From the Department of Oncology and Pathology
Karolinska Institutet, Stockholm, Sweden

PROGNOSIS BASED CLASSIFICATION AND TUMOR BIOLOGY OF UTERINE SARCOMAS

Elin Hardell

Karolinska Institutet

Stockholm 2020

Cover picture created with BioRender.com.
Published by Karolinska Institutet.
Printed by E-print AB 2020

ISBN 978-91-7831-815-5

Prognosis based classification and tumor biology of uterine sarcomas

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Elin Hardell

Principal Supervisor:
Associate Professor Joseph W. Carlson
Karolinska Institutet
Department of Oncology and Pathology

Co-supervisors:
Associate Professor Hanna Dahlstrand
Uppsala University
Department of Immunology, Genetics and Pathology
And
Karolinska Institutet
Department of Oncology and Pathology

Associate Professor Anders Isaksson
Uppsala University
Department of Medical Sciences

Opponent:
Professor Alexander J Lazar
University of Texas, MD Anderson Cancer Center
Department of Pathology

Examination Board:
Professor Georgios Rassidakis
Karolinska Institutet
Department of Oncology and Pathology

Associate Professor Hans Hagberg
Uppsala University
Department of Immunology, Genetics and Pathology

Professor Roger Henriksson
Umeå University
Department of Radiation Sciences

ABSTRACT

Uterine stromal sarcomas are a heterogenous group of tumors ranging from low-grade stromal sarcomas with a relatively good survival but high risk of recurrence, to undifferentiated uterine sarcomas with a far worse prognosis. Little is known about their biology, and prognostic markers are lacking. The treatment options are limited mainly to surgery. Chemotherapy and radiotherapy have little or no effect on survival. Recent success with immunotherapy has opened a promising research field with potential to gain new insights into the biology, prognosis and therapy of uterine stromal sarcomas.

In **paper I** we examined the correlation of clinicopathological factors, biomarkers and YWHAE-FAM22 translocation with prognosis in undifferentiated uterine sarcomas. Twenty-six cases from the Karolinska University Hospital's archive were included, out of which 22 of them had paraffin blocks for translocation status and immunohistochemical analysis of p53, p16, Ki67, Cyclin-D1, estrogen receptor, progesterone receptor and Anillin. Our findings show that the cases could be divided into two prognostic groups based on mitotic index; the high mitotic index group with a statistically significant worse prognosis than the group with low mitotic index.

In **paper II** we wanted to validate the results from paper I in an independent cohort of cases. A total of 40 cases of undifferentiated uterine sarcomas were included from the Norwegian Radium Hospital, the Mayo Clinic and Skåne University Hospital. Mitotic index was recorded and the cases were divided into high and low mitotic index groups based on the cut-off from paper I. The analysis showed that one-third of the patients survived beyond five years. In the adjusted survival analysis, mitotic index group and tumor stage were prognostic factors.

In **paper III** we identified molecular subgroups of undifferentiated uterine sarcomas and evaluated the possible correlation with different clinicopathological parameters. The cohort of paper III consisted of 50 cases with undifferentiated uterine sarcomas from six different institutions. All cases had formalin-fixed paraffin-embedded tumor material used for isolation of DNA and RNA. In total 50 cases were analyzed for gene expression, copy number variation, cell morphometry and protein expression. Four groups with different mRNA expression pattern were identified. Gene ontology analysis showed an activation of pathways related to genital tract development, extracellular matrix, muscle function and proliferation in the different groups. The result of the chromosomal copy number analysis showed a spectrum of variation, from cases that were diploid or near diploid to cases with

extensive chromosomal aberrations. The adjusted Cox Proportional Hazard model showed that the mitotic index group, the hormone receptor expression and the mRNA group had a statistically significant impact on overall survival. In the ontology analysis, the mRNA group with the worst prognosis showed overexpressed pathways related to the extracellular matrix (ECM). When further analyzed by image analysis the ECM group was characterized by reduced cell density and increased nuclear size compared to the other groups. The ECM group also showed higher expression of the four ECM related proteins matrix metalloproteinase 14, collagen 1, collagen 6 and fibronectin, when evaluated with immunohistochemistry.

In **paper IV** we analyzed the prognostic significance of different immune markers in low-grade endometrial stromal sarcomas (LGESS). The cohort consisted of 21 cases identified by searching the pathological database at the Karolinska University hospital and the Stockholm region cancer registry. All cases had formalin-fixed paraffin-embedded tumor material and follow-up data. Tissue microarrays consisting of two biopsies of tumor material from each case were constructed. Multiplex fluorescent immunohistochemistry in combination with digital image analysis in QuPath was used to quantitatively assess the expression of different immune markers, including CD8, FOXP3, CD68, CD163, IDO1, B7-H4 and PD-L1. A low ratio of CD8+/FOXP3+ cells was significantly associated with a favorable prognosis in LGESS. For patients with a high quantity of CD8+ T cells and B7-H4, a trend towards better survival was seen. The expression of B7-H4 is also a potential therapeutic target.

LIST OF SCIENTIFIC PAPERS

I. Gremel G, Liew M, Hamzei F, **Hardell E**, Selling J, Ghaderi M, Stemme S, Pontén F, Carlson JW.
A Prognosis Based Classification of Undifferentiated Uterine Sarcomas: Identification of Mitotic Index, Hormone Receptors and YWHAE-FAM22 Translocation Status as Predictors of Survival. *International Journal of Cancer.* 2015;136(7):1608–18.

II. **Hardell E**, Josefson S, Ghaderi M, Skeie-Jensen T, Westbom-Fremer S, Cheek EH, Bell D, Selling J, Schoolmeester JK, Måsbäck A, Davidson B, Carlson JW.
Validation of a Mitotic Index Cutoff as a Prognostic Marker in Undifferentiated Uterine Sarcomas. *The American Journal of Surgical Pathology.* 2017; 41(9):1231-1237.

III. Binzer-Panchal A, **Hardell E (shared first authorship)**, Viklund B, Ghaderi M, Bosse T, Nucci MR, Lee CH, Hollfelder N, Corcoran P, Gonzalez-Molina J, Moyano-Galceran L, Bell DA, Schoolmeester JK, Måsbäck A, Kristensen GB, Davidson B, Lehti K, Isaksson A, and Carlson JW.
Integrated Molecular Analysis of Undifferentiated Uterine Sarcomas Reveals Clinically Relevant Molecular Subtypes. *Clinical Cancer Research.* 2019;25(7):2155–65.

IV. **Hardell E**, Gonzales-Molina J, Gültekin O, Mitsios N, Lehti K, Carlson JW.
The immune microenvironment in low-grade endometrial stromal sarcomas: The ratio of CD8+ T cells/FOXP3+ Tregs (T regulatory cells) is a significant prognostic factor. *Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

ANLN	Anillin
APC	Antigen-presenting cell
CAR	Chimeric antigen receptor
CD10	Cluster of differentiation 10
CDK4	Cyclin-dependent kinase 4
CDK6	Cyclin-dependent kinase 6
cDNA	Complementary DNA
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte associated protein 4
ECM	Extracellular matrix
ER	Estrogen receptor
FDA	The Food and Drug Administration
FFPE	Formalin-fixed paraffin-embedded
FIGO	International Federation of Gynecology and Obstetrics
HPF	High power field
IDO	Indoleamine 2,3 dioxygenase
LGESS	Low-grade endometrial stromal sarcoma
MHC	Major histocompatibility complex
PCR	Polymerase chain reaction
PD1	Programmed cell death protein 1
PD-L1	Programmed cell death protein 1 ligand 1
PD-L2	Programmed cell death protein 1 ligand 2
PR	Progesterone receptor
qPCR	Quantivative PCR/real-time PCR
RT-PCR	Reverse transcript PCR
sTCR	Synthetic T cell receptor

TAM	Tumor associated macrophage
TCR	T cell receptor
TIL	Tumor infiltrating lymphocyte
TMA	Tissue microarray
Treg	Regulatory T cell
UES	Undifferentiated endometrial sarcoma
UUS	Undifferentiated uterine sarcoma
WHO	World Health Organization

1 INTRODUCTION

Uterine sarcomas comprise 1-3% of malignancies of the female genital tract. The most common histological type is leiomyosarcoma, followed by endometrial stromal sarcoma[1]. Because of the rarity of endometrial stromal sarcomas our knowledge about their biology and behavior is limited. The possibility to draw conclusions from studies are also affected by changes in the classification system over the years.

