts disease specific survival in patients with high-risk soft tissue sarcomas. Cancer 92: 869-74, 2001.
73. Hoos A, Urist MJ, Stojadinovic A, et al. Validation of tissue microarrays for immunohistochemical profiling of cancer specimens using the example of human fibroblastic tumors. Am J Pathol 158: 1245-51, 2001.
74. Hoppin JA, Tolbert PE, Flanders WD, et al. Occupational risk factors for sarcoma subtypes. Epidemiology 10: 300-6, 1999.
75. Huber AH ,Weis WI. The structure of the beta-catenin/E-cadherin complex and the molecular basis of diverse ligand recognition by beta-catenin. Cell 105: 391-402, 2001.
76. Huuhtanen RL, Blomqvist CP, Bohling TO, et al. Expression of cyclin A in soft tissue sarcomas correlates with tumor aggressiveness. Cancer Res 59: 2885-90, 1999.
77. Huuhtanen RL, Blomqvist CP, Wiklund TA, et al. S-phase fraction of 155 soft tissue sarcomas: correlation with clinical outcome. Cancer 77: 1815-22, 1996.
78. Issels RD, Schlemmer M. Current trials and new aspects in soft tissue sarcoma of adults. Cancer Chemother Pharmacol 49 Suppl 1: S4-8, 2002.
79. Iwaya K, Ogawa H, Kuroda M, et al. Cytoplasmic and/or nuclear staining of beta-catenin is associated with lung metastasis. Clin Exp Metastasis 20: 525-9, 2003.
80. Jensen V, Sorensen FB, Bentzen SM, et al. Proliferative activity (MIB-1 index) is an independent prognostic parameter in patients with high-grade soft tissue sarcomas of subtypes other than malignant fibrous histiocytomas: a retrospective immunohistological study including 216 soft tissue sarcomas. Histopathology 32: 536-46, 1998.
81. Jimenez RE, Zalupski MM, Frank JJ, et al. Multidrug resistance phenotype in high grade soft tissue sarcoma: correlation of P-glycoprotein immunohistochemistry with pathologic response to chemotherapy. Cancer 86: 976-81, 1999.
82. Joensuu H, Fletcher C, Dimitrijevic S, et al. Management of malignant gastrointestinal stromal tumours. Lancet Oncol 3: 655-64, 2002.
83. Kahara N, Ozaki T, Doi T, et al. CD44 expression in soft tissue sarcomas. Virchows Arch 436: 574-8, 2000.
84. Kalbfleisch J, RL. P, (1980) The Statistical Analysis of Failure Time DataNew York, U.S.A.: John Wiley & Sons.
85. Kallioniemi OP, Wagner U, Kononen J, et al. Tissue microarray technology for high-throughput molecular profiling of cancer. Hum Mol Genet 10: 657-62, 2001.
86. Karlsson P, Holmberg E, Samuelsson A, et al. Soft tissue sarcoma after treatment for breast cancer-a Swedish population-based study. Eur J Cancer 34: 2068-75, 1998.
87. Kashima T, Nakamura K, Kawaguchi J, et al. Overexpression of cadherins suppresses pulmonary metastasis of osteosarcoma in vivo. Int J Cancer 104: 147-54, 2003.
88. Kattan MW, Leung DH, Brennan MF. Postoperative nomogram for 12-year sarcoma-specific death. J Clin Oncol 20: 791-6, 2002.
89. Kawai A, Woodruff J, Healey JH, et al. SYT-SSX gene fusion as a determinant of morphology and prognosis in synovial sarcoma. N Engl J Med 338: 153-60, 1998.
90. Kempson RL, Fletcher C, Evans HL, Hendrickson MR, Sibley RK (2001) Tumors of the soft tissues. Vol. Third series, Washington D.C.: Armed Forces Institute of Pathology.
91. Khan J, Simon R, Bittner M, et al. Gene expression profiling of alveolar rhabdomyosarcoma with cDNA microarrays. Cancer Res 58: 5009-13, 1998.
92. Kilpatrick SE. Histologic prognostication in soft tissue sarcomas: grading versus subtyping or both? A comprehensive review of the literature with proposed practical guidelines. Ann Diagn Pathol 3: 48-61, 1999.
93. Kindblom LG, Widehn S, Meis-Kindblom JM. The role of electron microscopy in the diagnosis of pleomorphic sarcomas of soft tissue. Semin Diagn Pathol 20: 72-81, 2003.

