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Chapter 1

General introduction
Classification of benign and malignant soft tissue tumours

Soft tissue sarcomas are rare malignant tumours originating from the mesenchymal or neuroectodermal tissues, comprising less than 1% of all cancers. The annual incidence of soft tissue sarcomas is around 5 (4.74) per 100,000 with a 5-year survival rate of around 49% (1, 2). Nevertheless, there are more than 50 different histological subtypes described, which differ in biology and are characterized by a unique clinical behaviour and prognosis and therefore require specialized treatment (3). This emphasizes the pivotal importance of a correct diagnosis, which can be challenging for pathologists as these tumours are rare and due to the numerous different histological subtypes of which some have overlapping features. Uniform classification of bone and soft tissue sarcomas is performed on the international consensus guidelines of the World Health Organisation (WHO)(3). The biological potential of tumours varies and four main groups can be discerned: benign, locally aggressive, rarely metastasizing and malignant.

Soft tissue sarcomas can be grouped based on their line of differentiation, thus, based on the normal tissue that the tumour most closely resembles, including for example adipocytes, smooth muscle cells, striated muscle cells, fibroblasts, histiocytes, pericytes, endothelial cells, perineurial-like cells and Schwann cells. Some soft tissue tumours are composed of a combination of two or more cell types and there is also a group of tumours of uncertain differentiation. Mesenchymal tumours can occur anywhere in the body, although the majority is located in the extremities (up to 75%), the trunk wall (around 10%) and the retroperitoneum (10%). Some subtypes even have a strong predilection site, for instance, two-third of the myxoid liposarcomas develop in the thigh. Moreover, the age of patients is important to consider as some sarcomas have an age-related incidence, for example, embryonal rhabdomyosarcoma is encountered almost exclusively in young children while alveolar soft part sarcoma is most frequently detected in young adults and soft tissue leiomyosarcoma and undifferentiated pleomorphic sarcoma occur mainly in elderly patients (3). The overall male to female ratio is 1.1/1 for soft tissue sarcomas. In the subgroup of visceral sarcomas, including gastrointestinal stromal tumour (GIST), a female preponderance is observed (1.4/1) (4).

On the other end of the spectrum of malignant soft tissue tumours, are the benign mesenchymal lesions. This group of neoplasms is also
heterogeneous and consists of multiple different histological subtypes. The incidence of some of these lesions is relatively high, and is estimated to outnumber sarcomas by a factor of minimal 100. A well-known example of a benign soft tissue tumour is a conventional lipoma, responsible for around 30% of all benign soft tissue tumours (3, 5). Conventional lipomas are most frequently located subcutaneously or in the deep soft tissues on the trunk or extremities. Generally they are maximum a few centimetres in diameter, macroscopically well circumscribed and histologically composed of lobules of mature adipocytes without cytonuclear atypia (3). Other common benign neoplasms are for example leiomyomas, which will be discussed in more detail below, nodular fasciitis and schwannoma (3).

**Histological classification and grading of soft tissue neoplasia**

As mentioned before, soft tissue sarcomas, comprises a rare group of malignancies and accurate diagnosis is challenging for pathologist who are not familiar with the broad range of histological subtypes, therefore the risk of initial diagnostic error ranges from 8-25% (3, 4, 6). This has been investigated by performing a second opinion on diagnosed cases of soft tissue sarcomas by expert pathologists. In the majority of the cases there is either a full agreement on the diagnosis or a partial agreement, situation in which both pathologists diagnosed a sarcoma but with different histopathological grade, between the first and expert pathologist. Other examples of major discrepancies are cases in which benign lesions are mistaken for a sarcoma (10%) or the other way round, a sarcoma is mistaken for a benign neoplasm (4%)(4). Expert pathologists have extensive experience with these rare tumours and knowledge of and access to the latest molecular diagnostic tools like immunohistochemistry, FISH and (Next Generation) Sequencing. In a large series <10% of initial diagnoses were considered major misdiagnoses which were related to histological type or tumour grade (6). The correct diagnosis is crucial for determination of the right treatment, especially for tumours with novel targeted treatments, as this influences patient outcome. Therefore referral to a specialized sarcoma center is often advised for diagnostics and to make appropriate decisions on treatment. These multidisciplinary teams often include surgeons, pathologists,
radiologists, medical oncologists, radiation therapists and when applicable organ-based specialists.

The most widely used grading system of sarcomas and also advised by the WHO is the system of the Fédération Nationale des Centres de Lutte Contre le Cancer (FNCLCC) (3, 7). This system is reproducible and the grade is assessed based on three independent histological parameters: tumour differentiation, mitotic activity and necrosis. Tumour differentiation indicates how closely the tumour resembles normal mesenchymal tissue. Poorly differentiated sarcomas, like for example rhabdomyosarcoma, synovial sarcoma and clear cell sarcoma, are scored as undifferentiated per definition (3, 7).