1.1 HISTOPATHOLOGY AND WHO CLASSIFICTAION OF ENDOMETRIAL STROMAL TUMORS

The classification system of endometrial stromal tumors has changed several times the last half century. In the 2003 World Health Organization (WHO) classification of gynecological malignancies, endometrial stromal tumors were divided into endometrial stromal nodule, low-grade endometrial stromal sarcomas and undifferentiated endometrial sarcomas (UES)[2]. Previously, stromal sarcomas had been divided into low- and high-grade based on mitotic count, but since the high-grade endometrial stromal sarcomas lacked histological resemblance to endometrial stroma they were instead thought to be best regarded as undifferentiated sarcomas. The differentiation between low-grade endometrial sarcomas and UES was based on the low-grade tumors' resemblance to endometrial stroma and their lack of significant atypia and pleomorphism. UES on the other hand, lack specific differentiation and have marked cellular atypia[2].

In 2008, Kurihara *et al.* examined 18 cases of low-grade endometrial stromal sarcoma and 13 cases of UES. Based on nuclear atypia, they divided the cases of UES into one group with nuclear uniformity and one group with nuclear pleomorphism. They concluded that the uniform group shared histological characteristics with low-grade endometrial stromal sarcomas. However, the significance of this classification was unclear since there were no apparent differences in outcome between the groups[3].

In 2012, Lee *et al.* described a translocation, t(10;17)(q22;p13), resulting in a fusion between YWHAE and either FAM22A or FAM22B in endometrial stromal sarcomas[4]. When they later compared 13 cases of YWHAE-FAM22 endometrial stromal sarcomas with 20 cases of low-grade endometrial stromal sarcoma without the fusion, they concluded

that YWHAE-FAM22 rearrangements where associated with high-grade morphology and a more aggressive clinical behavior [5].

In the most recent WHO classification of gynecological tumors, the endometrial stromal tumors are once again divided into endometrial stromal nodules, low-grade endometrial stromal sarcomas, high-grade endometrial stromal sarcomas and undifferentiated uterine sarcomas (UUS)[6].

Endometrial stromal nodule is a tumor consisting of cells resembling the proliferative-phase of endometrial stroma. It has a well-circumscribed margin and is considered as a benign tumor.[6]

Low-grade endometrial stromal sarcoma (LGESS) is also composed of cells resembling proliferative-phase endometrial stroma, but it has an infiltrative, tongue-like growth pattern and is a malignant tumor. Immunohistochemically, the tumor is typically positive for cluster of differentiation (CD) 10, estrogen receptors (ER) and progesterone receptors (PR). It can also be positive for smooth-muscle actin and occasionally for desmin, but negative for h-caldesmon[6]. One study reported that the immunohistochemical marker interferon-induced transmembrane protein 1 (IFITM1) is a more specific marker compared to CD10 in differentiating low-grade endometrial stromal sarcomas from smooth muscle neoplasms[7]. Generally, patients with stage I disease have a good prognosis with 5- and 10-year survival of 98% and 89%, respectively. However, recurrence is common. The most common place for recurrence is the pelvis and abdomen[8].

High-grade endometrial stromal sarcoma is a malignant tumor consisting of a mixture of a high-grade round cell component and a low-grade spindle cell component. The mitotic activity is usually high, and necrosis is common. The tumor typically harbors the YWHAE-FAM22 (also known as YWHAE–NUTM2A/B) fusion previously described. Immunohistochemically, the high-grade component is CD10, ER and PR negative but shows strong diffuse cyclin D1 positivity. The low-grade spindle cell component is positive for CD10, ER and PR[6]. In a study by McCluggage and Lee, 19 of 19 cases were positive for CD56 and 17 of 20 cases were positive for CD99[9]. Abnormal bleeding is the most common presenting symptom[5]. High-grade endometrial stromal sarcomas are more aggressive than low-grade endometrial stromal sarcomas, but do not behave as aggressively as undifferentiated uterine sarcomas[10].

Undifferentiated uterine sarcoma (UUS) is a high-grade sarcoma with no specific differentiation. It is a diagnosis based on exclusion, and other uterine tumors must be ruled out. It typically presents in postmenopausal women as abnormal uterine bleeding, a pelvic mass or symptoms secondary to extra uterine spread[11]. Histologically, the tumors grow destructively into the myometrium in sheets. Immunohistochemically, the tumors are variably CD10 positive. ER and PR are typically weakly positive or negative[6]. The majority of the patients present with disseminated disease, and most patients die due to the disease within two years of diagnosis[12]. However, this is a heterogenous group of tumors and some patients survive longer.

1.2 TNM/FIGO STAGING

Until 2009 the International Federation of Gynecology and Obstetrics (FIGO) criteria for endometrial carcinoma was used for uterine sarcomas. To reflect the different biology and behavior of the tumors, separate criteria for uterine sarcomas were introduced in 2009[13]. The FIGO criteria are basically the same as American Joint Committee on Cancer TNM system. Both systems are based on three factors: the primary tumor size and if the tumor extends beyond the uterus (T), regional lymph node involvement (N) and presence of distant metastasis (M). Based on the TNM/FIGO classification, the stage of the tumor is defined[14,15].

Primary tumor		
TNM category	**FIGO stage**	**Criteria**
TX		Primary tumor cannot be assessed
T0		No evidence of primary tumor
T1	I	Tumor limited to the uterus
T1a	IA	Tumor 5 cm or less in greatest dimension
T1b	IB	Tumor more than 5 cm
T2	II	Tumor extends beyond the uterus, within the pelvis
T2a	IIA	Tumor involves adnexa
T2b	IIB	Tumor involves other pelvic tissues
T3	III	Tumor infiltrates abdominal tissues
T3a	IIIA	One site
T3b	IIIB	More than one site
T4	IVA	Tumor invades bladder or rectum
Regional lymph nodes		
TNM category	**FIGO stage**	**Criteria**
NX		Regional lymph nodes cannot be assessed
N0		No regional lymph node metastasis
N0(i+)		Isolated tumor cells in regional lymph node(s) no greater than 0.2 mm
N1	IIIC	Regional lymph node metastasis
Distant metastasis		
TNM category	**FIGO stage**	**Criteria**
M0		No distant metastasis
M1	IVB	Distant metastasis (excluding adnexa, pelvic and abdominal tissue)

Table 1. TNM and FIGO staging of uterine sarcomas, excluding adenosarcomas. Modified from AJCC Cancer staging manual 8th edition 2017[16]. Reprinted with permission from Springer International Publishing.

1.3 TREATMENT OF ENDOMETRIAL STROMAL SARCOMA

Standard treatment for all uterine sarcomas is hysterectomy[17,18]. Endometrial stromal sarcomas tend to recur locally or in the lungs. Systematic lymphadenectomy has not been found to improve survival, and is not indicated[19].

In low-grade endometrial stromal sarcomas, the ovaries are usually removed because the tumors typically express ER and PR. However, there are discrepancies in the results of studies examining how ovarian-sparing surgery impacts overall survival and progression-free survival[20]. Hormone replacement therapy on the other hand is associated with higher relapse rates, and not recommended[18,21]. Tamoxifen is contraindicated as it has a proliferative effect on the endometrial stroma[22]. Retrospective studies have shown effectiveness of hormonal therapy with progestins, aromatase inhibitors and gonadotropin-releasing hormone analogues as adjuvant and recurrent therapy[23,24]. Few studies have investigated response of endometrial stromal sarcoma to chemotherapy, but the response rates are low and chemotherapy should only be used when hormonal therapies are ineffective[17]. The role of radiotherapy is limited, and it does not improve overall survival. Palliative radiotherapy can be used for recurrent or metastatic disease[17,18].