94. Koea JB, Leung D, Lewis JJ, et al. Histopathologic type: an independent prognostic factor in primary soft tissue sarcoma of the extremity? Ann Surg Oncol 10: 432-40, 2003.

95. Komdeur R, Klunder J, van der Graaf WT, et al. Multidrug resistance proteins in rhabdomyosarcomas: comparison between children and adults. Cancer 97: 1999-2005, 2003.

96. Komdeur R, Plaat BE, Hoekstra HJ, et al. Expression of P-glycoprotein, multidrug resistance-associated protein 1, and lung resistance-related protein in human soft tissue sarcomas before and after hyperthermic isolated limb perfusion with tumor necrosis factor-alpha and melphalan. Cancer 91: 1940-8, 2001.

97. Komdeur R, Plaat BE, van der Graaf WT, et al. Expression of multidrug resistance proteins, P-gp, MRP1 and LRP, in soft tissue sarcomas analysed according to their histological type and grade. Eur J Cancer 39: 909-16, 2003.

98. Kononen J, Bubendorf L, Kallioniemi A, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 4: 844-7, 1998.

99. Kuhnen C, Herter P, Muller O, et al. Beta-catenin in soft tissue sarcomas: expression is related to proliferative activity in high-grade sarcomas. Mod Pathol 13: 1005-13, 2000.

100. Lack EE, Steinberg SM, White DE, et al. Extremity soft tissue sarcomas: analysis of prognostic variables in 300 cases and evaluation of tumor necrosis as a factor in stratifying higher-grade sarcomas. J Surg Oncol 41: 263-73, 1989.

101. Ladanyi M, Antonescu CR, Leung DH, et al. Impact of SYT-SSX fusion type on the clinical behavior of synovial sarcoma: a multi-institutional retrospective study of 243 patients. Cancer Res 62: 135-40, 2002.

102. Levine EA, Holzmayer T, Bacus S, et al. Evaluation of newer prognostic markers for adult soft tissue sarcomas. J Clin Oncol 15: 3249-57, 1997.

103. Lewis JJ, Leung D, Casper ES, et al. Multifactorial analysis of long-term follow-up (more than 5 years) of primary extremity sarcoma. Arch Surg 134: 190-4, 1999.

104. Lynch HT, Deters CA, Hogg D, et al. Familial sarcoma: challenging pedigrees. Cancer 98: 1947-57, 2003.

105. Mandahl N, Mertens F, Panagopoulos I, et al. Genetic characterization of bone and soft tissue tumors. Acta Orthop Scand (Suppl 311) 75: 21-8, 2004.

106. Mandard AM, Petiot JF, Marnay J, et al. Prognostic factors in soft tissue sarcomas. A multivariate analysis of 109 cases. Cancer 63: 1437-51, 1989.

107. Markhede G, Angervall L, Stener BA. multivariate analysis of the prognosis after surgical treatment of malignant soft-tissue tumors. Cancer 49: 1721-33, 1982.

108. Maula S, Huuhtanen RL, Blomqvist CP, et al. The adhesion molecule CD44v6 is associated with a high risk for local recurrence in adult soft tissue sarcomas. Br J Cancer 84: 244-52, 2001.

109. Meis-Kindblom JM, Bjerkehage B, Bohling T, et al. Morphologic review of 1000 soft tissue sarcomas from the Scandinavian Sarcoma Group (SSG) Register. The peer-review committee experience. Acta Orthop Scand Suppl 285: 18-26, 1999.

110. Mentzel T, Calonje E, Wadden C, et al. Myxofibrosarcoma. Clinicopathologic analysis of 75 cases with emphasis on the low-grade variant. Am J Surg Pathol 20: 391-405, 1996.