Clinical features and diagnosis

Most soft tissue sarcomas present as slowly growing, painless masses that are detected incidentally and do not, at first instance, interfere with the daily function or general health. Especially at certain locations, as for example the retroperitoneum, tumours can become quite large before they are detected. Due to this seemingly innocent behaviour tumours are often in the beginning characterized as benign lesions. Patients with a superficial lesion of >5 cm in diameter or with a deep soft tissue mass should preferably be directed to a referral center with a multidisciplinary team for diagnosis and treatment (2, 3).

Imaging of soft tissue neoplasia is important as it will provide key information on size of the tumour and relation with other structures, and specialized radiologists might give a good differential diagnosis on the images. Conventional X-ray is often performed in the first place and although it is not detailed it might be helpful in ruling out a primary bone tumour extending in the surrounding soft tissues. Also calcifications in a soft tissue mass, as seen in myositis ossificans, will be detected. Magnetic resonance imaging (MRI) is able to give detailed information on tumour size, tumour components like fat, necrosis, cystic degeneration, haemorrhage and oedema, and relation with surrounding structures, including for example bone and neurovascular bundles. The use of T1-weighted and T2-weighted images might help to discriminate between tumours with a high fat or blood content (high signal on T1W) and fibrous tumours (high signal on T2W). CT-scans are in some instances performed to detect deposition of calcifications.
and baseline chest CT might be performed for accurate staging at time of diagnosis (2, 3).

Following adequate imaging, the next step in the work-up is taking of multiple core needle biopsies of the soft tissue mass. In case of a deep seated lesion this might be done with image-guidance. The biopsy location must be chosen in deliberation with the surgeon in such a way that complete resection of the biopsy tract can be achieved during definitive surgery of the tumour (2, 3).

**Genetic classification of mesenchymal tumours**

Next to the histopathological classification, soft tissue neoplasms can be divided based on the molecular biology of the tumours. From the molecular genetic perspective, tumours are classified in accordance to their genomic profile. Development of cytogenetic and molecular techniques have resulted in the discovery of recurrent genetic aberrations in a large subset of soft tissue tumours. The identification of specific genetic alterations have led to an increased understanding of the biological mechanisms and cellular pathways that have a role in the tumorigenesis.

From a genetic point of view soft tissue tumours can be classified in three main categories: tumours with a specific, reciprocal translocation and often a near-diploid karyotype, tumours with specific mutations (activating or inactivating mutation or gene amplification) in a variable genomic background and tumours with an instable genome and as a consequence a complex karyotype (Table 1) (3, 8). Of the 117 soft tissue tumours listed in the WHO Classification of Tumours of Soft Tissue and Bone of 2013, approximately 45% harbour a recurrent cytogenetic or molecular aberration (3, 9). However, it is not inconceivable that this number will increase in the future with the ongoing development of molecular genetic techniques.

The overall fraction of soft tissue tumours with a reciprocal translocation is estimated at ~20% (3, 9, 10). The occurrence of a chromosomal translocation is considered a crucial genomic event for a cell and often plays an important role in tumorigenesis. A chromosome translocation is formed in a complex one-step event and is influenced by several factors, including for example the occurrence of double strand breaks (DSB), the concentration of repair proteins around the broken chromosome ends and the three-dimensional location of
chromosomes with DSB in the interphase nucleus (11, 12). Dependent on the exact location of the breakpoints and the resulting composition of the fusion product, translocations can have several consequences, it can form a chimeric fusion protein in case the coding sequence of two genes are fused, it can disrupt a tumour suppressor gene or it can place a tumour-promoting gene behind the promoter of a transcriptional active gene (promotor swapping). In all these three cases the cell can experience a proliferative advantage and this can subsequently lead to tumorigenesis (11, 13). An example of a soft tissue sarcoma with a chimeric fusion gene is myxoid liposarcoma characterized by an $FUS-DDIT3$ or $EWSR1-DDIT3$ translocation; which was first described at cytogenetic level in 1986 and the involved genes were identified more than six years later (3, 14, 15). Translocations and point mutations are not exclusively found in malignant tumours as also multiple benign neoplasm harbour recurrent cytogenetic aberrations (16). The translocations found in aneurysmal bone cyst (ABC), a locally aggressive neoplasm, are caused by juxtaposition of the $USP6$ gene to any of the highly transcriptional active promotors of the $CDH11$, $COL1A1$, $OMD$, $TRAP150$ or $ZNF9$ genes (12, 17). A subset of lipomas show rearrangements at 12q14.3, leading to deregulation of the high-mobility group AT-hook 2 ($HMGA2$) gene and several partner genes of $HMGA2$ have been identified (3).

The second category consist of tumours with specific in- or activating mutations or gene amplifications which often lead to alterations in important signalling pathways. A well-known example is gastrointestinal stromal tumour (GIST) characterized by oncogenic, activating mutations in $KIT$, $PDGFRA$ or $BRAF$, resulting in a continuous signalling of the KIT pathway (8). These tumours might harbour a variation in secondary genetic changes and in some cases DNA copy number changes seem to be related to the occurrence of metastasis (18).

