Prognostic value of adaptive and innate immune system in soft tissue sarcomas
A retrospective tissue microarray-based study

Sveinung Wergeland Sørbye
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Prognostic value of adaptive and innate immune system in soft tissue sarcomas

A retrospective tissue microarray-based study

by

Sveinung Wergeland Sørbye

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# TABLE OF CONTENTS

## ACKNOWLEDGEMENTS

## LIST OF PAPERS

## LIST OF ABBREVIATIONS

1. **BACKGROUND**
   
   1.1. Epidemiology and incidence
   
   1.2. Histopathology
   
   1.3. Pathogenesis
   
   1.4. Hereditary sarcoma
   
   1.5. Environmental factors
   
   1.6. Oncogenic viruses and immunologic factors
   
   1.7. Diagnostics
   
   1.8. Prognostic factors
      
      1.8.1. Grading
      
      1.8.2. Staging
      
      1.8.3. Other prognosticators in STS
   
   1.9. Treatment
      
      1.9.1. Surgery
      
      1.9.2. Chemotherapy
      
      1.9.3. Radiotherapy
   
   1.10. Molecular-genetic abnormalities in sarcomas
   
   1.11. Tumor proliferation and growth
   
   1.12. Molecular markers
      
      1.12.1. Markers of tumor growth, proliferation, and differentiation
      
      1.12.2. The adaptive and the innate immune system in STSs
      
      1.12.3. Tumor-infiltrating lymphocytes

   6

   8

   9

   11

   11

   15

   20

   20

   20

   21

   21

   22

   22

   24

   26

   27

   27

   27

   28

   29

   29

   30

   30

   31

   34
1.12.4. Cell cycle regulatory proteins 34
1.12.5. Female steroid hormone receptors 34
1.12.6. TGF-beta 35
1.13. Tissue microarray 35
2. AIMS OF THESIS 38
3. MATERIAL AND METHODS 39
  3.1. Study population and material 39
  3.2. Immunohistochemistry (IHC) 40
  3.3. Scoring 41
  3.4. Statistical analysis 42
  3.5. Ethical clearance 42
4. MAIN RESULTS 43
  4.1. Paper I 43
  4.2. Paper II 43
  4.3. Paper III 44
  4.4. Paper IV 44
5. DISCUSSION 45
  5.1. Methods 45
    5.1.1. Data collection and study population 45
    5.1.2. Representativity of Norwegian and Russian study populations 45
    5.1.3. Separate investigation of differently located sarcomas 46
    5.1.4. Heterogeneity of histological entities in the study population 46
    5.1.5. Conclusion on material representativity 47
    5.1.6. Tissue microarray 47
    5.1.7. Immunohistochemistry 48
    5.1.8. Antibodies 48
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1.9. Controls</td>
<td>49</td>
</tr>
<tr>
<td>5.1.10. Statistics</td>
<td>50</td>
</tr>
<tr>
<td>5.1.11. Significance level</td>
<td>50</td>
</tr>
<tr>
<td>5.1.12. Cut-off values</td>
<td>50</td>
</tr>
<tr>
<td>5.1.13. Survival analysis</td>
<td>51</td>
</tr>
<tr>
<td>5.2. Discussion of the results</td>
<td>52</td>
</tr>
<tr>
<td>5.2.1. Paper I</td>
<td>53</td>
</tr>
<tr>
<td>5.2.1.1. CD20 positive tumor-infiltrating cells</td>
<td>53</td>
</tr>
<tr>
<td>5.2.1.2. CD3 positive tumor-infiltrating cells</td>
<td>54</td>
</tr>
<tr>
<td>5.2.1.3. CD4 positive tumor-infiltrating cells</td>
<td>55</td>
</tr>
<tr>
<td>5.2.1.4. CD8 positive tumor-infiltrating cells</td>
<td>56</td>
</tr>
<tr>
<td>5.2.2. Paper II</td>
<td>58</td>
</tr>
<tr>
<td>5.2.2.1. CD68</td>
<td>58</td>
</tr>
<tr>
<td>5.2.2.2. M-CSF</td>
<td>58</td>
</tr>
<tr>
<td>5.2.2.3. CSF-1R</td>
<td>59</td>
</tr>
<tr>
<td>5.2.2.4. CD57</td>
<td>59</td>
</tr>
<tr>
<td>5.2.2.5. TGF-beta</td>
<td>60</td>
</tr>
<tr>
<td>5.2.2.6. Ki67</td>
<td>60</td>
</tr>
<tr>
<td>5.2.3. Paper III</td>
<td>61</td>
</tr>
<tr>
<td>5.2.3.1. Jab1</td>
<td>61</td>
</tr>
<tr>
<td>5.2.3.2. p16</td>
<td>61</td>
</tr>
<tr>
<td>5.2.3.3. p21</td>
<td>62</td>
</tr>
<tr>
<td>5.2.3.4. p62</td>
<td>62</td>
</tr>
<tr>
<td>5.2.3.5. Skp2</td>
<td>63</td>
</tr>
<tr>
<td>5.2.4. Paper IV</td>
<td>63</td>
</tr>
<tr>
<td>6. CONCLUSIONS AND IMPLICATIONS FOR FURTHER RESEARCH</td>
<td>65</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

The work presented in this thesis was conducted in the period from spring 2005 to spring 2013. I began collecting material in spring 2005. In 2009 I started the PhD program and became a member of translational cancer research at the University of Tromsø. In addition to research, I have worked in the Department of Clinical Pathology at the University Hospital of North Norway, where I have been employed as a consultant since 2002. The project is funded by the Northern Norway Regional Health Authority.

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LIST OF PAPERS


LIST OF ABBREVIATIONS

AJCC American Joint Committee on Cancer
BAD Bcl-2-associated death promoter
CDK Cyclin-dependent kinase
CD Cluster of differentiation
CK Cytokeratin
CT Computer tomography
DAB Diaminobenzidin
DAKO Dakota Manufacturing Company
DFSP Dermatofibrosarcoma protuburens
DSS Disease-specific survival
EDTA Ethylenediaminetetraacetic acid
EMT Epithelial-to-mesenchymal transition
ER Estrogen receptor
ESMO European Society for Medical Oncology
EWSR1-ETS Ewing sarcoma breakpoint region 1-E twenty six
FAP Familial adenomatous polyposis
FISH Fluorescent in situ hybridization
FKHR Forkhead homolog 1 in rhabdomyosarcoma
FNCLCC Fédération Nationale des Centres de Lutte Contre le Cancer
GSK3 Glycogen synthase kinase 3
Gy Grey
HHV8 Human herpes virus 8
HR Hazard ratio
IMRT Intensity-modulated radiation therapy
IHC Immunohistochemistry
Mab Monoclonal antibody
MAPK Mitogen-activated protein kinase
MFS Metastasis free survival
MPNST Malignant peripheral nerve sheath tumor
MRI Magnetic resonance imaging
MSKCC Memorial Sloan-Kettering Cancer Center
mTOR Mammalian target of rapamycin
m TORC Mammalian target of rapamycin complex 2
NCI National Cancer Institute
NF-κB Nuclear factor-kappa B
Non-GIST STS Non-gastrointestinal stromal tumor soft tissue sarcoma
OS Overall survival
p-Akt Ser\textsuperscript{473} Akt phosphorylated on serin 473
p-Akt Thr\textsuperscript{308} Akt phosphorylated on threonin 308
Par6 Partitioning protein 6
PCR Polymerase chain reaction
PDGF Platelet-derived growth factor
PDGFR Platelet-derived growth factor receptor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PET</td>
<td>Positron emission transmission</td>
</tr>
<tr>
<td>PGR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIP₃</td>
<td>Phosphatidylinositol trisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein-kinase C</td>
</tr>
<tr>
<td>PNET</td>
<td>Peripheral neuroectodermal tumor</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog deleted on chromosome 10</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SIN</td>
<td>Size, Invasion, and Necrosis</td>
</tr>
<tr>
<td>SMA</td>
<td>Smooth muscle actin</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for the Social Sciences</td>
</tr>
<tr>
<td>SSG</td>
<td>Scandinavian sarcoma group</td>
</tr>
<tr>
<td>STS</td>
<td>Soft tissue sarcoma</td>
</tr>
<tr>
<td>TGF-beta</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue microarray</td>
</tr>
<tr>
<td>TNGM</td>
<td>Tumor, nodule, grade, and metastasis</td>
</tr>
<tr>
<td>UICC</td>
<td>Union Internationale Contre le Cancer</td>
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<td>WHO</td>
<td>World health organization</td>
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</tbody>
</table>
1. BACKGROUND

1.1. Epidemiology and incidence

Soft tissue sarcomas (STS) represent a heterogeneous group of tumors that arise from mesenchymal tissues and consist of 50 histologic subtypes [1]. They are malignant tumors derived from nonepithelial extraskeletal tissue (except glia, the reticuloendothelial system, and the supporting tissue of different parenchymal organs) [1]. STSs occur at diverse sites of the body, and different subgroups of STSs have very different prognoses. Seventy-five percent are located in the extremities, most common in the thigh, and 10% each in the trunk wall and peritoneum. Three quarters of all STSs are histologically classified as liposarcoma, leiomyosarcoma, high grade pleomorphic sarcoma, synovial sarcoma, and malignant peripheral nerve sheet tumors. One fifth of the patients have local recurrence and one third have distant metastases [2]; however, this occurs more frequently in high-grade tumors. Despite treatment 30–40% of these patients will die of STSs [3].

STSs are rare tumors with an estimated annual incidence of around 30 new cases per 1,000,000 of population [4–7]. They comprise only 0.5–1% of all cancer types [8]. In Norway the number of new cases per year (incidence 2006–2010) was 81 males and 68 females. The proportion related to all cancers was 0.6% for males and 0.5% for females. The number of deaths per year (2006–2010) was 24 males and 25 females [9].

In children the incidence of STSs is relatively higher, at 1–3%, but cancer is not a childhood common disease. Like other malignancies, STSs become more common with increasing age, with 65 years being the median age of diagnosis [4, 5, 8]. The age-related incidence varies among the different histological subtypes. Embryonal rhabdomyosarcoma is found mostly in children; synovial sarcoma is more common in young adults. Liposarcoma, pleomorphic high-grade sarcoma, and leiomyosarcoma dominate in the elderly (Figure 1) [1].
Figure 1. Approximate relation of age to incidence of various types of sarcoma. Modified from Weiss SW, Goldblum R: Enzinger & Weis’s Soft Tissue Tumors, 5th edn. Philadelphia: Mosby, Elsevier Inc; 2008[1] Permission obtained from Elsevier Inc.