111. Meric F, Milas M, Hunt KK, et al. Impact of neoadjuvant chemotherapy on postoperative morbidity in soft tissue sarcomas. J Clin Oncol 18: 3378-83, 2000.

112. Miettinen M (2003) Diagnostic Soft Tissue Pathology. 1st ed. Philadelphia: Churchill Livingstone.

113. Miettinen M, Sarlomo-Rikala M, Kovatich AJ. Cell-type- and tumour-type-related patterns of bcl-2 reactivity in mesenchymal cells and soft tissue tumours. Virchows Arch 433: 255-60, 1998.

114. Mitelman F. Recurrent chromosome aberrations in cancer. Mutat Res 462: 247-53, 2000.

115. Mitelman F, Johansson B, Mertens F. Fusion genes and rearranged genes as a linear function of chromosome aberrations in cancer. Nat Genet 36: 331-4, 2004.

116. Moller Nielsen M Biological characterisation of soft tissue sarcomas - STS. PhD thesis. Faculty of Health Sciences. Odense University. Odense. Denmark p. 207, 2001.

117. Mounier N, Morel P, Haioun C, et al. A multivariate analysis of the survival of patients with aggressive lymphoma: variations in the predictive value of prognostic factors during the course of the disease. Groupe d'Etudes des Lymphomes de l'Adulte. Cancer 82: 1952-62, 1998.

118. Mucci NR, Akdas G, Manely S, et al. Neuroendocrine expression in metastatic prostate cancer: evaluation of high throughput tissue microarrays to detect heterogeneous protein expression. Hum Pathol 31: 406-14, 2000.

119. Murray AW. Recycling the cell cycle: cyclins revisited. Cell 116: 221-34, 2004.

120. Myhre-Jensen O, Kaae S, Madsen EH, et al. Histopathological grading in soft-tissue tumours. Relation to survival in 261 surgically treated patients. Acta Pathol Microbiol Immunol Scand [A] 91: 145-50, 1983.

121. Nakanishi H, Myoui A, Ochi T, et al. P-glycoprotein expression in soft-tissue sarcomas. J Cancer Res Clin Oncol 123: 352-6, 1997.

122. Nakanishi H, Ohsawa M, Naka N, et al. Immunohistochemical detection of bcl-2 and p53 proteins and apoptosis in soft tissue sarcoma: their correlations with prognosis. Oncology 54: 238-44, 1997.

123. Naor D, Nedvetzki S, Golan I, et al. CD44 in cancer. Crit Rev Clin Lab Sci 39: 527-79, 2002.

124. Nielsen TO, Hsu FD, O'Connell JX, et al. Tissue microarray validation of epidermal growth factor receptor and SALL2 in synovial sarcoma with comparison to tumors of similar histology. Am J Pathol 163: 1449-56, 2003.

125. Nielsen TO, West RB, Linn SC, et al. Molecular characterisation of soft tissue tumours: a gene expression study. Lancet 359: 1301-7, 2002.

126. Nilbert M, Meza-Zepeda LA, Francis P, et al. Lessons from genetic profiling in soft tissue sarcomas. Acta Orthop Scand (Suppl 311) 75: 35-50, 2004.

127. Nocito A, Bubendorf L, Maria Tinner E, et al. Microarrays of bladder cancer tissue are highly representative of proliferation index and histological grade. J Pathol 194: 349-57, 2001.

128. O'Brien JE, Stout AP. Malignant fibrous xanthomas. Cancer 17: 1445-55, 1964.

129. Oda Y, Naka T, Takeshita M, et al. Comparison of histological changes and changes in nm23 and c-MET expression between primary and metastatic sites in osteosarcoma: a clinicopathologic and immunohistochemical study. Hum Pathol 31: 709-16, 2000.

130. Oda Y, Takahira T, Kawaguchi K, et al. Altered expression of cell cycle regulators in myxofibrosarcoma, with special emphasis on their prognostic implications. Hum Pathol 34: 1035-42, 2003.