The last group comprise tumours with an instable genome and as a consequence a complex karyotype, these tumours often contain multiple non-recurrent numerical and structural chromosome alterations (3, 8). These alterations can accumulate gradually over time during cancer development, although complex rearrangements can also develop in a single catastrophic event of reshuffling of chromosomes, known as chromothripsis (19). Leiomyosarcoma, pleomorphic liposarcoma and undifferentiated pleomorphic sarcoma (UPS) are examples of karyotypically complex sarcomas (3).
The discovery of specific, recurrent translocations or mutations in certain soft tissue neoplasms are unique features that discriminates them from other lesions and have contributed significantly to the classification of mesenchymal neoplasms. The presence of a pathognomonic molecular genetic alteration has also proven to be of tremendous value in the diagnostic process (9). Molecular genetic techniques provide a helpful diagnostic tool, especially in discriminating tumours with similar histology, but different biological behaviour or clinical outcome (9, 10). Due to the ongoing development of genetic profiling techniques, for example the broad spectrum of sequencing options, including RNA, whole genome and transcriptome sequencing, tumours that are now classified under the same diagnosis, might in the future be split in different molecular subtypes. The seemingly ‘ever increasing’ number of molecular subtypes will inevitably lead to a discussion between the ‘splitters’ and the ‘lumpers. It is important to focus on those molecular subtypes that differ in clinical prognosis or that have alterations in molecular pathways which might be candidates for targeted therapies.

Table 1. The molecular genetic subtypes of soft tissue tumours.

<table>
<thead>
<tr>
<th>Genetic profile</th>
<th>Example</th>
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<tbody>
<tr>
<td>Specific translocation and relatively simple karyotype</td>
<td>Myxoid liposarcoma: t(12;16)(q13;p11) or t(12;22)(q13;q12)</td>
</tr>
<tr>
<td>Synovial sarcoma: t(X;18)(p11;q11)</td>
<td></td>
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<tr>
<td>Specific mutation in variable genomic background</td>
<td>Gastrointestinal stromal tumour: KIT, PDGFRA, BRAF</td>
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<tr>
<td>Dedifferentiated liposarcoma: amplification MDM2, CDK4</td>
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</tr>
<tr>
<td>Complex karyotype without recurrent aberration</td>
<td>Leiomyosarcoma of soft tissue</td>
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<td></td>
<td>Myxofibrosarcoma</td>
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Therapeutic strategies in soft tissue sarcomas

The cornerstone of the treatment of soft tissue sarcomas is surgery with a wide tumour resection with negative margins, which implies that the tumour needs to be surrounded by a rim of normal tissue or the tumour may not cross anatomical borders like muscular fasciae or epineurium. Radiation and chemotherapy, neo-adjuvant or adjuvant, are frequently part of the treatment regimen and the decision of these therapies are made in a specialised multidisciplinary team by taking into account several variables, including: specific sarcoma subtype, chemosensitivity of histological subtype, resection margins (if
possible to operate at all), tumour location, tumour size, tumour grade, stage of the disease (especially localized versus advanced and/or metastatic disease), patient’s general condition, relevant comorbidities, previous chemotherapy and treatment goal (2, 20-22). The first-line chemotherapy of choice is the anthracycline doxorubicin, often used as monotherapy but individual cases might benefit from the combination with ifosfamide (2, 23). Over the past years several trials have compared these two chemotherapeutic regimens in soft tissue sarcomas and there is no evidence that multi-agent chemotherapy leads to a statistical significant longer overall survival compared to doxorubicin alone, although in some studies a higher regression rate was observed (23, 24).

The specific alterations that have been identified in the genomes of sarcomas over the past years, can be used to develop molecular targeted therapy. In particular tumours with a pathognomonic genetic aberration are good candidates for targeted therapy. The most well-known example is GIST, which can be targeted by the KIT kinase inhibitor imatinib, which gives a partial response or stable disease in around 77% of patients with advanced or metastatic GIST (25). Other targeted (tyrosine kinase) inhibitors are being investigated in several sarcoma types. A thorough understanding of the different mechanisms of pathogenesis in soft tissue tumours is of pivotal importance in the search for novel therapeutic targets. Most translocation-associated sarcomas are characterized by gene-fusions which act as aberrant transcription factors, thereby influencing the expression of a multitude of genes promoting oncogenesis. In this way the chimeric transcription factor dysregulates downstream signalling of several cellular pathways. As a result, specific targeting of only one affected signalling pathway might have a limited effect in individual cases. Secondary changes occurring during tumour progression might serve as a potential target as well. For instance, ~18% of myxoid liposarcomas, characterized by FUS-DDIT3 fusion products, harbour additional PIK3CA mutations resulting in altered signalling of the mammalian target of rapamycin (mTOR) pathway. However, targeting the mTOR pathway alone might not be sufficient in myxoid liposarcoma, since the tumour cells are likely to show disturbances in other pathways as well due to altered signalling caused by the aberrant transcription factor (26). Occasionally, a translocation may lead to receptor or ligand activation leading to aberrant receptor tyrosine kinase signalling that more often have specific, inhibitory small molecules. An example of this type of translocation-associated sarcomas is
dermatofibrosarcoma protuberans. This locally aggressive neoplasm harbours a \textit{COL1A1-PDGFB} chimeric fusion gene and the fusion product is processed to the normal PDGFB ligand (3, 27). Tumour cells carry the membrane kinase receptor, resulting in autocrine signalling and stimulating tumorigenesis. This autocrine growth signalling loop can be disrupted by the PDGFR inhibitor imatinib.