The age-adjusted incidence rates of STSs in Norway have shown a slight increase in the last 50 years (Figure 2), recorded at January 2013 [6].

For the Russian Federation, this figure was 2.3 per 100,000 in 2007, but specifically in the Arkhangelsk region, where our research material was partly gathered from, it was 3.6 per 100,000 [5].

The incidence of STSs increases with advancing age and is approximately the same for male and female patients, with the exception of a drop in incidence in females during the age range 65–70 (Figure 3).
Figure 3. Age-specific incidence rates of STS in Norway per 100,000, 2006 to 2010. Modified from NORDCAN: Cancer Incidence, Mortality, Prevalence and Survival in the Nordic Countries, Version 5.0. Association of the Nordic Cancer Registries [9]. Permission obtained from The Cancer Registry of Norway.

Mortality due to STSs remains high at 30–40%, making STSs, prognostically speaking, one of the more unfavorable forms of cancer [3–5]. In Norway the survival has gradually increased during the last 50 years, from a 30–40% five-year survival during the 1960s to a 60–70% survival after 1990 (Figure 4). The relative five-year survival (1999–2003) was 66% for males and 68% for females (Nordcan 2013). This increase in survival rate is partially due to new and better treatment protocols for childhood STSs, giving the younger age groups a better overall prognosis [10]. Even so, the prognosis in the adult population has also improved, due to multidisciplinary teams with optimized diagnostic and treatment protocols (Figure 4) [11].
Figure 4. Age-standardized relative survival of STS in Norway, all ages. Modified from NORDCAN: Cancer Incidence, Mortality, Prevalence and Survival in the Nordic Countries, Version 5.0. Association of the Nordic Cancer Registries [9]. Permission obtained from The Cancer Registry of Norway.

1.2. Histopathology

STSs are usually classified according to their similarity to normal mature mesenchymal tissues [1]. However, high-grade lesions gradually lose resemblance to their tissue of origin. Moreover, some sarcomas have no obvious normal counterpart and therefore belong to a class of tumors of uncertain differentiation. Taking into consideration the rarity and variability of sarcomas, these tumors often represent a diagnostic challenge for a pathologist, who in many cases has to give a pathologic diagnosis based on small-sized biopsy specimens [12].

According to the current World Health Organization’s classification of tumors of soft tissue and bone, there are nine main groups of STSs [12]. Some examples of major STS types are demonstrated in Figure 5.
**Figure 5.** Examples of major STS types. A, Undifferentiated pleomorphic sarcoma; B, Round cell/myxoid liposarcoma. *Unpublished data. Valkov A.*
Figure 5 (continued). Examples of major STS types. C, Leiomyosarcoma; D, Biphasic synovial sarcoma. Unpublished data. Valkov A.
Figure 5 (continued). Examples of major STS types. E, Malignant peripheral nerve sheath tumor (MPNST); F, Angiosarcoma. *Unpublished data. Valkov A.*
When conducting studies on STSs it appears that some specific sarcomas differ greatly from others and should be investigated separately. This is particularly the case for skin sarcomas, gastrointestinal stromal tumors (GISTs), rhabdomyosarcomas, and Ewing/peripheral neuroectodermal tumor (PNET) sarcomas, as these have their own tailored treatments [10, 13, 14].

1.3. Pathogenesis

The pathogenesis of most STSs is still unknown [1]. Nevertheless, there are some recognized causes, which are listed below.

1.4. Hereditary sarcoma

A number of syndromes are associated with STS development. Syndromes with the ability to induce STSs are most often due to mutations in tumor suppressor-, growth factor-, and growth factor receptor genes and translocations forming new potent fusion-genes and proteins [15]. The list of the most common cancer syndromes leading to STSs includes Li Fraumeni, neurofibromatosis type I (Von Recklinghausen’s) and type II, familial adenomatous polyposis (FAP)/Gardner, Retinoblastoma, Werner, Lynch syndromes, and tuberous sclerosis/Burneville disease, among others [15]. This list will undoubtedly lengthen with an increased understanding of the molecular underpinnings of mesenchymal neoplasia [1].

1.5. Environmental factors

Among the environmental factors implicated in the development of STSs, trauma is most frequently mentioned. It is now clear, however, that trauma often seems to be an event that merely calls attention to the underlying neoplasm. But there are several well-documented reports of STS plainly linked to trauma [1, 16]. Radiation exposure can result in radiation-induced sarcomas, which in the majority of cases is represented by pleomorphic undifferentiated sarcoma [17]. In addition, there is an increased risk of subsequent sarcoma in survivors of childhood
cancers such as leukemia, retinoblastoma, Wilms’s tumor, Hodgkin’s lymphoma, and neuroblastoma [18, 19].

1.6. Oncogenic viruses and immunologic factors

Kaposi’s sarcoma is closely linked to human herpes virus 8 (HHV8) infection. However, very few healthy individuals infected with HHV8 develop Kaposi’s sarcoma, but in immunocompromised individuals many of those with HHV8 infection will develop Kaposi’s sarcoma [20, 21]. There is also a large body of literature supporting the role of Epstein-Barr virus in the pathogenesis of leiomyosarcoma in patients with suppressed immunity [22, 23]. In Stewart-Treves syndrome, angiosarcomas can arise in the setting of chronic lymphedema secondary to radical mastectomy [24, 25], which is often explained by the loss of regional immunosurveillance.

1.7. Diagnostics

Most patients with suspected sarcoma present with a growing, painless extremity lump. Pain is reported in only about one third of the cases. Because of the mostly painless presentation, the diagnosis of STSs is often delayed. Late diagnosis of patients with retroperitoneal sarcomas is especially common because of the large retroperitoneal space, generally slow growth rate, and the tendency of sarcomas to gradually displace rather than to invade adjacent tissues [26].

In Scandinavia, patients presenting with a superficial tumor or lump > 5 cm in greatest diameter or deep tumor irrespective of size should be referred to a sarcoma center as soon as possible and prior to any surgical intervention [27]. This is extremely important, as initial inadequate surgery leads to an unfavorable clinical course [28]. All patients with a suspected sarcoma are subjected to imaging procedures in order to establish the extent of the tumor (and eventual metastases) and hence determine the type of surgical procedure needed. Normal skeletal x-ray, CT, and MRI are used, although MRI gives the best impression of the soft tissues and therefore is the imaging modality of choice [29, 30]. In recent years positron emission tomography (PET) scans have become popular and have been implemented in the diagnostics for many types of cancer. The role of PET in STS diagnostics is yet to be elucidated and its use is
recommended only as a supplement to MRI [31]. PET scans are, as of today, more efficiently used to detect local recurrence after the completed therapy [31].

The necessity of pretreatment biopsy is a topic of discussion due to the risk of possible tumor contamination with further possible recurrence in the needle track after a core biopsy [32]. In Norway, a biopsy is recommended only in cases where initial wide resection is not feasible. The biopsy is used to determine the histological type and malignancy grade, and together with imaging procedures, also the stage of the tumor.

1.8. Prognostic factors

1.8.1. Grading

Since the first grading system for sarcomas was introduced by Broders et al. in 1939, a number of systems have been utilized in sarcoma diagnostics [33]. Several parameters have been used to grade sarcomas, such as cellular pleomorphism, cellularity, mitotic index, vascular invasion, tumor necrosis, surgical site, nuclear atypia, histologic type and subtype, tumor size, and tumor differentiation [34, 35]. The WHO manual on the Pathology and Genetics of Tumors of Soft Tissues and Bone recognizes two grading systems used on STSs: the FNCLCC and the NCI grading systems [12].

The FNCLCC grading system, reviewed by Coindre 2006 [34], is calculated from tumor differentiation, mitotic count, and tumor necrosis. Tumor differentiation and mitotic count are given a score from 1–3 and tumor necrosis is scored as 0–2 [1, 12, 33–36]. The histologic grade is derived from the total score, with 2–3 being grade 1, 4–5 being grade 2, and 6–8 being grade 3 (Table 1).
Table 1. Definitions of grading parameters for the FNCLCC system.

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

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

The NCI grade is derived from the histologic type or subtype and histopathological parameters, including necrosis (the most important), cellularity, pleomorphism, and mitosis, as described by Costa et al. in 1984 and modified in 1990 [37, 38].
In a comparative study of 410 patients diagnosed with STSs, Guillou et al. found the FNCLCC grading system to be marginally better at predicting metastasis and disease-specific survival (DSS) compared to the NCI grading system [1, 35]. However, both systems yielded prognostic groups and are recognized in the WHO manual as suitable for grading STS [12].

In addition to these well-recognized systems, both two-, and four-tiered (as for SSG) systems exist [35]. A proposed conversion between two-, three-, and four-tiered grading systems for STSs is presented in Table 2.

**Table 2.** Conversion table between different grading systems for soft tissue sarcomas

<table>
<thead>
<tr>
<th>Two-tiered system</th>
<th>Three-tiered systems</th>
<th>Four-tiered systems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low grade</td>
<td>Grade 1</td>
<td>Grade 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grade 2</td>
</tr>
<tr>
<td>High grade</td>
<td>Grade 2</td>
<td>Grade 3</td>
</tr>
<tr>
<td></td>
<td>Grade 3</td>
<td>Grade 4</td>
</tr>
</tbody>
</table>

Adapted from *The WHO Classification of Tumors: Pathology and Genetics of Tumors of Soft Tissue and Bone* [12]. Permission obtained from WHO IARC.

The three-tiered systems are considered most suitable for predicting survival and likelihood of treatment response, since they are able to predict the behavior of both low-grade, intermediate-grade, and high-grade tumors, which seem to be well-defined categories of STSs. Nevertheless, the recently proposed system, termed SIN by the SSG group, anticipated promising binary stratification that would help to simplify treatment strategy schemes [35, 39]. The system uses three factors, namely size, vascular invasion, and necrosis in a dichotomous fashion (size < or > 8 cm, and +/- vascular invasion and necrosis). The low-risk group (score 0–1) had an 81% five-year survival compared to the high-risk group (score 2–3) with a five-year survival of 32%.