Undifferentiated uterine sarcomas usually do not express hormone receptors, and hormonal therapies are generally not used[18]. External pelvic radiotherapy is reported to decrease regional recurrence, but impact on survival is unknown. The experience of adjuvant chemotherapy is also limited[25]. One randomized study compared adjuvant polychemotherapy (doxorubicin, ifosfamide, and cisplatin) followed by pelvic radiotherapy versus radiotherapy alone in uterine sarcomas (53 leiomyosarcomas, 9 undifferentiated sarcomas and 19 carcinosarcomas). Adjuvant polychemotherapy combined with radiotherapy increased the 3-year disease free survival, but had no effect on overall survival. Due to lack of recruitment of patients, the study was stopped earlier than planned[26,27].

2 TUMOR BIOLOGY

Hanahan's classic hallmarks of cancer consists of six capabilities: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis[28]. Since then, the awareness of the tumor cells' interaction with its microenvironment has increased. The tumor microenvironment consists of different cells including fibroblasts, endothelial cells and immune cells, but also non-cellular parts [29].

2.1 TUMOR MICROENVIRONMNET

Both the cellular and the non-cellular parts of the tumor microenvironment play important roles for cancer development. The extracellular matrix (ECM) consists of different biochemical components, including collagens, proteoglycans and glycoproteins. These biochemical components give the ECM unique physical, biochemical and biomechanical properties. The physical properties give the tissue its architecture and integrity, but is also functioning as a barrier and movement track, and can both have negative and positive roles in cell migration. The biochemical properties of the ECM affect the capability of cell signaling, and the cells' possibilities to interact with the microenvironment. The cytoskeleton of the cells is linked to the ECM via adhesion complexes. This works as a mechanosensing machinery, and allows the cell to react to the ECM's biomechanical properties[30].

2.2 THE CELL CYCLE

The cell cycle is divided into two parts, mitosis (M phase) and interphase. The M phase can be further divided into prophase, prometaphase, metaphase, anaphase and telophase representing different stages of mitosis. The interphase consists of the phases G_1, S and G_2. The G_1 phase (gap 1) represents the gap between mitosis and DNA replication. In this phase, the cell is metabolically active, but does not replicate its DNA. The DNA replication takes place during the S phase. In the G_2 phase (gap 2) DNA synthesis is finished and the cell prepares for mitosis[31].

The cell cycle is regulated by both extracellular signals and internal signals. A series of control points coordinate and regulate the progression through the different phases of the cell cycle. For example, if the appropriate growth factors are not available in G_1, the cell

cycle stops and the cell enters a quiescent stage called G_0. There are also several cell cycle checkpoints controlling that damaged DNA is not replicated and passed on to daughter cells[31].

In normal tissue the cell cycle is carefully controlled to maintain a homeostasis of the cell number, and one of the hallmarks of cancer is the cancer cells' ability to sustain proliferative signaling[28,32]. In contrast to most normal cell linages in the body, cancer cells also have an unlimited replicative potential[28].

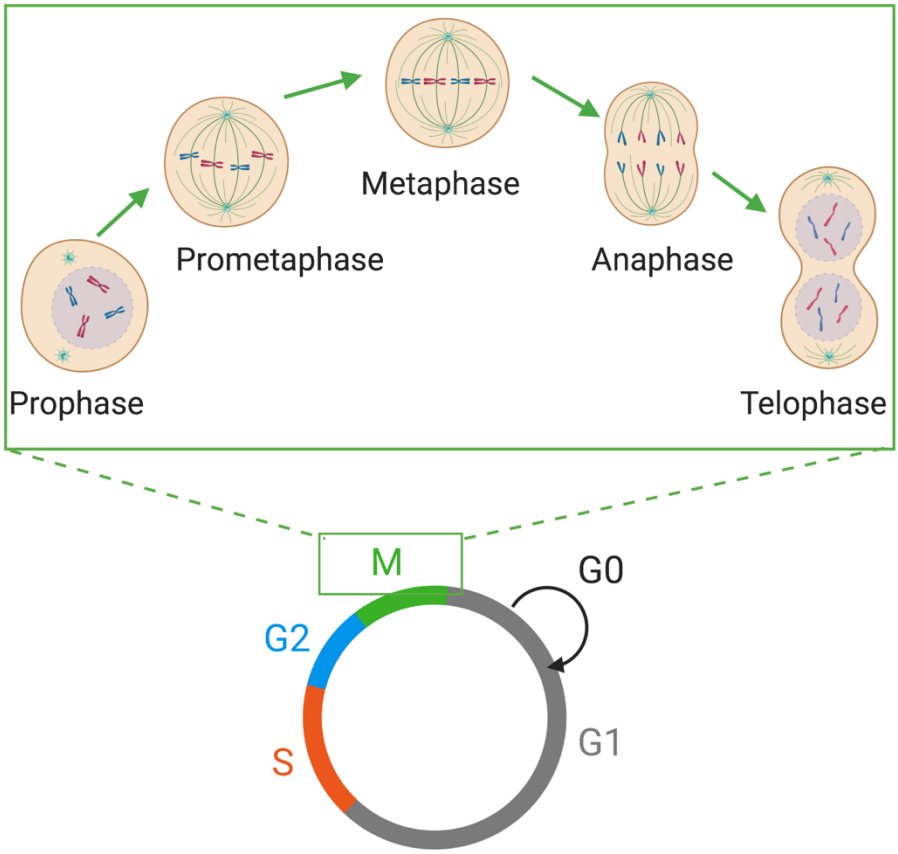

Figure 1. The cell cycle consists of the phases G1, S, G2 and M. The M-phase can be further divided into prophase, prometaphase, metaphase, anaphase and telophase. G0 is a quiescent stage. Created with BioRender.com.

2.3 TRANSFORMATION OF GENETIC INFORMATION

Transforming the information in the DNA to proteins requires a process sometimes called the central dogma. It is based on three steps:

1. DNA replication: copying DNA to DNA
2. Transcription of DNA to messenger RNA (mRNA)
3. Translation of mRNA to proteins

2.4 GENOMIC INSTABILITY

Cancer cells are characterized by genomic instability caused by mutations affecting different pathways regulating the cell cycle and repair of DNA damage. It increases the cell-to-cell variation leading to better chance to adaptation to the tumor microenvironment, as well as increasing the chances of beneficial mutations. Genomic instability both includes nucleotide mutations (base substitutions, deletions, insertions) and chromosomal instability (gains and losses of whole or parts of the chromosomes and chromosomal rearrangements). One result of chromosomal instability is aneuploidy, and the degree of chromosomal instability often correlates with karyotypic complexity[33]. However, the relationship and mechanisms of aneuploidy, chromosome instability and tumorigenesis is complex and largely unknown[34].

2.5 MOLECULAR CHARACTERISTICS OF SARCOMAS

Sarcomas in general can be classified into two categories: one with near diploid karyotypes and simple genetic alterations and one with complex and unbalanced karyotypes. In the first category of tumors, translocation-associated sarcomas are the most common variants and the tumors tend to arise de novo. The majority of the karyotypically complex sarcomas arise de novo, but they can also arise from dedifferentiated less aggressive forms[35].

In a study by the Cancer Genome Atlas Research Network, 206 cases of adult soft tissue sarcomas were characterized. One of the findings was that these sarcomas had low mutational burden, and only three significantly mutated genes were identified: TP53, ATRX and RB1. Copy number alterations were on the other hand frequent[36].

A number of gene rearrangements has been described in low-grade endometrial stromal sarcomas. The most common is the translocation t(7;17)(p15;q21) resulting in JAZF1-SUZ12 fusion. Many other fusions are described, including JAZF1-PHF1, EPC1-PHF1, MEAF6-PHF1, ZC3H7B-BCOR and MBTD1-CXorf67[12]. All these fusions are believed to

affect genes involved in transcriptional regulation in endometrial stromal progenitor cells, leading to oncogenic effects. Clinically and histologically, they are all similar[10].
High-grade endometrial stromal sarcomas are, as previously described, characterized by the YWHAE-FAM22 fusion. In 2017, Hoang *et al.* described a new type of high-grade endometrial stromal sarcoma[37]. The tumors mimicked myxoid leiomyosarcomas morphologically, and also had overlapping immunohistochemical profile. All cases harbored ZC3H7B-BCOR gene fusions. Clinically, the tumors seemed to have a more aggressive behavior than low-grade endometrial stromal sarcomas[37].