Recently, the field of cancer immunology has become more important and several immunotherapeutic approaches, like cytokine-based immunotherapy, immune checkpoint inhibitors, tumour vaccines and adoptive T-cell therapy, are under investigation (8, 28). The family of cancer-testes antigens constitute more than 200 proteins which are by definition expressed in the primitive spermatogonium of the testis, but not in other adult tissues (29). They have immunogenic properties, but as they are separated and protected by the blood-testis barrier, no natural immune response is provoked. There are different subgroups and some are more immunogenic than others. Well-known examples of cancer-testes antigens are MAGE, PRAME, GAGE and SSX2. New York oesophageal squamous cell carcinoma (NY-ESO-1) or CTAG1B belongs to the CTA family and was first detected in an oesophageal carcinoma. It is also highly expressed in the majority of myxoid liposarcomas and synovial sarcomas (30-32). Therefore it is regarded as an attractive target for immunotherapy. NY-ESO-1 can be targeted by dendritic vaccine based strategies resulting in CD8$^+$ and CD4$^+$ T-cell responses. Since the direct cytotoxic effect of CD8$^+$ T-cells against tumour cells is the most important, another option is adoptive cell therapy, involving the transfer of autologous cytotoxic T-cells or natural killer (NK-)cells which are genetically engineered to express T-cell receptors directed against NY-ESO-1 (28).

**Smooth muscle tumours**

**Leiomyomas**
Benign smooth muscle tumours are quite frequently encountered in the smooth muscle tissue of the uterus. Uterine fibroids have an estimated incidence of $\sim$70% of women in reproductive age and often more than one lesion is present (33, 34). The tumours are usually well-circumscribed and have a slight varying cell density of a population of uniform long spindle cells with
eosinophilic cytoplasm and elongated blunt-ended nuclei without substantial cytonuclear atypia.

Genetically, uterine leiomyomas can be classified in at least four different groups. The majority, around 70%, of the tumours harbour a mutation in mediator complex subunit 12 (MED12), located on chromosome Xq13.1 (33). Germline mutations in this gene were first described in patients with FG syndrome (Opitz-Kaveggia syndrome)(35). MED12 is a member of the mediator complex which is involved in regulation of transcription of RNA polymerase II-dependent genes. The molecular pathogenesis responsible for tumorigenesis in MED12 mutated fibroids is still not completely elucidated, but it has been postulated that interaction with β-catenin and increased canonical WNT signalling; and loss of repressor element 1 silencing transcription factor (REST) and increased signalling of PI3K/AKT/mTOR might have an important role (34, 36). Nuclear β-catenin is also known to directly interact with MED12, resulting in transcription of target genes of the canonical Wnt-signalling pathway. Markowski et al found high expression of wingless-type MMTV integration site family, member 4 (WNT4), also a member of the Wnt pathway, of which expression is regulated by estrogen in Müllerian mesenchymal tissues (37). Multiple chromosomal alterations have been described in leiomyomas, and the most frequent recurrent translocation is the t(12;14)(q15;q24), fusing high-mobility group AT-hook 2 (HMGA2) gene to the RAD51L1 gene (38). This is the second most common genetic aberration found in uterine leiomyomas. A small percentage of leiomyomas harbour a fumarate hydratase (FH) mutation or deletions affecting collagen, type IV, alpha 5 (COL4A5) and collagen, type IV, alpha 6 (COL4A6) (39). The HMGA2, FH, COL4A5-COL4A6 and MED12 alterations are mutually exclusive, while some other genetic aberrations including HMGAI alterations and 7q deletions can co-occur with MED12 mutations (37, 40, 41). An interesting observation is the occurrence of complex chromosomal rearrangements in some MED12 mutated and MED12 wild-type leiomyomas (40). The complex chromosomal rearrangements fulfil to the criteria of chromothripsis, which was first thought to be exclusively associated with malignant tumours, but is also encountered in genomes of some benign lesions and congenital disorders (42, 43).

Less frequently leiomyomas appear in the cutis, named cutaneous or piloleiomyomas, and these can occur either sporadic or in association with an inherited disorder. The most well-known is Reed's syndrome or hereditary
leiomyomatosis and renal cell cancer (HLRCC), an autosomal dominant disease, in which patients are predisposed to develop cutaneous and uterine leiomyomas and papillary renal cell cancer. HLRCC is caused by a heterozygotic germline mutation in the gene encoding the Krebs cycle enzyme fumarate hydratase (\(FH\)), located at 1q43 (44, 45). The accumulation of high levels of fumarate leads to aberrant succination of proteins by forming a chemical modification of cysteine residues to S-(2-succino)-cysteine (2SC) (46). In addition to HLRCC cutaneous leiomyomatosis has been associated with several other syndromes and conditions (47).