### 1.8.2. Staging

STSs are typically staged according to the tumor, nodule, grade, and metastasis (TNMG) system developed by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC), as devised by Russell et al. in 1977 (later revised and recently
published in the AJCC Cancer Staging Manual 7th edition [40, 41]). The TNGM system for STS includes tumor size, nodal metastasis, malignancy grade, and distant metastasis, yielding a stage ranging from I–IV. The system is designed to include two-, three-, and four-tiered grading systems using a conversion table (Table 2). Table 3 summarizes the current TNGM stages based on grades derived from a three-tiered grading system.

Table 3. Clinical staging and survival of soft tissue sarcoma according to the tumor, node, grade, and metastasis system

<table>
<thead>
<tr>
<th>Stage</th>
<th>Tumor</th>
<th>Node</th>
<th>Metastasis</th>
<th>Grade</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia</td>
<td>T1a</td>
<td>N0</td>
<td>M0</td>
<td>G1, GX</td>
<td>T1: Tumor ≤5cm in greatest dimension</td>
</tr>
<tr>
<td></td>
<td>T1b</td>
<td>N0</td>
<td>M0</td>
<td>G1, GX</td>
<td>T1b: Deep tumor</td>
</tr>
<tr>
<td>Ib</td>
<td>T2a</td>
<td>N0</td>
<td>M0</td>
<td>G1, GX</td>
<td>T1a: Superficial tumor</td>
</tr>
<tr>
<td></td>
<td>T2b</td>
<td>N0</td>
<td>M0</td>
<td>G1, GX</td>
<td>T2: Tumor&gt;5cm in greatest dimension</td>
</tr>
<tr>
<td>IIa</td>
<td>T1a</td>
<td>N0</td>
<td>M0</td>
<td>G2, G3</td>
<td>T2a: Superficial tumor</td>
</tr>
<tr>
<td></td>
<td>T1b</td>
<td>N0</td>
<td>M0</td>
<td>G2, G3</td>
<td>T2b: Deep tumor</td>
</tr>
<tr>
<td>IIb</td>
<td>T2a</td>
<td>N0</td>
<td>M0</td>
<td>G2</td>
<td>T2a: Superficial tumor</td>
</tr>
<tr>
<td></td>
<td>T2b</td>
<td>N0</td>
<td>M0</td>
<td>G2</td>
<td>T2b: Deep tumor</td>
</tr>
<tr>
<td>III</td>
<td>T2a, T2b</td>
<td>N0</td>
<td>M0</td>
<td>G3</td>
<td>N1: Regional lymph node metastasis</td>
</tr>
<tr>
<td></td>
<td>Any T</td>
<td>N1</td>
<td>M0</td>
<td>Any G</td>
<td>M1: Distant metastasis</td>
</tr>
<tr>
<td>IV</td>
<td>Any T</td>
<td>Any N</td>
<td>M1</td>
<td>Any G</td>
<td>G: Histological grade</td>
</tr>
</tbody>
</table>


In 2002, Kattan et al. published the Memorial Sloan-Kettering Cancer Center (MSKCC) nomogram for twelve-year sarcoma-specific deaths in which they utilized a subset of independent prognostic markers to predict the clinical cancer development [42, 43]. This approach was later adapted for several clinical situations (pre-/postoperative, after recurrence, etc.) and for specific
subsets of patients (specific sites and histology, etc.) [44–46]. If developed and used correctly, these nomograms seem to give a better prediction of the prognosis of each patient than the conventional staging systems [47].

1.8.3. Other prognosticators in STS

Primary tumor location has been previously reported as an important prognostic marker in STSs, with head and neck as well as retroperitoneal location greatly increasing STS-specific mortality [43, 48].

Traditionally, the specific histopathologic subtype has been considered to be of secondary importance since individual histologic subtypes of comparable histologic grade appear to behave similarly [48, 49]. However, several reports have established the independent adverse prognostic significance of specific histologic subtypes [50, 51]. Our data could not support the observation that different high-grade sarcomas possess discrepant biological behaviors.

Several studies suggest that margin positivity is a marker of adverse prognosis. For instance, the MSKCC group reported in 2002 [52] that a positive microscopic margin was correlated with a 1.6-fold increase in disease-specific survival. Our current data further support these observations; in the multivariate analysis, margin positivity was associated with a 2.9-fold increase in STS-related death (P < 0.001). Other clinical factors reported as a prognosticator in STSs include local and distant recurrence [42] and nodal status [53, 54].

Specific molecular prognostic markers may be particularly useful in this epoch of new insight into the molecular biology of cancer. The detection of such markers may be based on high-throughput assays. The main aim of this project is to investigate the prognostic impact of molecular markers of the innate and the adaptive immune system as well as cell cycle regulatory proteins in patients with STSs.
1.9. Treatment

1.9.1. Surgery

Surgery with wide resection margins is the main choice for treating STS patients [30]. Several studies show that surgery should be planned and implemented at a center with expertise in sarcoma surgery. Patients requiring re-excision, due to poorly planned surgery or when malignancy is found in lesions that were perceived as benign before surgery, have a greater risk of recurrence than patients with a well-planned primary surgery [55, 56].

Previously, amputation was perceived as necessary to obtain adequate resection margins when STSs is in the extremities, but in the last twenty years, limb-conserving surgery has become a good alternative to amputation and involves significantly less morbidity [57, 58]. A recently published study on the treatment of STSs of the extremities suggests that for tumors ≤ 3 cm in greatest diameter, surgery alone is adequate treatment [59]. For larger tumors and small tumors with marginal or uncertain resection margins, the recommended treatment is surgery in combination with radiotherapy and/or chemotherapy [30].

For STSs of the trunk, head, and neck, as well as visceral and retroperitoneal sites, the recommendation is surgery with wide resection margins. However, it is often a challenge to obtain wide resection margins for these places, and combinations with other treatment methods are often required [60, 61].

1.9.2. Chemotherapy

Pre- and postoperative chemotherapy is broadly used in treatment of bone sarcomas [62] and rhabdomyosarcomas. In STSs its usage is controversial as there have been conflicting reports regarding the treatment’s effects [63]. The ESMO clinical recommendations for STS diagnosis, treatment, and follow-up assess adjuvant chemotherapy as an option in cases of large or high-grade tumors rather than as a standard treatment [29].

Doxorubicin and Ifosfamide containing regimes are used both for adjuvant and for neoadjuvant treatment of advanced STSs [64–66]. Novel drugs such as gemcitabine and taxans, among others, are also used [11, 67]. Additionally, Trabectin® was recently approved by the FDA for palliative STS treatment [68].
Neoadjuvant chemotherapy is used for primary inoperable STSs in order to shrink the tumor and facilitate wide resection and elimination of subclinical disease [69]. Isolated limb perfusion and hyperthermic isolated limb perfusion are novel techniques available in some cancer centers for the treatment of primary unresectable extremity STSs. These techniques render the tumors operable in up to 40% of the cases, although often at the cost of considerable toxicity [70–72].

1.9.3. Radiotherapy

Primary radiotherapy is mostly used in cases where surgery is not possible, and the specific effect of this therapy is difficult to assess, as these tumors often have a dismal prognosis [73]. Intensity-modulated radiation therapy (IMRT) is a modern type of high-precision radiotherapy. Using computer technology, linear accelerators deliver defined radiation doses to a malignant tumor or specific areas within the tumor. Several studies recently demonstrated that IMRT can be administered safely and with promising efficacy, especially in patients with locally advanced STSs [74, 75].

Adjuvant radiotherapy is warranted for limb STSs where initial resection yields uncertain, marginal, or intralesional resection margins [76, 77]. The dosages are typically between 50 and 75 Gy, with higher radiation doses (63 Gy or more) yielding much better tumor control and survival [78]. The therapeutic window is between 63 and 68 Gy. An increase in complications occurs in patients that are given doses of 68 Gy or more. [78].

During the last 20–30 years, adjuvant radiotherapy has become more and more commonly used in the treatment of localized STSs. In a study of 1,093 patients with STSs in an extremity or trunk wall, adjuvant radiotherapy was shown to prevent local recurrence regardless of the malignancy grade, tumor depth, and surgical margin status. The effect was seen more clearly in deep-seated, high-grade tumors and in tumors treated with surgery with wide resection margins [79]. For STSs of other sites, adjuvant radiotherapy remains controversial [60, 74].
1.10. Molecular-genetic abnormalities in sarcomas

The molecular-genetic background of cancer in general is a hotspot in today’s research. Most STSs carry complex, but non-specific karyotypes, with numerous gains and losses [80], while ~15–20% of them—namely synovial sarcoma, Ewing sarcoma, and myxoid/round cell liposarcoma—have specific translocations and relatively simple karyotypes [81]. In addition, a minority of tumors have specific mutations, like c-kit mutation in GIST. The essential mechanisms of carcinogenesis were proposed in 2000 and considerably upgraded in 2011 by Hanahan and Weinberg [82, 83]. Each of these mechanisms is regulated by several intracellular signaling pathways that further interact in a complicated, cross-talk network. There is, however, growing evidence that certain molecular aberrations are more likely to influence the clinical behavior of a malignant tumor, including invasion and metastasis.

1.11. Tumor proliferation and growth

Tumor proliferation can be defined as an increase in tumor cell number due to altered balance between growth–antigrowth signaling and/or resistance to apoptosis and differentiation. Tumorigenesis is caused by abnormal cell proliferation. The rate of tumor cell proliferation depends on the rate of cell division, the growth fraction, and the rate of cell loss due to apoptosis or terminal differentiation. This is important since the aim of most cancer therapy strategies is to kill or reduce the growth of tumor cells.

The growth fraction of a tumor can be registered by several techniques. The easiest and most frequently used method is the mitotic count under light microscopy, which is incorporated in several STS grading systems, including the FNCLCC system [12, 35]. Alongside the advantages, this method has some drawbacks such as high intra- and interobserver variability and subjective estimation. This can be avoided by using immunohistochemical markers of proliferation, like Ki-67 or MIB-1 [84, 85]. Other methods of measuring the proliferation rate are identification of cells with active DNA synthesis [86], flow cytometry to find the approximate percentage of cells in S-phase, and the detection of cycle-linked markers.