131. O'Sullivan B, Davis AM, Turcotte R, et al. Preoperative versus postoperative radiotherapy in soft-tissue sarcoma of the limbs: a randomised trial. Lancet 359: 2235-41, 2002.

132. Ozzello L SA, Murray MR. Cultural charcteristics of malignant histiocytomas and fibrous xanthomas. Cancer 16: 331-44, 1963.

133. Pakos EE, Ioannidis JP. The association of P-glycoprotein with response to chemotherapy and clinical outcome in patients with osteosarcoma. A meta-analysis. Cancer 98: 581-9, 2003.

134. Panagopoulos I, Storlazzi CT, Fletcher CD, et al. The chimeric FUS/CREB3l2 gene is specific for low-grade fibromyxoid sarcoma. Genes Chromosomes Cancer 40: 218-28, 2004.

135. Peiper M, Sato T, Zurakowski D, et al. CD44s expression is associated with improved survival in soft tissue sarcoma. Anticancer Res 24: 1053-6, 2004.

136. Perez-Moreno M, Jamora C, Fuchs E. Sticky business: orchestrating cellular signals at adherens junctions. Cell 112: 535-48, 2003.

137. Pezzi CM, Rawlings MS, Jr., Esgro JJ, et al. Prognostic factors in 227 patients with malignant fibrous histiocytoma. Cancer 69: 2098-103, 1992.

138. Pisters PW, Harrison LB, Leung DH, et al. Long-term results of a prospective randomized trial of adjuvant brachytherapy in soft tissue sarcoma. J Clin Oncol 14: 859-68, 1996.

139. Pisters PW, Leung DH, Woodruff J, et al. Analysis of prognostic factors in 1,041 patients with localized soft tissue sarcomas of the extremities. J Clin Oncol 14: 1679-89, 1996.

140. Pisters PW, Pollock RE. Staging and prognostic factors in soft tissue sarcoma. Semin Radiat Oncol 9: 307-14, 1999.

141. Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol 4: 33-45, 2003.

142. Quantin C, Abrahamowicz M, Moreau T, et al. Variation over time of the effects of prognostic factors in a population-based study of colon cancer: comparison of statistical models. Am J Epidemiol 150: 1188-200, 1999.

143. Quirke P. Training and quality assurance for rectal cancer: 20 years of data is enough. Lancet Oncol 4: 695-702, 2003.

144. Ramanathan RC, A'Hern R, Fisher C, et al. Modified staging system for extremity soft tissue sarcomas. Ann Surg Oncol 6: 57-69, 1999.

145. Ray-Coquard I, Thiesse P, Ranchere-Vince D, et al. Conformity to clinical practice guidelines, multidisciplinary management and outcome of treatment for soft tissue sarcomas. Ann Oncol 15: 307-15, 2004.

146. Richter J, Wagner U, Kononen J, et al. High-throughput tissue microarray analysis of cyclin E gene amplification and overexpression in urinary bladder cancer. Am J Pathol 157: 787-94, 2000.

147. Rimm DL, Camp RL, Charette LA, et al. Tissue microarray: a new technology for amplification of tissue resources. Cancer J 7: 24-31, 2001.

148. Rooser B, Attewell R, Berg NO, et al. Prognostication in soft tissue sarcoma. A model with four risk factors. Cancer 61: 817-23, 1988.

149. Rooser B, Willen H, Gustafson P, et al. Malignant fibrous histiocytoma of soft tissue. A population-based epidemiologic and prognostic study of 137 patients. Cancer 67: 499-505, 1991.

150. Ruka W, Emrich LJ, Driscoll DL, et al. Prognostic significance of lymph node metastasis and bone, major vessel, or nerve involvement in adults with high-grade soft tissue sarcomas. Cancer 62: 999-1006, 1988.

151. Rydholm A, Gustafson P. Should tumor depth be included in prognostication of soft tissue sarcoma? BMC Cancer 3: 17, 2003.

152. Saito T, Oda Y, Sakamoto A, et al. Prognostic value of the preserved expression of the E-cadherin and catenin families of adhesion molecules and of beta-catenin mutations in synovial sarcoma. J Pathol 192: 342-50, 2000.