**Leiomyosarcomas**

Leiomyosarcomas are malignant tumours with features of smooth muscle differentiation and responsible for around 10 to 20% of all soft tissue sarcomas (3, 48, 49). Tumours are most frequently encountered in the 5th to 6th decade and are slightly more common in women compared to men. Based on the anatomic site the tumours can be classified in five major groups: leiomyosarcomas originating in the deep soft tissues, retroperitoneum, uterus, blood vessels and superficial dermis (3). Tumours confined to the dermis have an indolent clinical course and rarely metastasize, and are therefore better designated “atypical intradermal smooth muscle neoplasm” (50, 51).

Uterine leiomyosarcomas are considered as a separate entity, as they have a Müllerian origin and often express estrogen receptor (ER), progesterone receptor (PR) or Wilms tumour protein (WT1), supporting derivation of the genitourinary tract (52, 53). These tumours are not graded according to the FNCLCC, instead the Federation of Gynaecology and Obstetrics (FIGO) staging system is applied. The prognosis of uterine leiomyosarcoma is often poor and depends on the tumour stage. The 5-year overall survival (OS) rate is 40% (54).

Leiomyosarcomas of the deep soft tissues and the retroperitoneum are often large (>10 cm) at the moment of presentation (55). Histologically lesions are characterized by a population of long spindle cells with eosinophilic, fibrillar cytoplasm, arranged in intersecting bundles (Figure 1A and B). The cells contain cigar-shaped nuclei with often remarkable pleomorphism and hyperchromasia. Bizarre tumour giant cells, a high mitotic rate and necrosis can be present. Tumours are graded according to the FNCLCC system. Immunohistochemically, tumours frequently express smooth muscle actin (SMA), actin (HHF35) and vimentin. In addition, for the diagnosis of leiomyosarcoma focal expression of...
at least one of the two smooth muscle markers, desmin and heavy-caldesmon, is more supportive for the diagnosis (3). Tumour depth, tumour size (>5cm), high histological grade, age >60 years and metastasis at presentation are indicators of a poor prognosis (48, 55, 56). The overall disease-specific 5-year survival rate is around 54-64% (48, 55).

As mentioned above, leiomyosarcomas pertain to the sarcomas with an instable genome and complex karyotype (Figure 1C). Although no common cytogenetic aberrations have been identified in these tumours, over the past decades several genes were found to be mutated. Mono-allelic or bi-allelic TP53 alterations are found in up to 50% of leiomyosarcomas and p53 pathway inactivation is encountered in a minority of leiomyosarcomas by p14 loss (57, 58). Also loss of the tumour suppressor genes PTEN and RB1 is frequently found (59, 60). Myocardin (MYOCD), a transcriptional cofactor regulating smooth muscle differentiation, is amplified in a subset of soft tissue and retroperitoneal tumours (61). ATRX mutations, ROR2 overexpression and MYC overexpression are correlated with poor clinical outcome (58, 62, 63). Deregulation of the apoptosis pathway can occur under influence of MYC which affects death-associated protein (DAP) kinase or via high expression of anti-apoptotic proteins of the Bcl family (64).

More recently, molecular profiling including gene expression profiling and array comparative genomic hybridization (array-CGH) identified three molecular subtypes of leiomyosarcoma (49). One subtype demonstrated high expression of multiple muscle-associated genes and is regarded as the ‘muscle-enriched’ group. This subtype can be identified immunohistochemically with the smooth muscle marker leiomodin 1 (LMOD1) and is associated with an improved disease specific survival (49, 65). High expression of ARL4C, a member of the GTP-binding proteins, can distinguish the leiomyosarcomas of the second molecular genetic subtype, which are associated with a worse disease specific survival (65). The third group had an intermediate outcome and until now no specific marker is discovered to differentiate this group (65).

Leiomyosarcomas are treated with the conventional chemotherapeutics used for most soft tissue sarcomas. Single-agent doxorubicin is the treatment of choice in soft tissue sarcomas and although the combination of doxorubicin with ifosfamide or with dacarbazine has been suggested, there is no randomized trial that proves the effect of these drug combinations in leiomyosarcoma. Docetaxel plus gemcitabine is active in uterine and soft
tissue leiomyosarcomas (66). Trabectedin alone has been used as second-line treatment. The combination of doxorubicin with trabectedin is being investigated, but has not been approved for first-line treatment yet (67, 68).

As the driver mechanism responsible for leiomyosarcoma genesis is poorly understood and only a limited number of functional studies have been performed, at present, no targeted therapy is present for leiomyosarcomas.