The transition between cell cycle phases is regulated by checkpoints that, in turn, require an expression of a variety of proteins. These include regulating cyclin-dependent kinases
(CDKs), regulatory proteins, and transcription factors like Ras oncogene, retinoblastoma tumor-suppressor protein (Rb), transforming growth factor beta (TGF-beta), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF-1), and a host of others [87–90]. Many of these are known molecular biomarkers and current objects for research both in epithelial tumors and in STSs.

### 1.12. Molecular markers

Molecular markers are biological molecules found in blood, other bodily fluids, or tumor tissue [91–93]. They can be classified as those that can establish more accurate and definitive diagnoses, those that can predict responses to specific therapies, and those that can give a survival prognosis. [94–100].

There can be considerable overlap for a marker’s role across functional categories. For instance, an immunohistochemical testing of tumor tissue for female steroid hormone receptors can be used both as a diagnostic procedure in differential diagnostics of metastasis and as a predictor of tamoxifen or aromatase inhibitor therapy success in breast cancer [101, 102]. In addition, some prognostic value of these receptors has also been reported in gynecological cancers [103, 104]. The evidence for the efficacy of anti-estrogens in desmoid tumor growth is based on non-placebo-controlled trials. Tamoxifen is the most common antiestrogen agent used for treating desmoid tumors [105]. Molecular markers may offer great promise in the care of cancer patients, especially with respect to individual, tailored cancer treatment [106, 107].

#### 1.12.1. Markers of tumor growth, proliferation, and differentiation

Several studies show a close interaction between the malignant tumor cells and cells in the tumor stroma (see Figure 6 below). Here we investigate the expression profiles of STS tumor cells and the surrounding stoma.
**Figure 6.** Paracrine interactions between neoplastic cancer cells and supporting cells in tumor stroma (E. Richardson, 2008).

### 1.12.2. The adaptive and the innate immune system in STSs

Tumor-infiltrating lymphocytes (TIL) are often found in tumors, indicating that cancer triggers an immune response.

The growth of malignant cells can be suppressed by activation of the adaptive immune system, or tumor growth may be promoted by a variety of innate immune cells [108]. The adaptive immunity consists of antigen-specific T- and B-lymphocytes and can inhibit tumor growth by a combination of cytokine-mediated and antibody-mediated tumor cell lysis or direct killing by cytotoxic T-lymphocytes [108]. Recently, it became clear that it is important to study the anti-tumor effects of the innate system in the tumor stroma [109]. Efficient tumor eradication requires cancer infiltration by tumor-reactive T-lymphocytes [110]. However, there are many
mechanisms by which cancer cells can escape the immune surveillance, such as accumulation of myeloid suppressor cells and suppression of cytotoxic T-cells by regulatory T-cells [110–112].

In general we can divide tumor-infiltrating lymphocytes into three groups: a) epithelial lymphocytes, b) stromal lymphocytes, and c) peritumoral lymphocytes [113]. Infiltration of CD8+ lymphocytes in malignant tumors is associated with improved survival in different types of cancer [116–123]. The role of CD8+ cells in soft tissue sarcomas is controversial, and many publications either have a small number of cases and/or neglect the stromal component. In addition, CD4+ T- and B-lymphocytes may both promote or inhibit tumor growth [124], and their role is controversial in many cancers, including STSs [125, 126].

The most important components of the innate immune system are macrophages, granulocytes, dendritic cells (DCs), NK-cells, their receptors, and growth factors [108]. In contrast to the adaptive immune system, the innate immune system lacks “memory” when re-exposed to the same antigen. The innate immune system is important in the limitation and elimination of foreign threats to the host [108, 127].

The NK-cell plays a major role in tumor rejection in many different types of cancers [128–130]. The way these immune cells identify tumor cells has provided valuable information on tumor immunosurveillance. Based on this insight new strategies in the treatment of human cancer have been developed [131, 132].

DCs represent the most potent antigen-presenting cells and are important in the activation, recruitment, and stimulation of T-lymphocytes [133]. CD1 + DC is one of the major steps in the innate immune response against cancers. A high number of DCs in the tumoral or peritumoral area have been shown to correlate with better survival for patients with various solid tumors [134–137] and are used in therapeutic vaccination against cancer [138].

Tumor-associated macrophages are a double-edged sword. They may help tumor eradication by production of cytotoxic cytokines (IL-1, IL-6, and TNF-α). On the other hand, macrophages may favor tumor progression by TGF-beta production and by contributing to the formation of tumor stroma and angiogenesis through the release of angiogenic factors [114]. Macrophage Colony Stimulating Factor (M-CSF) is the major regulator of the mononuclear phagocytic lineage and plays a major role in innate immunity [139]. M-CSF mediates its effect
with a high affinity trans-membrane tyrosine kinase receptor (CSF-1R). Substantial evidence exists in different cancers, especially those of the breast and female reproductive system, that overexpression of CSF-1R is associated with poor survival [140]. The expression and role of M-CSF and its receptor in both the malignant and stromal components of STSs are not well studied.

To better understand the prognostic impact of the innate immune system in soft tissue sarcomas, we will analyze the degree of infiltration of cell subsets, growth factors, and their corresponding receptors belonging to the innate immune system, both in the malignant mesenchymal compartment and the stromal compartments, and study their relations to their clinicopathological variables and survival. The figure below shows schematic interactions between cells belonging to the immune system and the neoplastic cells during cancer progression.

**Figure 7.** Visser KE et al. Nature Reviews Cancer 2006:6; 24–37.
1.12.3. Tumor-infiltrating lymphocytes

Tumor-infiltrating lymphocytes are considered to be an indication of the host immune reaction to tumor antigens [141], and their clinical significance has been reported in a variety of human solid tumors.

1.12.4. Cell cycle regulatory proteins

The loss of cell cycle control is a critical step in the development of neoplasia. The cell cycle is a series of carefully coordinated and regulated steps that govern cellular proliferation. Cyclin-dependent kinases (CDK) phosphorylate the retinoblastoma (Rb) protein, a classic tumor suppressor and key component of the G1/S checkpoint. This allows DNA replication to proceed. Inhibitors of CDK, such as p16(INK4A), p21, and p27, act as brakes on progression through the cell cycle.

1.12.5. Female steroid hormone receptors

Estrogen receptors (ER) are a group of mostly intranuclear receptors activated by the hormone 17beta-estradiol (estrogen). There are two separate but highly homologous isoforms of ER, ERα, and ERbeta, which have completely different tissue distributions [171]. They are encoded by two separate genes, ESR1 and ESR2. ER, mostly in α isoform, mediates the action of estrogens and is responsible for growth and differentiation of target cells.

These steroid hormone receptors act as ligand-activated transcription factors. There are several mechanisms with such action, including (1) classic, when transcription starts after receptor-ligand complex binding to the specific response element in the gene promoter, (2) response element-independent pathway via binding to a transcription factor which in turn directly contacts the target gene promoter, (3) ligand-independent genomic action, when different growth factors induce phosphorylation of the hormone receptor followed by binding to the specific response element in the gene promoter and transcription/translation/protein synthesis, and (4) non-genomic actions, involving extranuclear fraction of hormone receptors [173].

Both ER and, to a lesser degree, PGR are well known predictive markers of endocrine therapy in breast cancer [174, 175]. They are also shown to have a slight positive prognostic
effect irrespective of endocrine therapy [103]. Steroid hormone receptors are known to be expressed to some extent by soft tissue tumors. In leiomyomatous tumors of the uterus, their expression level correlates inversely with tumor malignancy grade [176, 177]. In addition, effect of hormone-ablation therapy such as tamoxifen has been a reported in aggressive intraabdominal fibromatosis [178, 179].

1.12.6. TGF-beta

TGF-beta is a family of three highly homologous proteins, called TGF-beta-1, TGF-beta-2, and TGF-beta-3, which have very similar functions. They are natural tumor-suppressive agents and induce G1 to terminate proliferation, promote apoptosis, and induce differentiation in normal cells. However, in cancer development, this mediator initiates dedifferentiation through activation of SMAD and non-SMAD (DAXX) signaling pathways [180]. The TGF-beta pathway activation is associated with poor survival in epithelial tumors [183, 184] and in mesenchymal bone [185] and soft tissue tumors [186–188].

1.13. Tissue microarray

Tissue microarrays (TMAs) represent a powerful technology tool designed to explore molecular targets, on the DNA, RNA, or protein level, from several tissue specimens assembled in a single microscope slide [194]. This method implies the extraction of small tissue cylinders from a donor tissue block to be embedded in a recipient block (Figure 7).
Figure 7. Tissue microarray method. Cores punched from the donor blocks and embedded into the recipient block. The TMA block can then be sectioned and used for various staining methods. Adapted from Chen W, Foran DJ: Advances in cancer tissue microarray technology: Towards improved understanding and diagnostics. Anal Chim Acta 2006 [195]. Permission obtained from Elsevier Inc.

This block can then be cut into thin slices available for immunohistochemistry (IHC), in situ hybridization (ISH), etc. Once constructed, one block can potentially yield tissue for several hundred analyses, depending on its thickness [196, 197].

The method was first introduced by Battifora in 1986 as a so-called “multitumor (sausage) tissue block” [198] and further modified in 1990, referred to as “the checkerboard tissue block” [199]. Although offering significant benefits even at this early stage, the TMA technique was not embraced on a large scale before 1998, when Kononen et al. devised an instrument able to standardize the TMA construction process [200]. Adaptation has also allowed the use of material
other than paraffinized tissues, including frozen tissue, cell-lines, and needle biopsies. This has led to a vast increase in TMA studies, and in 2007 nearly 10% of all biomarker studies were conducted using TMA as the principal method of investigation [196].
2. AIMS OF THESIS

The aim of our study is to look into the role of different essential molecular markers of the innate and the adaptive immune system as predictors for disease-specific survival (DSS) in patients with STSs.

More specifically, the aims were to:

 ✓ explore the prognostic impact of lymphocytes in STSs by using immunohistochemistry to evaluate the expression of CD3+, CD4+, CD8+, CD20+, and CD45+ lymphocytes in tumors.

 ✓ evaluate the prognostic significance of macrophages (CD68), their growth factor macrophage colony-stimulating factor (M-CSF), its receptor colony-stimulating factor-1 receptor (CSF-1R), natural killer cells (CD57), and the general immunomodulating molecule (TGF-beta) in tumors and peritumoral capsule.

 ✓ investigate the prognostic significance of Jab1, p16, p21, p62, Ki67, and Skp2 in STSs.