Due to the rarity of undifferentiated uterine sarcomas, there is limited genomic data. The data available show complex karyotype and frequent p53 alteration[10]. Most lack chromosomal translocations, but JAZF1-SUZ12 fusion has been reported and probably represents dedifferentiated low-grade endometrial stromal sarcomas[6].

3 BIOMARKERS

In medicine, a biological marker (or biomarker) is an indicator that can be measured to give information of a normal or abnormal process or disease. In cancer diseases, biomarkers are used in three main ways: for diagnosis, for prognosis and to predict or monitor treatment response. It can also be used for risk assessment, for example BRCA mutation, as a risk factor for ovarian and breast cancer.

Often used biomarkers in pathology include analysis of different histological parameters, immunohistochemistry and genetic analysis.

3.1 MITOTIC INDEX

Mitotic counting is used in different types of tumors both for diagnostic and prognostic purposes. In invasive breast cancer, the number of mitoses per 10 high power fields (HPF) is a part of the Nottingham histological grade[38]. The Nottingham grade has been shown to be an important prognostic factor[39,40].

The most common method is to count the number of mitotic figures in a specific number of high-power fields in hematoxylin and eosin stained histological slides, resulting in a mitotic index. Different tumors have different cellularity, which will affect the mitotic index. It has been proposed that the mitotic count should be based on the number of cells in the specimen instead of HPF[41]. A volume corrected mitotic index has also been proposed to correct for differences in the thickness of the histological sections[42]. However, these methods are time consuming and have not been widely used.

Several other factors are also known to affect the mitotic index, such as time to fixation and adequate macroscopic and microscopic sampling[43–45]. It is also of importance to define the area to count, not only the number of HPF, because of variations of the visual field areas between different microscopes[46]. Mitotic index has been shown to be reproducible if a protocol for counting is followed[47].

3.2 ESTROGEN AND PROGESTERONE RECEPTORS

The estrogen receptor (ER) exists in two forms, ERα and ERβ. The ER is located in the nucleus and acts as a binding protein for the hormone estrogen. ERα and ERβ are encoded

by the genes *ESR1* and *ESR2*, respectively, and numerous mRNA splice variants exist. Little is known about the exact function of these different splice types. Estrogen has been associated with the development of different types of cancer, including breast cancer, endometrial cancer and ovarian cancer.[48] The progesterone receptor is also located in the nucleus where it regulates hormone response target genes and acts as a transcription factor[49].

In endometrial stromal sarcomas, ER and PR are expressed mainly in the low-grade tumors and to a lesser extent in the high-grade tumors[6,50].

3.3 P53

P53 coordinates transcription programs that contribute to tumor suppression[51]. P53 can both activate and repress expression of genes involved in apoptosis, senescence and cell cycle arrest, but also for instance metabolism, necrosis and stem cell maintenance[52]. In many human cancers the p53 signaling pathway is mutated or functionally inactivated, but the p53 gene can still be wilde-type[53]. Mutations in p53 itself or disruptions in pathways signaling to p53, lead to loss of p53 wild-type function. However, mutations in p53 in itself can also lead to gain of new functions such as induction of angiogenic factors, metastasis or inactivation of the tumor suppressors p63 and p73[52].

3.4 KI67

Ki67 is a protein associated with cell proliferation. It is present in the cell cycle phases G1, S, G2 and M, but not in resting cells in the G0 phase[54]. It has been suggested that Ki67 functions as a surfactant that enables chromosome motility by preventing them from collapsing after nuclear envelope disassembly[55]. Immunohistochemical evaluation of Ki67 can provide prognostic and predictive information in for example breast cancer[56,57].

3.5 CYCLIN-D

There are three forms of D cyclins in mammalian cells, D1, D2 and D3. They regulate the transition of the cell cycle from G1 to S phase by acting as activators of cyclin-dependent kinase (CDK) 4 and CDK6[58]. CDK4/6 phosphorylates the retinoblastoma protein, priming it for inactivation. The inactivation leads to activation of genes required for initiation of the S phase[59].

Cyclin D1 is the most studied D cyclin. Overexpression of cyclin D1 leads to dysregulation of CDK activity resulting in cell growth and contribution to neoplastic growth. Cyclin D1 is frequently overexpressed or amplified in different types of malignancies, e.g. pancreatic cancer, non-small cell lung carcinoma, endometrial cancer and mantle cell lymphoma[58].

In endometrial stromal sarcomas, it has been shown that cyclin D1 is a sensitive and specific diagnostic immunohistochemical marker for t(10;17)(q22;p13) high-grade endometrial stromal sarcomas[60].

3.6 P16

P16 acts as a tumor suppressor by inhibiting the effect of CDK4 and 6, resulting in cell cycle arrest at the G1-S boundary[61]. P16 is also involved in cellular senescence which is a special form of a stress-induced, durable cell-cycle arrest that prevents cancer. Senescent cells are characterized by expression of anti-proliferative molecules, e.g. p16[62]. Loss of p16 function is frequent in many human tumors[63].

3.7 ANILLIN (ANLN)

Anillin is a protein that interacts with cytoskeletal components and is involved in organizing the cytoskeleton during cytokinesis. After the cell division is completed, anillin is degraded[64]. Anillin expression has been shown to be upregulated in different cancer types, and it has also been linked to metastatic potential and poor prognosis[65].

4 TUMOR IMMUNOLOGY

4.1 BACKGROUND

The immune system has a dual role in the progression of tumors and can kill early tumor cells, but it can also select tumor cells that can evade surveillance[66]. The immune system recognizes different antigens expressed by the tumor cells and can separate them from normal cells. Some of the antigens are shared between different tumor types, others are unique to the tumor[67]. Avoiding recognition and destruction by the immune system is one of the hallmarks of cancer[28].

The immune system is regulated by stimulatory and inhibitory signals that normally regulate self-tolerance, wound-healing and different homeostatic mechanisms[68]. To avoid recognition and destruction, the tumor can either turn off the immune response, or create resistance mechanisms in the local microenvironment[69].

4.2 TUMOR INFILTRATING LYMPHOCYTES

All types of immune cells can be present in tumors, including B cells, T helper cells, T regulatory cells, macrophages, dendritic cells and cytotoxic T cells. The type of immune cells, the quantity of cells and also the location of the cells (e.g. the center of the tumor or the invasive margin) varies in different tumor types[70].

The major histocompatibility complex (MHC) class I and II is necessary for antigen presentation. MHC class I is expressed on all nucleated cells and binds endogenous proteins. MHC class II is mainly expressed on antigen-presenting cells (APCs) and presents exogenous proteins[71].

For tumor destruction mediated by cytotoxic T lymphocytes (CTLs) MHC class I is crucial. However, in tumors, MHC I is frequently downregulated, and tumor cells usually do not express MHC class II[71].

CD8+ T cells have the capacity to kill tumor cells through recognition of antigenic peptides presented by MHC I on the tumor cells. The recognition of an antigenic peptide by the T cell receptor (TCR) initiates cytotoxic effector functions. The cytotoxic functions are either

direct through exocytosis of cytotoxic granules into the target cell, or indirect through secretion of cytokines[72].

Even though CD8+ CTLs are considered the most important T cells for tumor elimination, CD4+ T cells are also of importance. CD4+ T cells help with the priming of CD8+ T cells to optimize the effector and memory functions of CTLs. This is partly done via interaction with dendritic cells[73].

A high amount of tumor infiltrating lymphocytes (TILs) is associated with a positive clinical outcome in several tumor types, including melanoma, head and neck, urothelial, breast, bladder, colorectal, ovarian, prostatic and lung cancer[70]. In sarcomas, TILs have been reported mainly in gastrointestinal stromal tumors, where it correlates with survival. In other soft tissue sarcomas, the presence of TILs varied. Most studies are limited by small sample sizes and by the vast spectrum of subtypes of soft tissue sarcomas[74].