Figure 1. Histology and molecular cytogenetic representation of leiomyosarcoma.
(A) H&E stained section of a soft tissue leiomyosarcoma, grade II, with characteristic elongated spindle cells with fibrillar and eosinophilic cytoplasm arranged in bundles. A moderate variation in size and chromasia is observed and several mitotic figures are present. Inset shows desmin positivity in the majority of the tumour cells. (B) Grade III leiomyosarcoma of soft tissue with increased cytonuclear pleomorphism and atypical mitotic figures (inset). (C) Hypotetraploid karyotype of leiomyosarcoma with multiple translocations.
**Liposarcomas**

**Different subtypes of liposarcomas**

Liposarcomas are malignant mesenchymal neoplasms characterized by a varying degree of lipogenic differentiation and can be classified into three subtypes, each with a distinctive histopathology, genetic profile and clinical behaviour (3). The most common subtype is the well-differentiated/dedifferentiated liposarcoma, consisting of areas resembling well-differentiated fat with an often abrupt transition to high-grade, non-lipogenic areas. This subtype is characterized by amplification of various segments of 12q most consistently involving the amplification of *MDM2* and *CDK4* genes, located herein (69). Pleomorphic liposarcoma is rare and resembles histologically an undifferentiated high grade sarcoma with varying amounts of lipoblasts. Genetically this tumour has a complex karyotype without recurrent aberrations (3, 69). Myxoid liposarcoma is responsible for around 20-30% of the liposarcomas and 5% of all soft tissue sarcomas (3).

**Myxoid liposarcomas**

Myxoid liposarcomas most commonly arise in the deep soft tissues of the thigh (two-third of the cases). The peak age is between the 3rd and 5th decade and no gender predilection is observed (3). Tumours are histologically composed of a variably cellular proliferation of stellate spindle cells with some signet-ring lipoblasts intermingled and embedded in a myxoid matrix with a characteristic plexiform, “crow’s feet”, vascular network (Figure 2A and B). Areas of undifferentiated small blue round cells with scarce cytoplasm and no matrix might be present and if they compose more than 5% of the total tumour volume, the tumor is considered high grade (round cell variant) and is associated with a higher metastatic potential and a worse prognosis (70-75). The presence of necrosis, male gender, increasing age and multifocal disease are other poor prognostic factors (76-78).

The cytogenetic hallmark of myxoid liposarcoma is the reciprocal translocation t(12;16)(q13;p11) which generates a fusion transcription factor oncogene by fusing *FUS* (fused in sarcoma; also known as *TLS*) on 16p11 with *DDIT3* (DNA-damage-inducible transcript 3; also known as *CHOP*) on 12q13. To date, twelve different fusion transcripts have been described (Figure 2C)(79, 80). In less than 5% of the cases a t(12;22)(q13;q12) is identified resulting in an
**EWSR1-DDIT3** fusion, of which 4 different transcripts are known (81). The fusion type in myxoid liposarcoma does not impact clinical outcome, which is similar to the translocation-associated sarcomas Ewing and synovial sarcoma (77, 81-83).

The exact mechanism via which the chimeric fusion product exerts its oncogenic potential remains to be elucidated, however it is postulated that it functions as an aberrant transcription factor by stimulating proliferation while inhibiting adipogenic differentiation (84, 85). The fusion product interferes with heterodimerization of DDIT3 with CCAAT/enhancer-binding protein-β (C/EBPβ). FUS-DDIT3 represses the promotors of C/EBPa and PPARγ, resulting in inhibition of the adipocyte differentiation program in mesenchymal cell progenitors (85). Investigation of C/EBPa and PPARγ at mRNA and protein level revealed under- and overexpression, respectively in myxoid liposarcoma tumour samples, which might be explained by the fact that both factors exert their effect at another stage of the differentiation process. Additionally, the fusion protein stimulates expression of elf4E, which is able to downregulate the afore mentioned C/EBPa pathway and further inhibits adipogenic differentiation (85). In line with these data, the mRNA expression profile of myxoid liposarcoma is consistent with an immature adipogenic status (86). The fusion product also interacts with NF-kappa-B inhibitor zeta (NFKBIZ), which could contribute to oncogenesis through deregulation of nuclear factor-kB-controlled genes (87, 88). The expression of the **FUS-DDIT3** oncogene is tightly regulated at both the mRNA and protein levels. Also the level of the aberrant transcription factor has shown to differ between individual tumour cells. This once more illustrates that tumorigenesis is a complex process and not all regulatory mechanisms and role of downstream targets are understood yet (89).

MicroRNAs (miRNAs) are small, non-coding RNAs that are able to negatively regulate expression of target genes and they are associated with various cellular processes as differentiation and proliferation. In myxoid liposarcoma the FUS-DDIT3 fusion product is found to suppress miR-486 resulting in increase of cell growth (90). The miR-135b is higher expressed in the round cell component and associated with worse prognosis (91, 92).
Figure 2. Histology of myxoid liposarcoma. (A) Low grade, hypocellular area of stellate spindle cells set in a myxoid background. The fine branching vascular network (crow’s feet) is visible. (B) Moderate cell rich area with characteristic pools of myxoid matrix. (C) High grade area with closely packed round cells. (D) Partial karyotype of myxoid liposarcoma with t(12;16).