153. Sakamoto A, Oda Y, Adachi T, et al. Beta-catenin accumulation and gene mutation in exon 3 in dedifferentiated liposarcoma and malignant fibrous histiocytoma. Arch Pathol Lab Med 126: 1071-8, 2002.

154. Salo JC, Lewis JJ, Woodruff JM, et al. Malignant fibrous histiocytoma of the extremity. Cancer 85: 1765-72, 1999.

155. Schmitt M, Thomssen C, Ulm K, et al. Time-varying prognostic impact of tumour biological factors urokinase (uPA), PAI-1 and steroid hormone receptor status in primary breast cancer. Br J Cancer 76: 306-11, 1997.

156. Schoenfield D. Partial residuals for the proportional hazards regression model. Biometrika 69: 239-241, 1982.

157. Schraml P, Kononen J, Bubendorf L, et al. Tissue microarrays for gene amplification surveys in many different tumor types. Clin Cancer Res 5: 1966-75, 1999.

158. Segal NH, Pavlidis P, Antonescu CR, et al. Classification and subtype prediction of adult soft tissue sarcoma by functional genomics. Am J Pathol 163: 691-700, 2003.

159. Shapiro L. Beta-catenin and its multiple partners: promiscuity explained. Nat Struct Biol 8: 484-7, 2001.

160. Sherr CJ. Principles of tumor suppression. Cell 116: 235-46, 2004.

161. Shtutman M, Zhurinsky J, Simcha I, et al. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc Natl Acad Sci U S A 96: 5522-7, 1999.

162. Simon MA, Enneking WF. The management of soft-tissue sarcomas of the extremities. J Bone Joint Surg Am 58: 317-27, 1976.

163. Skubitz KM, Skubitz AP. Characterization of sarcomas by means of gene expression. J Lab Clin Med 144: 78-91, 2004.

164. Stamey TA, Donaldson AN, Yemoto CE, et al. Histological and clinical findings in 896 consecutive prostates treated only with radical retropubic prostatectomy: epidemiologic significance of annual changes. J Urol 160: 2412-7, 1998.

165. Stojadinovic A, Leung DH, Allen P, et al. Primary adult soft tissue sarcoma: time-dependent influence of prognostic variables. J Clin Oncol 20: 4344-52, 2002.

166. Suit HD, Spiro I. Role of radiation in the management of adult patients with sarcoma of soft tissue. Semin Surg Oncol 10: 347-56, 1994.

167. Taubert H, Koehler T, Meye A, et al. mdm2 mRNA level is a prognostic factor in soft tissue sarcoma. Mol Med 6: 50-9, 2000.

168. Tetsu O, McCormick F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 398: 422-6, 1999.

169. Torhorst J, Bucher C, Kononen J, et al. Tissue microarrays for rapid linking of molecular changes to clinical endpoints. Am J Pathol 159: 2249-56, 2001.

170. Trassard M, Le Doussal V, Hacene K, et al. Prognostic factors in localized primary synovial sarcoma: a multicenter study of 128 adult patients. J Clin Oncol 19: 525-34, 2001.

171. Trojani M, Contesso G, Coindre JM, et al. Soft-tissue sarcomas of adults; study of pathological prognostic variables and definition of a histopathological grading system. Int J Cancer 33: 37-42, 1984.

172. Trovik CS. Local recurrence of soft tissue sarcoma. A Scandinavian Sarcoma Group Project. Acta Orthop Scand (Suppl 300) 72: 1-31, 2001.

173. Trovik CS, Bauer HC Local recurrence of soft tissue sarcoma a risk factor for late metastases. 379 patients followed for 0.5-20 years. Acta Orthop Scand 65: 553-8, 1994.

174. van Unnik JA, Coindre JM, Contesso C, et al. Grading of soft tissue sarcomas: experience of the EORTC Soft Tissue and Bone Sarcoma Group. Eur J Cancer 29A: 2089-93, 1993.