 ✓ explore the prognostic significance of Skp2 related to ER and PGR in male and female patients with STSs.
3. MATERIAL AND METHODS

3.1. Study population and material

Figure 8 shows the inclusion and exclusion of patients in the different studies. We conducted a retrospective search for patients with sarcoma diagnosis in archival material at the University Hospital of North Norway (1973–2006) and hospitals in Arkhangelsk County, Russia (1993–2004). In the Russian material we searched for patients within a ten-year period, since the archival system before the selected time period was less organized. A total of 959 patients were found (Norwegian, n = 632; Russian, n = 337).

Formalin-fixed and paraffin-embedded samples from primary tumor tissues were obtained. All biopsies were re-evaluated by two experienced pathologists. The tumors were graded according the FNCLCC system and histologically subtyped according to the World Health Organization guidelines. For the Russian material new slides were made of all paraffin blocks. For the Norwegian material new slides were made when necessary. All biopsies were immunostained with actin, CK, CD34, CD117, SMA, and vimentin. Some slides were also stained with the S100 when it was necessary to exclude or verify the differential diagnosis. Other molecular methods were not used in our study, but in some cases PCR or FISH were performed at the time of the initial diagnosis. About 10% of the initial diagnoses were revised due to changes in classification systems and the creation of new entities such as GIST. Non-sarcoma, other sarcomas not classified as STSs, and GIST were excluded. Exclusions based on this were as follows: carcinosarcomas (n = 81), dermatofibrosarcoma protuberans (n = 78), GIST (n = 47), osteosarcomas (n = 42), chondrosarcoma (n = 30), Kaposi’s sarcoma (n = 30), endometrial stromal tumors (n = 27), benign tumors (n = 18), malignant mesothelioma (n = 11), and other sarcomas/unknown (n = 99).

In total, 496 non-GIST STSs (Norwegian, n = 299; Russian, n = 197) were registered. However, 247 patients were excluded due to inadequate paraffin-embedded fixed-tissue blocks (n = 161) or missing clinical data (n = 86). Thus, 249 non-GIST STS patients (Norwegian, n = 167; Russian, n = 82) were eligible and included in the study.
Figure 8. Flowchart visualizing inclusion and exclusion of patients in the study.

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

3.2. Immunohistochemistry (IHC)

The applied antibodies were subjected to in-house validation by the manufacturer for IHC analysis of the paraffin-embedded material. The antibodies used in the study are summarized in Table 4. All stainings were performed in the Ventana Benchmark XT automated slide stainer (Ventana Medical System, Illkirch, France). Before staining, the sections were incubated over night at 60 degrees Celsius. Tissue sections were incubated with primary mouse monoclonal antibodies as well as rabbit polyclonal antibodies recognizing the different antigens (Table 4).
Table 4. Schematic overview of the antibodies used in the studies.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Dilution</th>
<th>Antibody</th>
<th>Clone</th>
<th>Source</th>
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<td>2GV6</td>
<td>Ventana Medical Systems</td>
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</tr>
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</table>

3.3. Scoring

The ARIOL imaging system (Genetix, San Jose, CA) was used to scan the slides for antibody staining of the TMAs. Representative and viable tissue sections were scored manually and semi-quantitatively on a computer screen for nuclear and/or cytoplasmic staining. (Figure 1). The number of CD3, CD4, CD8, CD45, CD57, and CD68 positive cells in tumors were scored as 0 (no cells), 1 (1–5 cells), 2 (6–19 cells), or 3 (20+ cells) per 0.6 mm core. Expressions of M-CSF,
CSR-1R, TGF-beta, Jab1, p16, p21, p62, Ki67, Skp2, ER, and PGR were scored as: 0, negative; 1, weak; 2, intermediate; 3, strong. Each patient’s score was based on the mean score of cores from one or several biopsies. High expression was defined as mean score ≥ 0 for CD57, M-CSF, CSF-1R, p21, Skp2, ER, and PGR, ≥ 0.30 for CD68, ≥ 0.33 for p62, ≥ 0.50 for CD20, ≥ 0.75 for p16 and TGF-beta, ≥ 1.00 for CD4, ≥ 1.50 for CD3 and CD8, and ≥ 2.00 for CD45, Jab1, and Ki67. All samples were anonymized and independently scored by two pathologists (AV and SWS). When disagreements occurred, the slides were re-examined and a consensus was reached by the observers. When assessing a variable for a given score, the scores of the other variables and the outcome were hidden from the observers.

3.4. Statistical analysis

For statistical analyses we used the SPSS (Chicago, IL) statistical package. The chi-square test and Fisher’s exact test were used to examine the association between the expression of molecular marker and various clinicopathological parameters. Marker expression correlation was measured with the Pearson correlation (2-tailed) at the 0.05 and 0.01 levels. For univariate analyses we used the Kaplan–Meier method. Statistical significance between survival curves was assessed by the log rank test. Disease-specific survival (DSS) was determined from the date of histological-confirmed STS diagnosis.

For multivariate analysis we used the Cox proportional hazards model to assess the specific impact of each pre-treatment variable on survival in the presence of other variables. Only variables of significant value from the univariate analysis were entered into the Cox regression analysis. Probability for stepwise entry and removal was set at 0.05 and 0.10, respectively. The significance level used was P < 0.05. IHC scores from each observer were compared for interobserver reliability by the use of a two-way random effect model with absolute agreement definition. The intraclass correlation coefficient (reliability coefficient) was obtained from these results.

3.5. Ethical clearance

Our study was approved by the Regional Committee for Research Ethics (REK Nord) and the National Cancer Data Inspection Board.
4. MAIN RESULTS

4.1. Paper I

Tumor-infiltrating lymphocytes (TIL) are often found in tumors, which indicates that tumors trigger immune responses. The immune status at the time of the diagnosis of the tumor may be important, but the prognostic significance of TIL is controversial since the immune system may both promote and reduce tumor growth. The aim of this study was to investigate the prognostic significance of TIL in STSs. The number of tumor-infiltrating CD3+, CD4+, CD8+, CD20+, and CD45+ lymphocytes was analyzed in 249 patients with STSs in relation to other clinicopathological variables.

In univariate analyses increased numbers of CD4+ (P = 0.008) and CD20+ (P = 0.006) inflammatory cells were positively associated with a better disease-specific survival (DSS) in patients with wide resection margins (n = 108). For patients with non-wide resection margins (n = 141), increased numbers of CD3+ (P = 0.028) lymphocytes in a tumor was negatively associated with DSS. In multivariate analyses a high number of CD20+ lymphocytes (HR = 5.5, CI 95% = 1.6–18.6, P = 0.006) in the tumor was an independent, positive prognostic factor for DSS in patients with wide resections margins.

4.2. Paper II

This study was focused on exploring the prognostic impact of the presence of cells and growth factors belonging to the innate immune system in STSs. In univariate analyses high expressions of M-CSF (P = 0.034), Ki67 (P < 0.001), and TGF-beta (P = 0.003) in tumor were negatively associated with DSS. An increased expression of Ki67 in the peritumoral capsule tended to correlate with a shorter DSS (P = 0.057). An increased expression of CD68 in tumor correlated significantly with malignancy grade (P = 0.016) but not DSS (P = 0.270). In multivariate analyses co-expressions of M-CSF and TGF-beta (P = 0.022) in tumor and a high expression of Ki67 (P = 0.019) in peritumoral capsule were independent, negative prognostic factors for DSS.
4.3. Paper III

The purpose of this study was to clarify the prognostic significance of expressions of Jab1, p16, p21, p62, Ki67, and Skp2 in STS. In univariate analyses a high expression of Skp2 (P = 0.050) and a high expression of Ki67 (P = 0.007) were negatively associated with DSS. In a subgroup analysis, a negative correlation between Skp2 and DSS was seen in patients with malignancy grade 1 or 2 (P = 0.027), tumor size >5 cm (P = 0.018), no radiotherapy given (P = 0.029), and no chemotherapy given (P = 0.017). High expression of Ki67 was strongly positively associated with high malignancy grade (P = 0.001). In multivariate analyses, Skp2 was an independent negative prognostic factor for DSS in women (P = 0.009) and in patients without administered chemotherapy or radiotherapy (P = 0.026).

4.4. Paper IV

This study focused on clarifying the prognostic significance of Skp2 expressions related to gender, estrogen receptor (ER), and progesterone receptor (PGR) in STS. In subgroup analyses expressions of PGR in males (p = 0.010) and in patients older than 60 years (p = 0.043) were negative prognostic factors for DSS. A high expression of ER in females was a positive prognostic factor for DSS (p = 0.041). In co-expression analyses of the whole cohort, a low expression of Skp2 in combination with a low expression of ER was positive for DSS (p = 0.049). In females, a high expression of Skp2 in combination with a low expression of ER was a negative prognostic factor (p = 0.021). In the multivariate analyses malignancy grade (p<0.001), age (p = 0.012), wide resection margins (P = 0.010), ER negative/PGR positive co-expression profiles (p = 0.002), and ER positive/PGR negative co-expression profiles (p = 0.015) were independent, negative prognostic factors for DSS. In females expressions of Skp2 (p = 0.006) were associated with shorter DSS.
5. DISCUSSION

5.1. Methods

5.1.1. Data collection and study population

We have included patients from two countries, Norway and Russia, to achieve adequate statistical power for the analyses. The representativity of the studied population may be a problem in studies. The risk of heterogeneity in the population may also be a disadvantage. In terms of ethnicity and geography, however, northern Norway and Russia are quite close, as seen in the corresponding distribution of clinicopathological variables. Despite possible differences in diagnosis or treatment traditions, the histopathological reassessment of all tumors and the relatively limited and rough classification of treatment strategies are meaningful to study in both the Norwegian and Russian patients in a cohort. The study focuses on the natural biology of the STSs and not on treatment.

5.1.2. Representativity of Norwegian and Russian study populations

STSs represent about 0.5–1% of all cancer cases. Of the total of 21,000 cancer cases reported annually in Norway, sarcomas represents more than 160 cases, of which two thirds are STSs and third bone sarcoma. The proportion living in northern Norway accounts for about 10% of the Norwegian population. In view of this, it is estimated a total of 350 new STS patients during a period of 33 years (1973–2006), of which some had GIST and some had sarcomas of the skin (for example, dermatofibrosarcoma).