4.3 T REGULATORY CELLS

Regulatory T cells (Tregs) are a subset of T cells that can either develop in the thymus by antigen stimulation (natural/thymic Tregs), or differentiate from naïve T cells in peripheral tissues (peripherally induced Tregs). However, it is unclear if the peripherally induced Tregs are functionally stable[75]. The main regulatory transcription factor for Tregs is FOXP3[75].

In the tumor microenvironment, Tregs are recruited by different chemokines produced by tissue associated macrophages (TAMs) but also as a result of hypoxia. In certain tumor types, for example follicular lymphoma, it has also been shown that conventional CD4+ T cells can be converted to Tregs[76].

4.3.1 Immunosuppressive mechanisms of Tregs

There are several ways Tregs exhibit their suppressive activity. Among the most important ones are inhibition of APCs through the cytotoxic T-lymphocyte associated protein 4 (CTLA-4) pathway, secretion of inhibitory cytokines and downregulation/killing of APCs and effector T cells by expression of granzyme and perforin. In addition, FOXP3 directly affect transcription of different genes, such as upregulating CTLA-4 transcription[75].

4.3.2 Tregs as a prognostic marker

For most tumors high numbers of Tregs are associated with a poor prognosis[71]. A high ratio of CD8+ T cells/Tregs has been shown to be a beneficial prognostic marker in several types of cancer, such as ovarian cancer[77]. However, in some tumors high numbers of Tregs have been associated with an improved survival[78,79].

4.4 TUMOR ASSOCIATED MACROPHAGES

Macrophages can perform different functions important for the immune system. Depending on the stimuli, macrophages can be driven into different functional phenotypes. Two main types have been suggested, M1(classic activation) and M2 (alternatively activated). M1 has an inflammatory phenotype, expresses inflammatory chemokines and can produce inflammatory cytokines such as TNF, IL-12, IL-23 and IL-6. In addition, M1 promotes Th1 response and has microbicidal and tumoricidal functions. M2 has an anti-inflammatory phenotype, expresses non-inflammatory chemokines and produces anti-inflammatory cytokines such as IL-10. M2 promotes Th2 response and tumor growth, but is also important for tissue repair and remodelling[80].

A long-held view is that TAMs are recruited to the tumor microenvironment from the blood by signals from normal cells and tumor cells. Another theory from mouse models is that TAMs originate from resident precursor macropaghages[81].

Classically activated macrophages participate in the elimination of early tumors by killing tumor cells and mediate tissue destruction. When the tumor progresses, the macrophages are driven towards a M2 phenotype by signals from tumor cells, B cells and stromal cells. These TAMs have functions related to tumor progression, such as angiogenesis and suppression of immunity [81].

4.4.1 Protumoral mechanisms of TAMs

TAMs in the tumor microenvironment support tumor growth in many different ways. One mechanism is by inducing angiogenesis[28]. The induction of angiogenesis is necessary to provide oxygen and nutrition when the tumor growth is above a certain size, and also for disposal of waste. Macrophages can also promote tumor cell migration and invasion into the circulation[82].

Macrophages also express MHC I molecules and can inhibit the activation of NK cells and certain types of activated T cells. In addition, macrophages also express ligands to PD-1 and CTLA-4. Another way of suppressing CD4+ and CD8+ T cells is by the secretion of cytokines, chemokines and enzymes by TAMs[82].

4.4.2 TAMs as a prognostic marker

A high number of TAMs has been associated with a poor prognosis in several solid malignancies[83]. In a meta-analysis by Zhang *et al.,* high density of TAMs was associated with a shorter overall survival in for example breast cancer, ovarian cancer and bladder cancer. However, for patients with colorectal cancer high density of TAMs was associated with longer overall survival[84].

4.5 IMMUNE CHECKPOINTS

The T cell response is initiated when an antigen is recognized by the TCR. Immune-checkpoints are ligand-receptor pairs with stimulatory or inhibitory effects on the magnitude of the immune response[85]. Anti-tumor drugs composed of monoclonal antibodies that inhibit inhibitory immune checkpoints have been developed and antibodies working as agonists of stimulatory immune checkpoints are under development[86].

One group of immune-checkpoint molecules is the B7 family, which is a group of transmembrane proteins including for example B7-H1 (also called programmed cell death protein 1 ligand 1), B7-DC (programmed cell death protein 1 ligand 2), B7-H4, B7-1 (CD80) and B7-2 (CD86)[86].

4.5.1 B7-H4

Although B7-H4 mRNA is expressed in many different normal tissues including brain, heart and skin, the B7-H4 protein is not usually present on the surface of normal cells[87]. B7-H4 has been shown to be overexpressed in different types of cancer. The expression has been linked to different clinicopathological factors in for example ovarian cancer, gastric cancer, melanoma, pancreatic cancer and colorectal cancer[87–92].

Anti-B7-H4 monoclonal antibodies are under development[87].

4.5.2 Cytotoxic T-lymphocyte associated protein 4 (CTLA-4)

In the lymphoid organs, CTLA-4 is expressed on T cells and regulates the early stages of T cell activation. After T cell activation CTLA-4 is upregulated, leading to downregulation of T cell function through different mechanisms[93]. Normally, TCR signaling is reinforced through the co-stimulatory receptor CD28. However, CD28 and CTLA-4 share the same ligands CD80 and CD86. Upregulation of CTLA-4 will outcompete CD28 and suppress the T cell activation[85].

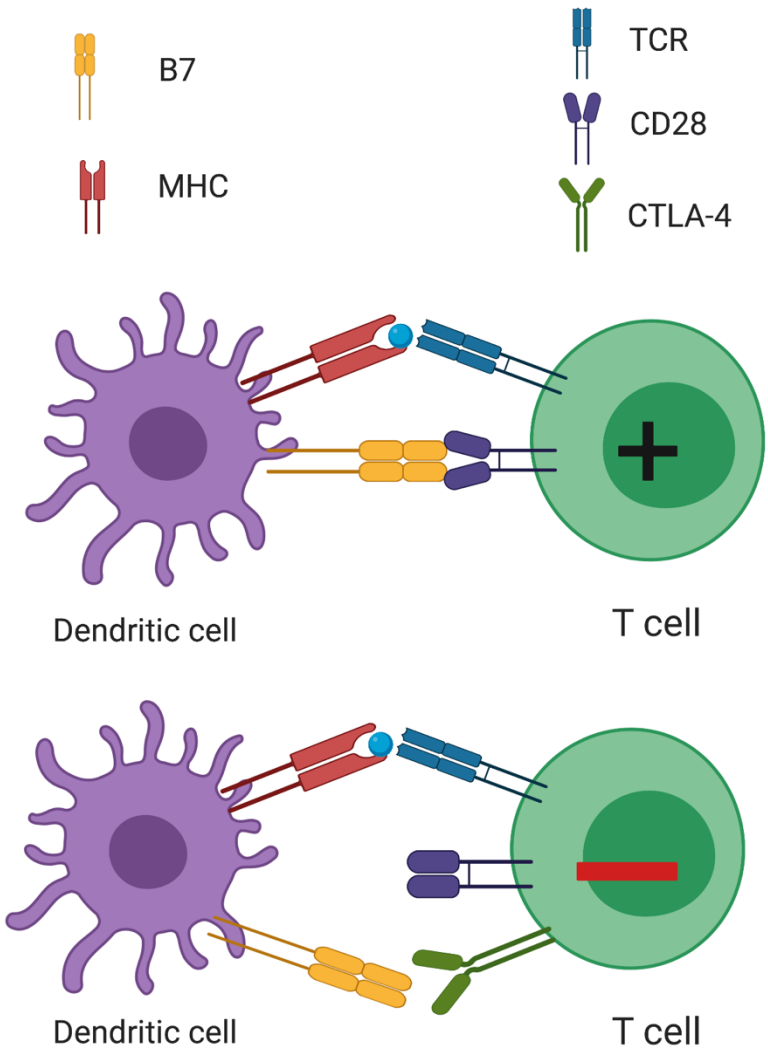

Figure 2. In the lymph node, T cell activation is reinforced by co-stimulation of CD28 binding to B7. When CTLA-4 is upregulated, CD28 is outcompeted and the T cell activation is suppressed. Created with BioRender.com.