Biomarker analysis of myxoid liposarcoma specimens has identified alterations in the IGF/Akt/mTOR axis, involved in crucial cellular processes such as cell survival, proliferation and growth. Overexpression of the receptor tyrosine kinases RET and IGF1R, and the ligand IGF1, are negative prognostic biomarkers known to stimulate the PI3K/Akt pathway (86, 93). PIK3CA, the catalytic subunit of phosphatidylinositol 3-kinase (PI3K), harbours activating mutations in 14-18% of the tumours. Loss of PTEN expression, a negative regulator of PI3K, is found in 12% of the cases and is mutually exclusive from PIK3CA mutations (94, 95). Increased PI3K/Akt signalling has been demonstrated by high expression of downstream targets like phosphorylated 4EBP1, PRAS40 and S6. The PIK3CA mutation rate, IGF1R expression and loss of PTEN were higher in tumors with a round cell component suggesting that this pathway might be involved in round cell transformation and tumor progression (86, 93). Also TP53 mutations and reduced protein expression of p16INK/p14ARF have been identified in a subset of the tumors, especially in round cell areas (93). Hotspot mutations in the TERT (telomerase reverse transcriptase) promoter region were recently reported to be recurrent in myxoid liposarcoma, as well as
in several other solid tumours like for example glioblastomas, melanomas and urothelial carcinomas (96-99). These mutations lead to increased TERT mRNA and protein expression and have been implicated in telomerase dysregulation and the resultant proliferative capability of tumor cells (100). In summary, most of the above listed biomarkers are progression markers rather than myxoid liposarcoma specific markers, again highlighting the need for further research to identify tumour specific, targetable, alterations.

Surgery or a combination of surgery with radiotherapy is an effective treatment for localized disease, however 25-50% of patients develop recurrence or metastases (73, 74, 78, 101). The metastatic pattern is unconventional with spread to sites in soft tissue or bone (retroperitoneum, abdominal wall, thoracic wall, spine) occurring before the tumour metastasizes to the lungs (3, 73, 101). The 5-year disease specific survival rate depends on the histology and is for purely myxoid tumours higher than for tumours with a round cell component (around 91-100% versus 73-79%) (70, 71, 73, 74, 77, 78, 101, 102). Cases with advanced disease at time of diagnosis have a worse disease specific survival of 8% (75).

Compared to other soft tissue sarcomas, myxoid liposarcoma is quite sensitive to radiotherapy and chemotherapy. In the search for new therapeutic agents the pure form of the natural tetrahydroisoquinolone alkaloid was isolated from the Caribbean tunicate *Ecteinascidia turbinata* in 1986. This substance, known as trabectedin or ET-743 was found to be a DNA alkylating agent, it binds the DNA minor groove in GC-rich sequences and the adducts are reversible upon DNA denaturation (103). Trabectedin was shown to block the cell cycle in late S and G2 phases and it is able to disorganize the microtubule network in the cell (104). *In vitro* and *in vivo* studies demonstrated a potent antitumour activity at a relatively low dose in several solid tumours (104, 105). The first, phase 1, clinical trial with advanced, pretreated sarcoma patients revealed an effect of treatment in patients with soft tissue sarcomas (106). Over the past decade several trials were conducted and especially myxoid liposarcoma patients do respond well to treatment with trabectedin (107-109). Therefore, next to doxorubicin also trabectedin has been approved for the treatment of unresectable or metastatic myxoid liposarcoma or leiomyosarcoma (110). In the European Union trabectedin has been approved in 2007 and in the USA the Food and Drug Administration approved the use of trabectedin in 2015 after a large phase III trial trabectedin (67, 111). It was postulated that the drug is able to dislocate the oncogenic FUS-DDIT3
transcription factor from the DNA (112). Interestingly trabectedin has shown to have an effect in other translocation-associated sarcomas as well, such as alveolar rhabdomyosarcoma and mesenchymal chondrosarcoma (113). Worth-mentioning is that trabectedin also demonstrates antitumour activity in leiomyosarcoma and platinum-sensitive ovarian cancer, which are both, as far as we know yet, not characterized by an oncogenic chimeric transcription factor (67, 114). The exact mechanism of trabectedin has been subject of multiple studies over the past years and is currently not entirely elucidated, ongoing research demonstrated that the mechanism of action of trabectedin is complex and involves multiple aspects: binding to DNA-minor groove, interaction with homologous recombination and transcription-coupled nucleotide-excision repair (TC-NER), modulation of transcription regulation and induction of micro-environment changes (115). Recently eribulin has been registered for the treatment of liposarcomas (116).

Table 2. Clinico-pathological characteristics of leiomyosarcoma and myxoid liposarcoma.