There were 299 Norwegian cases of STSs observed in our population. We excluded 132 cases due to lack of clinical data or inadequate paraffin-embedded fixed-tissue blocks (Figure 8). Since the lack of paraffin-embedded material is random, it can be argued that the patient group is representative of the population.

Nevertheless, there is more reason to question the representativeness of the Russian material. The population of Arkhangelsk Oblast is about three times larger than northern Norway.
As the number of Russian cases in our study (n = 82) is about a third of the Norwegian material, there is certainly a need for more patients in the Russian material, although the duration of enrolment period for the Russian sample (1993–2004, a total of 11 years) was a third (1973–2006, a total of 33 years) of the Norwegian. This potential selection bias should be considered when our results are analyzed. We also see that the subsets of the Russian patients have significantly poorer prognoses than the Norwegian cohort. However, when comparing the clinicopathological variables, only the distribution of histologic grade is significantly different in the Russian versus the Norwegian material. A larger proportion of Russian patients with malignancy grade 3 may partially explain the reduced survival of the Russian population. A significant part of the Russian material was from Arkhangelsk Regional Oncology Centre, while patients with less aggressive tumors can potentially be cured locally by the local district hospitals. In short, we cannot rule out a selection bias in the Russian material.

5.1.3. Separate investigation of differently located sarcomas

Sarcomas located on extremities and trunk (ET), versus retroperitoneal and visceral tumors (VR), may be regarded as distinct STS entities based on clinical and prognostic data. Metastases are the main cause of sarcoma-related death in patients with visceral tumors, while local relapse is a more common cause of sarcoma-related death in patients with STSs in extremities and trunk. We have stratified patients according to ET (n = 115) versus VR (n = 66) subgroups (patients with head and neck STS (n = 13) were excluded from these analyses). Significant differences and trends from the original papers were persistent in patients with STSs located on extremities and trunk. The number of patients with visceral tumors was insufficient for conducting reliable analyses.

5.1.4. Heterogeneity of histological entities in the study population

Heterogeneity with regard to the histological units included in the analyses may be a problem. It is possible that different subtypes of STSs have different expressions of prognostic molecules. We conducted subgroup analyses of the histological units in terms of expression of
different markers, and we found the same trends in the major subgroups compared to the smaller subgroups.

5.1.5. Conclusion on material representativity

STSs are rare tumors, and there are many sarcoma subtypes. In our study, it was difficult to collect a sufficient number of similar patients with similar tumors that have received the same treatment. This is a known issue in the implementation of patients in STS studies. But our study is largely focused on generating hypotheses rather than testing them, so patient similarities are less crucial. To be more conclusive, future STS studies should be based on large, multi-institutional and multi-national studies designed to collect the highest possible number of STS patients to ensure a sufficient number in each subgroup. At the same time, all tumors we examined were of mesenchymal differentiation and they belong to the same generic group. Moreover, we examined the role of some important molecular markers of the innate and the adaptive immune system as predictors for DSS in patients with STSs. Similar findings are found in many different epithelial and non-epithelial malignant tumors of diverse histological locations and devices and do not seem to depend on the tumor type.

5.1.6. Tissue microarray

A tissue microarray (TMA) assembles on a single histologic slide several small representative tumor cores from many different patients, thus making it possible to analyze multiple specimens in one staining [201]. Two pathologists (AV and SWS) reviewed the histology of all STSs. TMAs were constructed for high-throughput molecular pathology research [10]. The most representative areas of viable tumor cells were carefully selected and marked on the hematoxylin and eosin (HE) slides for the corresponding donor blocks and sampled for the TMA collector blocks. The TMAs were assembled using a tissue-arraying instrument (Beecher Instruments).

TMA is a valuable tool for high-throughput analyses of tissues to identify prognostic markers and possible targets for therapy in human cancers [201]. Obvious advantages of the TMA technique, versus whole slide assessments, include the high throughput, robust benefits at a lower cost, the possibility for large cohorts simultaneously, supreme staining standardization,
reproducibility, and relative simplicity. It is also possible to use the donor specimens for further analysis and to share the material between institutions.

Along with these apparent benefits, there are some disadvantages often discussed with regard to the use of TMAs. A common question is whether a few core samples are representative for large tumor specimens. Instead of 0.6 mm cores, some investigators have used larger cores (2–4 mm or more) to increase the representativity [202–204]. Others suggest that multiple, small cores from different regions offer better coverage of tumor heterogeneity [194]. After reviewing all the original tumor sections and taking heterogeneity into consideration, we decided to use duplicate 0.6 mm cores that were selected to maximize representativeness. Studies reveal a 95% correlation when comparing evaluations of tumors in duplicate 0.6 mm cores versus the whole slide [194]. To include all core samples, we constructed 12 tissue array blocks.

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

5.1.7. Immunohistochemistry

Immunohistochemistry is one of many techniques used to analyze the tissue for expressions of proteins and other molecules. In addition to standard HE staining, immunohistochemistry is one of the most widely used techniques in routine diagnosis of pathological laboratories. Immunohistochemistry is also commonly used in research. It is reliable, well developed and familiar, easy to interpret, and widely available. Unlike a number of more modern techniques, immunohistochemistry visualizes the final protein product, localization of protein, and not just an up or down regulated gene, etc.

5.1.8. Antibodies

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

The Sarcoma Study Group in Tromsø is a part of a larger Translational Cancer Research Group. All the immunomarkers we used were chosen from published literature and validated by the manufacturer and by the group’s previous studies of lung cancer [205, 206].

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

5.1.9. Controls

The principle of immunochemical staining is that a specific antibody will combine with its specific antigen, making a unique antibody–antigen complex. Antibody specificity was ensured by a western blot showing binding of a protein of the expected size. In the case of the antibodies used in our studies, this was done by the manufacturer and presented in the data leaflets of the antibodies.

The use of staining controls helps to reduce false positive and false negative results and make it easier to read the results of the immunochemical staining. Negative controls are conducted by replacing the primary antibody with a primary antibody diluent to check for unspecific staining in the absence of the antibody. Negative controls could be made even more stringent by introducing isotype controls to check for unspecific binding. A positive control may be any tissue that contains the antigen of interest. We used tissue controls with other tumor groups and normal tissue on each TMA slide, representing both positive and negative controls.
5.1.10. Statistics

Almost every time we make a decision based on data, there is some chance we will make an error. There are many approaches to statistical analysis of survival data, and no optimal method of analysis exists. In order not to over- or under-interpret the significance of their data, investigators have to be vigilant when choosing an approach. The objective of the hypothesis test will be to make a decision about the null and alternate hypothesis statements. The possibility of error comes in because we make this decision regardless of whether the null hypothesis is actually true or false. We believe that we in our analyses have found a reasonable balance between type I and type II errors. A short discussion of the statistical methods used in our studies is presented below.

5.1.11. Significance level

Type I errors occur in a situation where the null hypothesis is true but our statistical test rejects it anyway. This is a situation when inappropriate significance levels are used. Type II errors occur in a situation where the null hypothesis is a false statement and we should reject it. However our hypothesis test fails to reject the null. In biological studies it has become a norm to use $P < 0.05$ as the cut-off point where a difference is considered significant. This shows that one in twenty tests for the same difference will be a type I error. When conducting a large number of tests the chance of an erroneous positive result thus increases. Several approaches have been developed for reducing the chance of a type I error in the setting of multiple testing. The drawback of these approaches is the increased chance of a type II error. There is no consensus whether such methods should be used in prognostic studies. We chose not to conduct a correction of multiple testing, as we see our studies as hypothesis generating. This increased the risk of type I errors but decreased the chance of type II errors.

5.1.12. Cut-off values

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

5.1.13. Survival analysis

Survival analysis is used to analyze datasets in which the response variable denotes time until an event occurs. These events often refer to time between diagnosis and death or time until relapse and recovery [207]. There are several different statistical methods for analyzing survival data. One well-proven method is the Kaplan–Meier (KM) analysis, which tests the difference between groups in time to event data. However, the KM method does not adjust for the presence of other clinical variables. To address this point we used the Cox proportional hazards method to adjust for clinical variables found to be significant when using the KM method. This stringent method works to ensure that the variables found in our studies are in fact independent of known
demographic, clinical, or pathological variables and could therefore contribute when calculating the prognosis of STS patients.

An issue is which endpoint to use. In prognostic studies there are a variety of endpoints, such as overall survival (OS), metastasis-free survival (MFS), time to recurrence (TTR), time to progression (TTP), and, as we have chosen, disease-specific survival. DSS is a well-established endpoint. In our study we excluded from the survival analysis patients with non-sarcoma-related deaths.

5.2. Discussion of the results

There are few studies with large cohorts of STS patients because sarcomas are rare tumors. Our study population is quite large compared to similar studies. Fully reassessed histology, scrutinized staining, visualizing and scoring processes, as well as comprehensive clinical data for each patient and rather long follow-ups ensure objectivity to the study performance and assessment. Our research group has investigated the prognostic impact of several families of proteins that are responsible not only for tumor growth, proliferation, and differentiation, but also for angiogenesis and local immunity, and estimate possible co-expressions within and between these marker families.

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

In summary, the results of our studies suggest the involvement of these molecular markers in the innate and the adaptive immune system, as well as cell cycle regulatory proteins as predictors for treatment response, metastasis, and treatment strategies within subgroups of STSs. The exact mechanisms of such involvement are, however, yet to be elucidated.
5.2.1. Paper I

TILs are considered to be a response of the host immune reaction to tumor antigens [141], and their clinical significance has been reported in several different cancer subgroups. The purpose of this study was to explore the prognostic significance of TILs in STSs. We used immunohistochemistry to evaluate the CD3+, CD4+, CD8+, CD20+, and CD45+ TIL. In univariate analyses, high numbers of CD4+ (P = 0.008) and CD20+ (P = 0.006) TIL were positively associated with DSS in patients with wide resection margins (n = 108). High numbers of CD3+ (P = 0.028) TIL were negatively associated with DSS in patients with non-wide resection margins (n = 141). In multivariate analyses, a high number of CD20+ TIL (HR = 5.5, CI 95% = 1.6–18.6, P = 0.006) was an independent, positive prognosticator for DSS in patients with wide resections margins. In conclusion high density of CD20+ TIL is an independent, positive prognostic indicator for STS patients with wide resection margins.