4.5.3 Programmed cell death protein 1 (PD1)

Programmed cell death protein 1 (PD1) is a inhibitory regulator of T cells in peripheral tissues and the tumor microenvironment where it is expressed on different cells including TILs, B cells, natural killer cells and Tregs[94].

When PD1 binds to one of its two ligands, programmed cell death protein 1 ligand 1 (PD-L1) or programmed cell death protein 1 ligand 2 (PD-L2), it will ultimately lead to a decrease of the amount of active T cells through different signaling pathways[85].

PD-L1 is often upregulated on the surface of tumor cells, but can also be expressed in the tumor microenvironment. PD-L2 has been reported to be overexpressed in certain B cell lymphomas and is also expressed by antigen presenting cells[85,94].

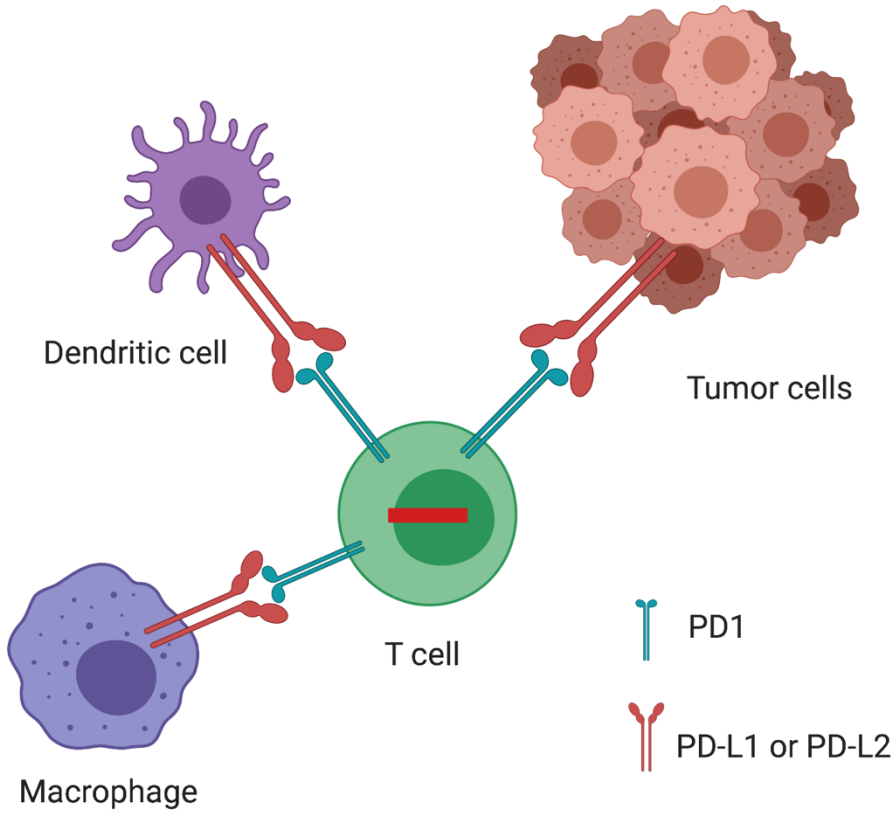

Figure 3. Expression of PD-L1/PD-L2 in the tumor microenvironment or by dendritic cells will inhibit T cells by binding to PD1. Created with BioRender.com.

4.5.4 Indoleamine 2,3 dioxygenase

Indoleamine 2,3 dioxygenase (IDO) is an enzyme that has an important role in immunoregulation by degrading the essential amino acid tryptophan and producing the breakdown product kynurenine. Two different enzymes are described, IDO1 and IDO2, with similar but not completely identical effects. Most studies do not separate them, and inhibitors of IDO affect both enzymes[95].

IDO1 has been linked to both reduced cytotoxic effect of T cells and increased activation of Tregs. The reduced access of tryptophan activates a pathway leading to cell cycle arrest and inability to normal immune response induction. The accumulation of kynurenine can also directly induce T cell apoptosis. IDO also has an indirect effect in the tumor microenvironment by increasing the amount of proinflammatory cytokines. IDO has been shown to be dysregulated in different malignancies[96].

5 TUMOR IMMUNOTHERAPY

The basic concept of tumor immunotherapy is to stimulate and use the immune system to attack tumor cells. The main methods for doing this are cytokines, monoclonal antibodies, cell-based therapies and vaccines[97,98].

5.1 CYTOKINES

Cytokines are polypeptides or glycoproteins and can be released in response to stimulus. They are usually short-lived, and have important roles in signaling pathways related to differentiation, growth and inflammation/anti-inflammation. In cancer treatment, cytokines can either have a direct anti-proliferative or pro-apoptotic effect, or they can work indirectly by stimulating immune cells. However, the clinical effect of cytokines is limited. This is mainly due to short half-life, problems to achieve effective concentrations within the tumor and toxicity. Interleukin-2 and interferon-alpha have shown some effect for treatments of malignancies, including metastatic renal cell carcinoma and metastatic melanoma, and are approved by The Food and Drug Administration (FDA). Several studies are ongoing trying to improve the effectiveness by improving pharmacokinetics or by combining other treatments with cytokines[99–101].

5.2 ADOPTIVE CELL THERAPY

Adoptive T cell therapy is using *ex vivo* manipulated T cells to eradicate tumor cells. This is done by first isolating lymphocytes from the patient's peripheral blood or tumor tissue. The lymphocytes are then manipulated and expanded *ex vivo* before they are re-infused to the patient. The methods for manipulation can include selection and expansion of specific TILs. The lymphocytes can also be genetically manipulated to express a synthetic T cell receptor (sTCR). The sTCR is designed to recognize a target antigen on the tumor cells, and requires that the tumor expresses antigens through the MHC. Another method is to transfer a chimeric antigen receptor (CAR) to the T cells. CAR T cells target tumor antigens independent of the MHC[98,102,103].

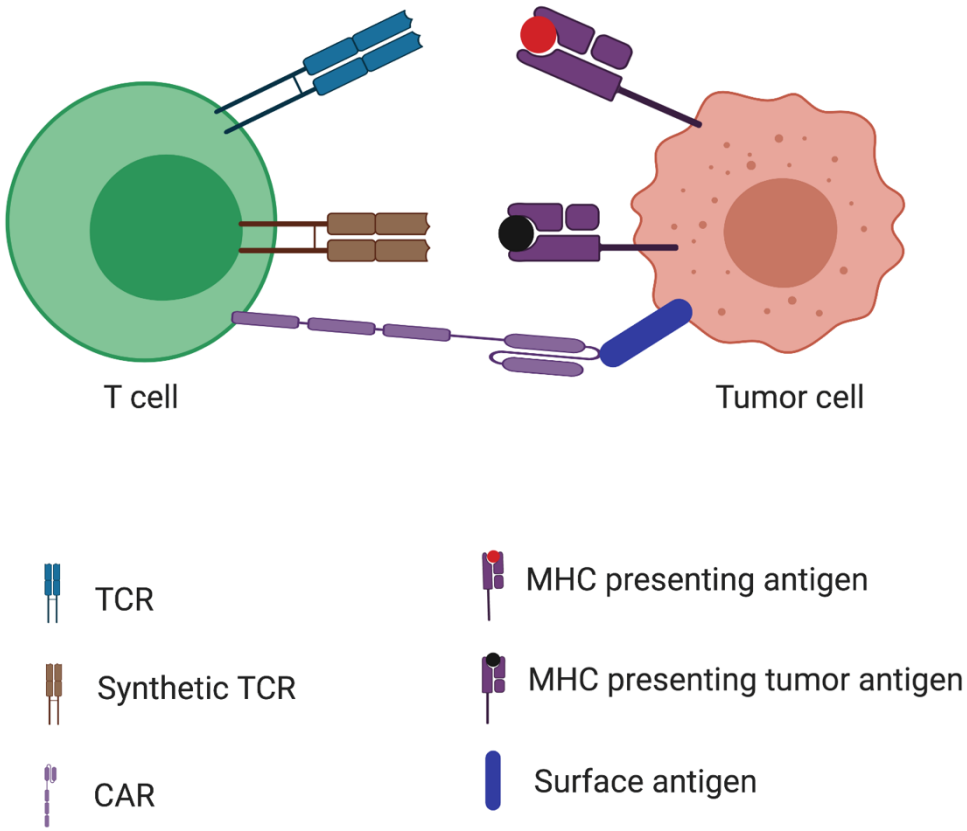

Figure 4. Different types of adoptive cell therapy. The T cells can be genetically manipulated to express a synthetic T cell receptor (TCR) or a chimeric antigen receptor (CAR). Created with BioRender.com.