175. Watanabe T, Oda Y, Tamiya S, et al. Malignant peripheral nerve sheath tumours: high Ki67 labelling index is the significant prognostic indicator. Histopathology 39: 187-97, 2001.

176. Weber GF, Bronson RT, Ilagan J, et al. Absence of the CD44 gene prevents sarcoma metastasis. Cancer Res 62: 2281-6, 2002.

177. Weiss SW, Goldblum JR, (2001) Enzinger and Weiss's soft tissue tumors. 4th edition, ed. M. Strauss, St. Louis: C.V. Mosby Co.

178. Weitz J, Antonescu CR, Brennan MF. Localized extremity soft tissue sarcoma: improved knowledge with unchanged survival over time. J Clin Oncol 21: 2719-25, 2003.

179. Vermund H, Krajci P, Eide TJ, et al. Laryngectomy whole organ serial sections--histological parameters correlated with recurrence rate. Acta Oncol 43: 98-107, 2004.

180. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature 408: 307-10, 2000.

181. Wong NA, Pignatelli M. Beta-catenin-a linchpin in colorectal carcinogenesis? Am J Pathol 160: 389-401, 2002.

182. Wunder JS, Bull SB, Aneliunas V, et al. MDR1 gene expression and outcome in osteosarcoma: a prospective, multicenter study. J Clin Oncol 18: 2685-94, 2000.

183. Wunder JS, Healey JH, Davis AM, et al. A comparison of staging systems for localized extremity soft tissue sarcoma. Cancer 88: 2721-30, 2000.

184. Wurl P, Meye A, Berger D, et al. Prognostic relevance of C-terminal Mdm2 detection is enhanced by p53 positivity in soft tissue sarcomas. Diagn Mol Pathol 6: 249-54, 1997.

185. Wurl P, Meye A, Lautenschlager C, et al. Clinical relevance of pRb and p53 co-overexpression in soft tissue sarcomas. Cancer Lett 139: 159-65, 1999.

186. Wurl P, Meye A, Schmidt H, et al. High prognostic significance of Mdm2/p53 co-overexpression in soft tissue sarcomas of the extremities. Oncogene 16: 1183-5, 1998.

187. Xie Y, Skytting B, Nilsson G, et al. The SYT-SSX1 fusion type of synovial sarcoma is associated with increased expression of cyclin A and D1. A link between t(X;18)(p11.2; q11.2) and the cell cycle machinery. Oncogene 21: 5791-6, 2002.

188. Yang JC, Chang AE, Baker AR, et al. Randomized prospective study of the benefit of adjuvant radiation therapy in the treatment of soft tissue sarcomas of the extremity. J Clin Oncol 16: 197-203, 1998.

189. Yang P, Hirose T, Hasegawa T, et al. Prognostic implication of the p53 protein and Ki-67 antigen immunohistochemistry in malignant fibrous histiocytoma. Cancer 76: 618-25, 1995.

190. Zagars GK, Ballo MT. Significance of dose in postoperative radiotherapy for soft tissue sarcoma. Int J Radiat Oncol Biol Phys 56: 473-81, 2003.

191. Zagars GK, Ballo MT, Pisters PW, et al. Preoperative vs. postoperative radiation therapy for soft tissue sarcoma: a retrospective comparative evaluation of disease outcome. Int J Radiat Oncol Biol Phys 56: 482-8, 2003.

192. Zagars GK, Ballo MT, Pisters PW, et al. Prognostic factors for disease-specific survival after first relapse of soft-tissue sarcoma: analysis of 402 patients with disease relapse after initial conservative surgery and radiotherapy. Int J Radiat Oncol Biol Phys 57: 739-47, 2003.

193. Zagars GK, Ballo MT, Pisters PW, et al. Prognostic factors for patients with localized soft-tissue sarcoma treated with conservation surgery and radiation therapy: an analysis of 1225 patients. Cancer 97: 2530-43, 2003.

194. Zahm SH, Fraumeni JF, Jr. The epidemiology of soft tissue sarcoma. Semin Oncol 24: 504-14, 1997.