<table>
<thead>
<tr>
<th>Features</th>
<th>Leiomyosarcoma of soft tissue</th>
<th>Myxoid/round cell liposarcoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age range</td>
<td>5th - 6th decade</td>
<td>3rd - 5th decade</td>
</tr>
<tr>
<td>Frequency</td>
<td>10%-20% of soft tissue sarcomas</td>
<td>~5% of soft tissue sarcomas</td>
</tr>
<tr>
<td>Preferential location</td>
<td>Retroperitoneum and other deep soft tissues</td>
<td>Deep soft tissues of extremities (2/3 in thigh)</td>
</tr>
<tr>
<td>Survival</td>
<td>5-years survival 54%-64%</td>
<td>5-years survival ~10%-90%</td>
</tr>
<tr>
<td>Histomorphology</td>
<td>Spindle cells in intersecting bundles</td>
<td>Stellate cells in myxoid matrix with &quot;crow's feet&quot; vasculature</td>
</tr>
<tr>
<td>Histological grading</td>
<td>FNCLCC</td>
<td>Myxoid: low grade; &gt;5% round cell: high grade</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>Desmin, h-caldesmon</td>
<td>No specific stain</td>
</tr>
<tr>
<td>Normal counterpart</td>
<td>Smooth muscle</td>
<td>Adipose tissue</td>
</tr>
<tr>
<td>Genomic profile</td>
<td>Complex karyotype with multiple non-recurrent aberrations</td>
<td>t(12;16)(q13;p11) or t(12;22)(q13;q12)</td>
</tr>
<tr>
<td>Cell line models</td>
<td>LMS04, LMS05, IB133, IB140</td>
<td>402-91, 1765-92, DL-221</td>
</tr>
</tbody>
</table>

Aim and outline of the thesis

The aim of this thesis is to gain more insight in the molecular pathology of soft tissue sarcomas, especially leiomyosarcoma and myxoid liposarcoma. As mentioned above, the group of soft tissue sarcomas constitute a large number
of different, mostly rare, neoplasms, each with their own histopathology, genetic profile and clinical features. Here we focus on two different tumours, on one hand the leiomyosarcomas characterized by a complex genetic profile and on the other hand the myxoid liposarcoma with one well-defined recurrent translocation. Over the past decade, we have gained increasing insight into the molecular pathogenesis of leiomyosarcoma and myxoid liposarcoma; however, translating this knowledge into specific therapies has been challenging. Here we focus on the molecular profile of these two soft tissue tumours and performed studies to identify potential targets for specific therapeutic strategies.

**Part 1. Leiomyosarcoma**

To study leiomyosarcomas we used collected tumour specimens, primary tumour cultures and four well-established cell lines (LMS04, LMS05, IB133 and IB140) that were available through our international collaborators.

In **chapter 2** we investigate the presence of MED12 exon 2 mutations in both benign and malignant smooth muscle tumours of soft tissues as well as the mutation rate in uterine smooth muscle tumours. Also the possible role of the Wnt-signalling pathway in the pathogenesis of these tumours is investigated by use of β-catenin immunohistochemistry.

In **chapter 3** primary cell cultures of soft tissue leiomyosarcomas are characterized in depth by array-based comparative genomic hybridization and COBRA-FISH karyotyping in order to investigate the genetic alterations in leiomyosarcomas and to search for a common molecular genetic aberration. This might improve the understanding of this malignant tumour and identify candidate targets for future therapeutic regimens.

In **chapter 4** we investigated whether the anti-apoptotic Bcl family proteins play a role in the responsiveness to conventional chemotherapy in leiomyosarcomas. The expression of the Bcl proteins in a large patient series is investigated. Next, we determined whether the Bcl-2 pathway inhibitor ABT-737 sensitises leiomyosarcoma cells to chemotherapy *in vitro*. 
Part 2. Myxoid liposarcoma
The studies on myxoid liposarcoma were performed within an international consortium, encouraged and partly funded by the Liddy Shriver Sarcoma Initiative, and led by Dr. T. Nielsen, University of British Columbia, Vancouver.

Chapter 5 is a large, multicenter, validation study in which we investigate the immunohistochemical expression of the cancer-testis antigen NY-ESO-1 (CTAG1B) in myxoid liposarcomas as well as multiple other soft tissue and bone neoplasms. The aim is to identify tumour subtypes with a high expression rate, which might benefit from NY-ESO-1 targeted immunotherapy.

Chapter 6 reports the detailed molecular cytogenetic profile, phenotypic properties and xenograft properties of a newly established myxoid liposarcoma cell line (DL-221). Reliable \textit{in vitro} and \textit{in vivo} models are of pivotal importance to investigate novel therapies, and to study the mechanisms of action and causes of resistance. Although myxoid liposarcoma is a relatively common soft tissue sarcoma subtype, only few model systems are available to study the disease. Thus far only two cell lines were available (402-91 and 1765-92), both of which are immortalized by transfection with the SV40 large T-antigen. One of the goals of the consortium was to increase the number of available models. We were able to establish one new cell line (DL-221) with xenograft model, which is so far the only spontaneously immortalized cell line model of myxoid liposarcoma.

In chapter 7 we use this newly established cell line, in addition to the two cell lines that were already available, to perform a high-throughput drug screen exploring the effectivity of 273 different drugs. Our aim is to identify auspicious candidate targets which might serve as a new therapeutic option for myxoid liposarcoma.

Finally, chapter 8 summarizes and discusses the results of the studies described in the preceding chapters.
References


82. ten Heuvel SE, Hoekstra HJ, Bastiaannet E, et al. The classic prognostic factors tumor stage, tumor size, and tumor grade are the strongest predictors of outcome in synovial sarcoma: no role for SSX fusion type or ezrin expression. Appl Immunohistochem Mol Morphol. 2009;17(3):189-95.
Chapter 1