5.3 MONOCLONAL ANTIBODIES

Antibodies for treatment of cancer is an important and effective therapeutic approach. They can either target antigens expressed by the tumor or target the tumor microenvironment. The targets expressed by the tumor include growth factor receptors, and by blocking the receptor or their signal pathway, apoptosis can be induced or growth rate decreased. Targets in the microenvironment include blocking vascular endothelial growth factors to inhibit angiogenesis. Antibodies can also be used to enhance immune response, for example previously described immune checkpoint blockade[104].

5.4 TUMOR VACCINES

Therapeutic cancer vaccines are designed to activate and stimulate the patients T cells *in vivo*. The most common types of vaccines consist of tumor specific antigens. Dendritic cells

can be used as adjuvants to increase the effectiveness. However, so far the clinical effect of therapeutic vaccines has been limited. In 2010, Sipuleucel-T was approved by FDA for treatment of metastatic castrate-resistant prostate cancer[105].

5.5 IMMUNOTHERAPY AND SARCOMAS

The treatment options for most sarcomas are limited to surgical resection, radiation and chemotherapy and new therapies are needed. Many trials have investigated the effect of different immunotherapies, but so far most of them have shown limited effect[74].

Expression of both PD-1 and PD-L1 has been reported in varying levels in different types of sarcomas. Torabi *et al*. showed overexpression of PD-1 in most cases of osteosarcoma, chondrosarcomas, liposarcomas and rhabdomyosarcomas[106]. In a study by Vanderstraeten *et al.,* expression of PD-L1 was seen in 100% of primary uterine sarcomas. Expression of PD-L2 was seen in 32% and B7-H4 in 100% of primary uterine sarcomas. However, the type of uterine sarcoma was not specified[107].

D'Angelo *et al.* investigated the expression of PD-L1 in 50 cases of different types of soft tissue sarcomas and found that 12% expressed PD-L1 in tumor cells, 30% in lymphocytes and 58% in macrophages. However, there was no association with overall survival[108]. Toulmonde *et al.* published a clinical trial investigating the efficacy and safety of targeting PD-1 in combination with metronomic chemotherapy in sarcomas. Leiomyosarcomas, undifferentiated pleomorphic sarcomas, other sarcomas and gastrointestinal stromal sarcomas were included. Of 50 patients, three were progression-free at six months, one of those hade endometrial stromal sarcoma[109]. Several clinical trials using check point blockade are ongoing[74].

Tawbi *et al*. published a clinical trial in 2017 examining the effect of Pembrolizumab (anti-PD-1 antibody) in advanced soft-tissue and bone sarcoma. Of 40 patients with soft-tissue sarcoma 18% had an objective response. The best effect had patients with undifferentiated pleomorphic sarcoma, four of ten patients had an objective response (partial response or better according to Response Evaluation Criteria In Solid Tumors (RECIST) version 1.1)[110].

D'Angelo *et al*. investigated the effect of Nivolumab (anti-PD-1 antibody) with or without Ipilimumab (CTLA-4 inhibitor) in 85 patients with locally advanced, metastatic or unresectable sarcoma. In the group with patients receiving nivolumab as monotherapy 5% (2/38) had a response, and in the group receiving nivolumab plus ipilimumab 16% (6/38) had a response[111].

Vaccines are a promising therapy for sarcomas. Many sarcomas have unique chromosomal translocations, genetic abnormalities and expression of specific antigens that could work as targets for vaccines[74]. Several vaccine trials investigating different target molecules in sarcomas are ongoing[112].

6 AIMS OF THE THESIS

The overall aim of the thesis is to analyze different clinical, histological and biological parameters of endometrial stromal sarcomas in relation to patient survival.

Aims of paper I: To investigate the correlation of clinicopathological parameters, biomarkers, YWHAE-FAM22 status and prognosis in undifferentiated uterine sarcomas.

Aims of paper II: To validate the result from paper I in an independent cohort of undifferentiated uterine sarcomas, and to investigate the relation of other clinicopathological parameters in relation to mitotic index group.

Aims of paper III: To identify molecular subgroups of undifferentiated uterine sarcomas and evaluate the possible correlation to different clinicopathological parameters.

Aims of paper IV: To analyze the prognostic significance of different immune markers in low-grade endometrial stromal sarcomas.

7 MATERIAL AND METHODS

7.1 PATIENT COHORTS

Paper I

The cohort of paper I consisted of 26 cases of undifferentiated uterine sarcoma identified by searching the pathological database at the Karolinska University Hospital and the Stockholm region cancer registry for patients diagnosed with undifferentiated endometrial sarcoma and endometrial stromal sarcoma between January 1, 1987 and January 1, 2008. All hematoxylin and eosin stained slides were reviewed and pathological parameters were recorded. Clinical records were reviewed for clinical parameters, including status at last follow-up, age at diagnosis and stage at diagnosis. For 22 cases tissue blocks were available and a tissue microarray was constructed for immunohistochemical analysis of expression of different proteins.

Paper II

In paper II we reviewed 92 cases of uterine sarcoma from three institutions: the Norwegian Radium Hospital, the Mayo Clinic, and Skåne University Hospital. Of these, 40 cases of undifferentiated uterine sarcoma were included. Hematoxylin and eosin stained slides were reviewed before inclusion, and a detailed histological evaluation was done, including dividing the cases into our previously (from paper I) defined high and low mitotic index groups. All cases had clinical follow-up data and all cases were analyzed for the presence of YWHAE-FAM22 and JAZF1-JJAZ1 translocations.

Paper III

The cohort of paper III consisted of cases from the two previously described cohorts of undifferentiated uterine sarcoma in paper I and II, and was complemented by additional cases of undifferentiated uterine sarcoma from Vancouver General Hospital and Brigham and Women's Hospital. In total 50 cases were analyzed for gene expression, copy number variation, cell morphometry and protein expression. Histopathological parameters and follow-up data for the cases not previously included were recorded. All cases had formalin-fixed paraffin-embedded (FFPE) tumor material used for isolation of DNA and RNA at the Karolinska University Hospital.

Paper IV

The cohort of paper IV consisted of 21 cases of low-grade endometrial stromal sarcomas diagnosed at the Karolinska University Hospital. All cases had follow-up data and FFPE tumor blocks. Tissue microarrays consisting of two biopsies of tumor material from each case were constructed. The TMAs were stained with immunofluorescence labeled antibodies targeting CD8, FOXP3, CD68, CD163, IDO1, B7-H4 and PD-L1. The slides were scanned for digital image analysis in QuPath.

7.2 TUMOR TISSUE

When a tumor is surgically removed and sent to the pathology department the tissue is fixated in 4% formaldehyde. After fixation the tumor is cut and pieces are selected for embedding in paraffin blocks. After embedding, the tumor can be cut into thin sections and mounted on glass slides. The slides can then be stained with for example hematoxylin and eosin or used for immunohistochemical stains.

The tissue blocks with FFPE tumor material as well as the stained slides are saved in archives and can be used for research after ethical approval.

7.3 TISSUE MICROARRAY

A tissue microarray (TMA) is a paraffin block containing tumor cores from multiple tumors. To create the TMAs used in these projects, the hematoxylin eosin slides were used to find a representative tumor area, and then used as a guide when taking two cores from the corresponding tissue block. The cores were then inserted into a paraffin block in a specific pattern to create a TMA. The TMAs were then cut and stained with hematoxylin eosin to verify the presence of representative tumor tissue.