5.2.1.1. CD20 positive tumor-infiltrating cells

CD20+ TILs are associated with a better survival in lung cancer, cervical cancer, prostate cancer, and ovarian cancer [208–212]. CD20+ cells in metastatic lymph nodes are positively correlated with better prognosis in patients with oro- and hypopharyngeal carcinoma [213]. A high density of CD20+ cells was associated with a good clinical outcome prognostic factor for stage IIIa gastric cancer [214]. The presence of both CD20+ and CD8+ tumor-infiltrating lymphocytes correlated with increased patient survival compared with CD8+ TIL alone [215]. In contrast, using flowcytometry with CD19, high B-cell infiltration was correlated with poor prognosis in metastatic ovarian carcinoma [216]. In a series of 3,261 prostate cancers, the number of CD20+ cells per tissue spot was not associated with other clinical and histopathological parameters [217]. In our material a high number of CD20+ TILs was an independent, positive prognostic indicator.
5.2.1.2. CD3 positive tumor-infiltrating cells

Several studies show that tumor-infiltrating CD3+ lymphocytes are strongly correlated with improved survival in epithelial tumors [218–222]. Our study did not uncover any such association in the mesenchymal tumors in patients with wide resection margins, but a high number of CD3+ TILs was correlated with reduced DSS in patients with non-wide resection margins. Combining both patients with wide and non-wide resection margins, the results were not statistically significant. Our results were included in forest plots in a meta-analysis of various cancer types [223] (Figure 9).
**Figure 9.** Forest plots of studies on CD3+ TILs. Hazard ratios and 95% confidence intervals from individual studies are depicted as squares and horizontal lines, respectively. The pooled estimate is shown as a diamond shape, where the center represents the pooled HR and the horizontal borders represent the 95% CI. Hazard ratios are defined as high CD3 versus low CD3 counts; therefore a hazard ratio < 1 represents a lower risk of death or progression associated with high CD3 counts [223]. *Permission obtained from British Journal of Cancer.*

In Figure 9 only two studies used disease-specific survival as the main endpoint, but with opposing results. Nosho et al. studied tumor-infiltrating CD3+ T-cells in colorectal cancer [224]. We studied tumor-infiltrating CD3+ T-cells in STSs. Different tumor biology in epithelial and mesenchymal tumors may explain the differences. However, the two studies had overlapping 95% confidence intervals, and both confidence intervals included the number 1.

### 5.2.1.3. CD4 positive tumor-infiltrating cells

CD4 is a glycoprotein that is expressed on the surface of regulatory T-cells, T helper cells, macrophages, monocytes, and dendritic cells. CD4+ T helper lymphocytes (Th) are a heterogeneous cytokine-secreting class of T-lymphocytes. T helper type 1 lymphocytes (Th1) have a crucial role in activating cytotoxic T-lymphocytes (CTL). T helper type 2 lymphocytes activate eosinophils and stimulate humoral immunity. In terms of antitumor immunity, Th1 activation is more effective than Th2 activation [225]. In cancer, Tregs preferentially move to the tumor by chemotaxis because of chemokines from tumor cells and microenvironmental macrophages [226].

The role of CD4+ T- and B-lymphocytes is controversial in many cancers including STS; CD4+ cells in the absence of the CD8+ cytotoxic T-cells are critical and sufficient for NKT cell-dependent rejection of experimental tumors [227]. In lung cancer the prognostic impact of CD4 is controversial [208, 228], but in our material CD4+ cells were a positive prognostic factor in univariate analyses.

In a meta-analysis of six publications from different cancers studying overall survival in CD4+, the pooled HR is 0.82 (95% CI: 0.69–0.98), which is statistically significant (P = 0.03). In
a pooled analysis, disease-specific survival [208, 232] and progression-free survival [229–231] were not influenced by CD4+ TIL [223].

5.2.1.4. CD8 positive tumor-infiltrating cells

CD8+ TIL has been positively correlated with better survival in a variety of cancers, including carcinomas of the bile duct, colon, endometrium, esophagus, follicular lymphoma, lung, urothelium, and uveal melanoma [116–122, 208, 233]. The prognostic impact of CD8+ TIL in sarcomas is controversial. Most of these studies are based on relatively few cases. There was an association between a high number of stromal CD4+ and CD8+ lymphocytes and favorable prognosis in non-small-cell lung cancer [208]. In our material CD8 was not a significant prognostic factor (P = 0.15). Gooden et al. included 23 studies in the meta-analysis below [223]. Here, the presence of CD8+ results in better prognosis for all survival endpoints tested (Figure 10).
Figure 10. Forest plots of studies on CD8+ TILs. Hazard ratios and 95% confidence intervals for death or progression associated with high versus low CD8 counts [223]. Permission obtained from British Journal of Cancer.
5.2.2. Paper II

The purpose of this study was to evaluate the innate immune system in STS. We used immunohistochemistry to study the expression of CD68, M-CSF, CSF-1R, CD57, TGF-beta, and Ki67 in tumor and peritumoral capsule. High co-expressions of M-CSF and TGF-beta in tumor and a high expression of Ki67 in the peritumoral capsule were associated with poor DSS.

5.2.2.1. CD68

Macrophages are the first line of defense against pathogens and are frequently present in the tumor stroma of carcinomas [139, 234, 235]. The majority of tumor-associated macrophages (TAM) produces anti-inflammatory factors and promotes tumor growth. TAMs have a major impact in cancer development because they can adopt tropic roles and are educated by the tumor microenvironment to facilitate tumor cell motility, matrix breakdown, and angiogenesis [236]. Altogether, this gives malignant tumors the capacity to infiltrate the surrounding normal tissues and metastasize to other parts of the body [236, 237]. The pan-macrophage marker CD68 is commonly used to identify TAMs in diagnostic biopsies. Several studies show a negative correlation between high density of TAMs and survival in women with breast cancers [238]. The same is found in thyroid cancer, liver cancer, and non-gynecological leiomyosarcomas [239–241]. No such relationship was observed in malignant melanoma or prostate cancer [242, 243]. High influx of CD68+ TAM improved survival in colon cancer and lung cancer [244, 245]. In our material a high number of CD68+ cells was positivity associated with malignancy grade (P = 0.016) but showed no correlation with disease-specific survival (P = 0.270).

5.2.2.2. M-CSF

The proliferation and differentiation of monocytes to macrophages is regulated by the hematopoietic growth factor macrophage stimulation factor (M-CSF). In inflammation M-CSF induces macrophages to produce proteases and cytokines, thereby enhancing the macrophages’ ability to combat microbial infections [246]. In our study a high M-CSF expression in tumor was positively associated with a high malignancy grade, increased Ki67, and DSS in univariate
analyses. However, the expression of M-CSF in the peritumoral capsule was not correlated with DSS.

5.2.2.3. **CSF-1R**

The macrophage colony stimulation factor 1 receptor (CSF-1R) is one of the growth factor receptors that regulate proliferation and differentiation of monocytes [253]. The expression of CSF-1R and/or CSF-1 is strongly associated with poor survival in several epithelial tumors, such as breast, ovarian, and prostate cancer [140, 254–256]. A high expression of CSF-1R in peritumoral liver tissue is correlated with poor prognosis in hepatocellular carcinoma [257]. Expression of CSF-1R is associated with histological grade in STSs [252]. In our material the expression of CSF-1R did not correlate with histological grade or DSS, but high CSF-1R was correlated with a high expression of Ki67 (p = 0.001, data not shown).

5.2.2.4. **CD57**

CD57 positive cells are important in the defense against malignant and virally infected cells. Presence of these cells is a positive prognostic marker for DSS in lung cancer [258][259], as well as in other malignant tumors like gastric and colon cancers [128, 129]. A high number of stromal CD57+ cells was positively associated with DSS in patients with lung cancer, whereas a high density of CD57 positive cells within the epithelial tumor cell was not [249].

The location of infiltrating inflammatory cells may be important. There are major differences between 1) CD57 positive cells within epithelial cancer cell nests in carcinomas; 2) CD57 positive cells present in the stroma of epithelial tumors, 3) peritumoral CD57 positive cells present along the invasive margins, and 4) CD57 positive cells in the peritumoral capsule of stromal tumors such as STSs. Even though CD57 may have a favorable impact on DSS in carcinomas, this may not be the case in STSs. In our material there was no such correlation in tumor or peritumoral capsule.
5.2.2.5. TGF-beta

TGF-beta belongs to a TGF-beta/BMP family of growth factors and is basically a tumor-suppressive agent whose functions include proliferation hampering and promotion of apoptosis in both normal and tumor cells. There is, however, broad evidence of its negative influence on prognosis, described mostly in epithelial [183, 184] but also in mesenchymal tumors [185–188]. The possible mechanisms of such pro-neoplastic action include receptor-inactivating mutations, selective inactivation of the tumor-inhibiting arm of this pathway [181], and TGF-beta induced systemic immune suppression [182]. Other proposed modulators of TGF-beta function are factors in the tumor microenvironment, particularly inflammatory cells, cancer-associated fibroblasts [182], and angiogenetic factors [193]. We found TGF-beta to be an important prognostic marker. High TGF-beta expression was negatively associated with DSS in STSs [260]. In our study co-expression of M-CSF and TGF-beta was an even stronger negative prognostic factor.

5.2.2.6. Ki67

Ki67 expression increases with increasing malignancy grade in many different cancers [165–169]. In Ewing’s sarcoma, high Ki67 expression was negatively associated with progression-free survival and overall survival [170]. For patients with STSs of the extremity and trunk wall, tumor proliferation can be assessed by Ki67 expression and used in statistical decision-tree models that give prognostic information [261]. In our study high expression of Ki67 in tumor was negatively associated with DSS in patients with STSs, but Ki67 expression was dependent on malignancy grade. Ki67 did not appear as an independent prognosticator in the multivariate analysis.

Ki67 as a predictive and prognostic biomarker has been extensively studied in breast cancer [264]. An expression level of Ki67 above 10%–14% defines a group of women with aggressive breast tumors. Using this definition in future studies may make for more reliable comparisons [265]. In 2009 the panel of experts at the St. Gallen Consensus conference considered the Ki67 labeling index to be imperative for selecting patients with hormone receptor-positive breast cancers for treatment with a combination of chemotherapy and endocrine therapy. The tumors were classified as low, intermediate, and highly proliferating based on the Ki67 expression [266].
In Norway Ki67 analysis has been introduced as a routine in breast cancer and is vital for therapy selection.

### 5.2.3. Paper III

This study sought to clarify the prognostic significance of the Jab1, p16, p21, p62, Ki67, and Skp2 expressions in STS. A high expression of Skp2 in patients with STSs is associated with poor DSS in women and in STS patients not treated with chemotherapy or radiotherapy.

#### 5.2.3.1. Jab1

Some studies suggest that Jab1 may interact with the protein form of the CDK inhibitor 27 and shuttle p27 from the nucleus to the cytoplasm, and, moreover, Jab1 may decrease the cellular amount of p27 by accelerating p27 degradation via the ubiquitin-proteasome system [143, 144]. Other reports have shown that a high expression of Jab1 and low expression of p27 are correlated with poor survival in a variety of cancers [145–148]. Expression of Jab1 protein in epithelial ovarian borderline tumors was significantly higher than in benign tumors [267]. Overexpression of Jab1 was associated with poor survival in patients with malignant glioma [268]. Tsuchida et al. [269] suggested that Jab1 may play an important role in determining the differentiation stage of rhabdomyosarcoma cells by modulating the activity of CDK inhibitor p27. In our material Jab1 expression was not associated with malignancy grade and had no prognostic impact on DSS.

#### 5.2.3.2. p16

Epigenetic silencing of p16 is probably an important event in the development of Ewing sarcoma [275], and p16 has been shown as a sensitive and specific marker for distinguishing atypical lipomatous tumors, well-differentiated liposarcomas, and dedifferentiated liposarcomas from benign adipocytic neoplasms [276]. In mammary phyllodes tumors high expressions of p16 and pRb are correlated with high tumor grade [167]. High expression of p16 was associated with good response of chemotherapy in osteosarcoma [277]. In a series of 38 pediatric osteosarcomas
there was an inverse correlation between pRB loss and p16 expression, where the absence of p16 expression significantly correlated with poor survival [278]. Low expression of p16 was correlated with poor survival in malignant peripheral nerve sheath tumor [279]. In our study p16 expression was not associated with malignancy grade or DSS.

5.2.3.3. p21

Using in vivo RNA interference, Young et al. implicated the p53 target gene p21 as an important factor in STS development [280]. The expression of p21 was positively correlated with tumor malignancy grade and therefore used as prognostic markers in a series of 152 patients with STSs [90]. In patients with Ewing’s sarcoma the expression of p21 (P = 0.015) was higher in disseminated as opposed to localized disease tumors, but p21 was not correlated with progression-free or overall survival [170]. In a series of 36 patients with leiomyosarcoma p21 was not correlated with time to recurrence or overall survival [281]. In a series of 169 primary soft tissue sarcomas of the extremities and the trunk wall, expression of p21 was not associated with prognosis [261]. Similarly, in our material p21 was not correlated with malignancy grade or DSS. This can be due to other bypass molecules involved in p53 suppression functions.

5.2.3.4. p62

There are few publications regarding p62 and STSs. Rolland et al. demonstrated that high expression of p63 in breast cancer is associated with tumor progression, but not DSS [282]. In a series of 109 NSCLC, high expression of p62 was correlated with poor survival [283]. Kitamura et al. demonstrated cytosolic overexpression of p62 in prostate adenocarcinoma and high-grade PIN, suggesting that p62 might be a useful marker for prostatic malignancy [284]. In a series of 59 colorectal carcinomas, however, p62 had no prognostic value [285]. High expression of p62 in our material was positively associated with high malignancy grade, but not DSS.
5.2.3.5. **Skp2**

High expression of Skp2 is negatively associated with overall survival in patients with myxofibrosarcoma [287, 288]. Di Vizio et al. [289] found that a high expression of Skp2 is negatively correlated with GIST survival. Oliveira found that a high expression of Skp2 is associated with high cell proliferation and poor prognosis in 182 STSs [290]. High expression of Skp2 in our material was a negative prognostic factor for DSS. Interestingly, this correlation was statistically significant in women only (P = 0.009) (men, P = 0.577). This may be related to differences in expression of sexual hormone receptors (ER and PGR) in male and female STS patients [291, 292]. An inverse correlation between Skp2 expression and the expression of ER and PGR has been reported by others investigating breast cancer [293]. Other studies suggest that Skp2B may modulate the activity of the estrogen receptor [294, 295]. High expression of Skp2 in breast cancer is correlated with p-Akt1 and associated with poor survival [296].

5.2.4. **Paper IV**

The purpose of this study was to clarify the prognostic significance of Skp2 expression in relation to gender, estrogen receptor (ER), and progesterone receptor (PGR) in STSs. We found diverse prognostic impacts by expression of Skp2, ER, and PGR on DSS in male and female patients with STSs. In men, but not women, an ER positive/PGR negative co-expression profile was an independent, negative prognostic factor for DSS. In women, but not men, Skp2 expression was associated with poor DSS.

Steroid hormones, and therefore their receptors too, are known to stimulate the progression of breast cancer as well as other gynecological tumors. ER served for decades as a predictor of the success of hormone-ablation therapy for ER-positive in contrast to ER-negative breast cancers [174, 175]. A diversity of soft tissue tumors expresses both ER and PGR [176, 301–303], but there is much uncertainty concerning the steroid hormone receptor expression value in the mesenchymal tumors. This is probably due to vagueness of the positivity cut-off point for non-gynecological tumors, which is as high as 10% in most of studies. We have modified the Allred score [304] for STSs and used 1% positivity as the cut-off value. The strong and moderate (score 3 and 2, respectively) hormone receptor expression occurred mostly in
uterus, pelvic, and breast sarcomas, while the weak (score 1) expression of both ER and PGR was surprisingly evenly distributed across location, gender, and age. Generally, 36% of the tumors expressed ER and 30% expressed PGR in our material.

The rate of ER and PGR expression in leiomyomatous tumors of the uterus was frequently demonstrated to rise with the grade of differentiation of malignant tumors from benign leiomyoma to high-grade malignant leiomyosarcoma [176, 177]. However, the information concerning steroid hormone receptor expression in soft tissue tumors outside the gynecological area is scarce and controversial. In our study ER expression (using a positivity threshold at 1%) had a positive impact on survival in women (univariate analysis) but failed to show any significant value in the Cox proportional-hazards analysis. PGR expression showed a clearly negative impact on DSS in men and slightly positive, but not significant influence on survival in women.

The value of ER/PGR co-expression profiles is well studied in breast carcinoma. In few words, any hormone receptor positivity gives a better prognosis for success of antihormonal therapy [305, 306]. In our study the ER-/PGR+ profile (14% of the tumors) was a significantly unfavorable factor for the whole patient cohort both in univariate and in multivariate analyses.

This study is, to our knowledge, the first to elucidate the distribution and prognostic value of steroid hormone receptors in STSs. Both ER and PGR were surprisingly frequently expressed in sarcomas irrelatively to the patient’s gender and location of the tumor. Their prognostic significance is not much of a surprise, since both of them in essence are growth factors.

We found diverse prognostic DSS impacts from gender-related expression of Skp2, ER, PGR, and DSS in STSs. In men, but not women, an ER positive/PGR negative co-expression profile was an independent, negative prognostic factor for DSS. In women, but not men, high expression of Skp2 was associated with reduced DSS. High expression of ER reduced the negative impact of Skp2 in women. While women with the Skp2+/ER+ phenotype had improved survival, the Skp2+/ER- had poor survival. To the best of our knowledge, this is the first prognostic evaluation of Skp2 related to the female hormone receptors ER and PGR in STSs.
6. CONCLUSIONS AND IMPLICATIONS FOR FURTHER RESEARCH

We have investigated markers of the adaptive and the innate immune system and cell cycle regulatory proteins in STS patients. Several markers and interesting co-expressions proved to be independent prognostic factors. Although the precise molecular interactions in STSs are still unclear, our findings may help to identify a subgroup of patients with aggressive tumors that require adjuvant therapy. Moreover, the biomarkers indicating such aggressiveness can represent molecular targets with the future development of small-molecule targeted therapy.

Adjuvant chemotherapy for patients with STSs remains controversial, while improvement in survival has never been conclusively demonstrated for metastatic STSs. In a series of 2,382 patients with resected STS, 106 (4.5%) received chemotherapy. High tumor grade, larger tumor size, and malignant fibrous histiocytoma subtype were associated with chemotherapy receipt [307]. In our material ER and PGR positivity, found to be surprisingly common in STSs, could possibly identify patients who may benefit from endocrine therapy. Among STS patients who have had wide resection margins, it will be essential to identify those who will relapse and succumb to this disease, as these patients may benefit from adjuvant therapy, including immunotherapy. Patients with the ER negative/PGR positive phenotype have especially poor DSS, while men with the ER negative/PGR negative phenotype have better DSS. Women with the ER positive/PGR positive phenotype also have favorable prognosis.

In our material Skp2 was an independent, negative prognostic factor for DSS in women and in patients without administered chemotherapy or radiotherapy. Further studies are warranted to explore if adjuvant chemotherapy or radiotherapy improve the poor prognosis of STSs with high Skp2 expression.

The human immune system contains specialized cells that are able to eliminate cancer cells [110], and tumor-infiltrating B-cells are able to produce tumor-specific antibodies [308]. Through external stimulation of the immune response, these cells may have the potential to aid the immune system in destroying single tumor cells and micro-metastases after surgery. This topic is investigated in the ongoing international osteosarcoma protocol EURAMOS, where those who respond well to chemotherapy are randomized to receive interferon or no interferon, in an attempt to improve the immune response.
TMA and IHC have proven to be reliable and feasible methods for biomarker studies on tissues. While these methods might not be the most novel, they are well proven and highly reliable when one takes into account their limitations. Our group will continue to conduct TMA and IHC studies on STSs. We would particularly like to explore factors responsible for TGF-beta modulation, such as matrix metalloproteinases, integrins, angiogenic and inflammatory agents, as well as the isoforms and specific receptor of this enigmatic growth factor. This also concerns ER and PGR isotypes.

In addition, we have started to measure proliferation-related micro-RNAs by in situ hybridization in paraffinized tissue from STS patients. We hope to further clarify prognostic factors in STS patients and to explore the impact of immune system, cycle regulatory proteins, and other prognostic markers in this patient group.
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74
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85


Paper 1
Paper 2
Paper 3