7.4 IMMUNOHISTOCHEMISTRY AND IMMUNOFLUORESCENCE

Immunohistochemistry and immunofluorescence are used to evaluate the expression of different proteins (antigens) in a tissue. Both techniques use antibodies with affinities to the proteins of interest, but the technique to visualize the antigen-antibody interaction differs. Both direct and indirect methods can be used in both techniques. In the direct method one labelled antibody is used to bind the antigen of interest, and can then be detected. With the indirect method an unlabeled, primary antibody is used for binding to the protein of interest. A secondary antibody is then used to bind the primary antibody. The secondary

antibody can be either directly bound to a reporter molecule, or conjugated to a linker molecule that then recruits reporter molecules.

The type of reporter molecule varies depending on the detection method. A chromogenic reporter uses an enzyme (for example alkaline phosphatase or horseradish peroxidase) to create a color product that can be detected with a light microscope. In immunofluorescence the antibody is instead labeled with a fluorophore that can be detected with a fluorescent microscope.

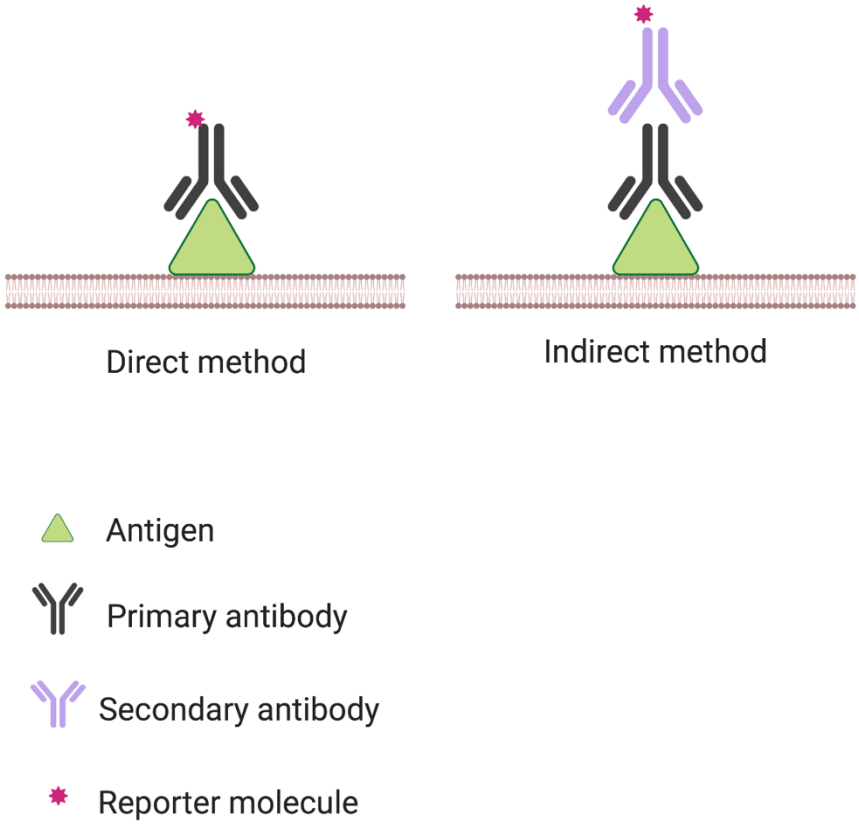

Figure 5. Direct and indirect method. The indirect method uses a secondary antibody for detection. Created with BioRender.com.

7.5 POLYMERASE CHAIN REACTION

Polymerase chain reaction (PCR) is a method to amplify a specific DNA region. The DNA template is mixed with a DNA polymerase, specific DNA primers for the target region and deoxynucleoside triphosphates (dNTPs). Cycles of temperature changes amplifies the DNA region of interest into millions of copies by several repeated steps, including denaturation,

annealing and elongation. The use of fluorescent DNA probes allows for detection of PCR products in real-time by measuring the fluorescent signal, which is called real-time PCR or quantitative PCR (qPCR)[31].

As PCR requires DNA, normal PCR cannot be used for detection of mRNA. For detection of mRNA, reverse transcription polymerase chain reaction (RT-PCR) can be used. In RT-PCR, the RNA template is first transcribed into complementary DNA (cDNA) by a polymerase and primers. The cDNA can then be amplified by PCR[113]. The combination of RT-PCR and qPCR is called real-time RT-PCR or qRT-PCR.

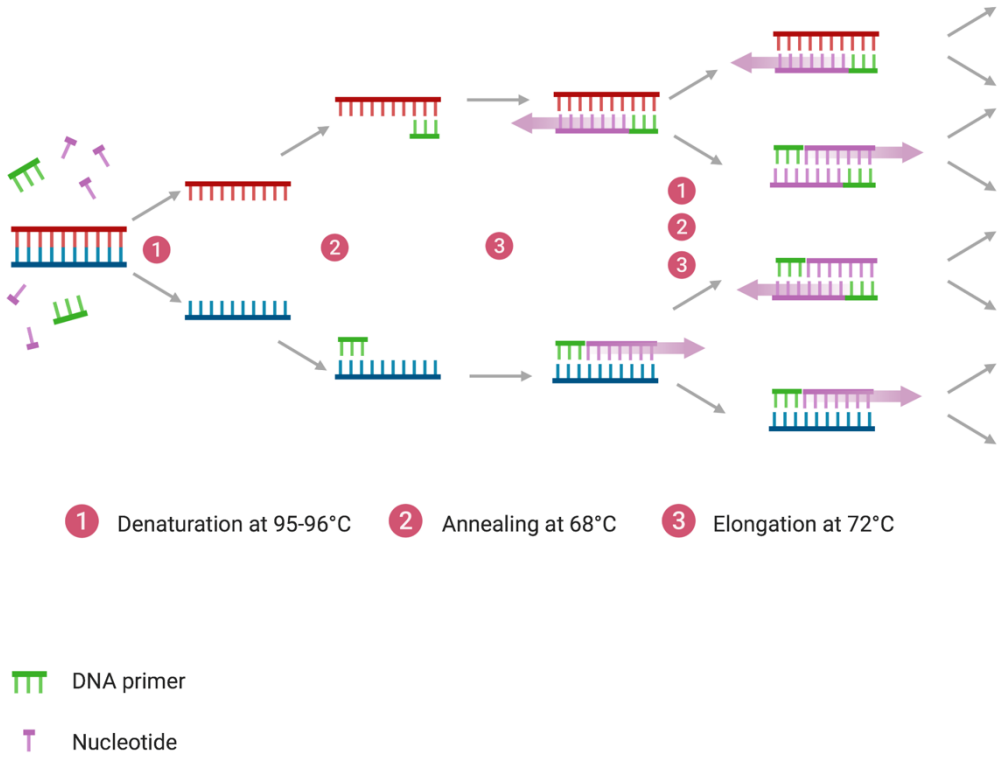

Figure 6. Polymerase chain reaction (PCR). Primers, nucleotides and DNA polymerase are used to amplify a DNA region. Created with BioRender.com.

7.6 DNA MICROARRAYS

DNA microarrays are used to measure expression levels of many genes at the same time or for genotyping. The basic principle is to use short, specific sections of DNA (probes) attached to a chip. The sample to be analyzed is labeled with fluorescence and then hybridized to the probes on the chip. Sequences with non-specific bindings are washed off, and fluorescence signals from the sequences bound to the probes can be analyzed. The intensity of the signal will be proportional to the amount of sample that has bound[31].

As different genes are turned on and off in different cells and under different conditions, expression profiling techniques used to measure the amount of mRNA expressed will tell us other things about the cells than only looking at the DNA. In the third paper, RNA expression was evaluated with GeneChip Human Gene 2.1 ST Array Plate, which is an array that measures more than 30 000 coding transcripts[114].

For analysis of DNA copy number variation in the third paper, Affymetrix OncoScan Array was used. This is a microarray-based method for whole-genome copy number analysis including copy number gain and loss, and loss of heterozygosity. A panel of somatic mutations is also included, but was not analyzed in these projects[115].

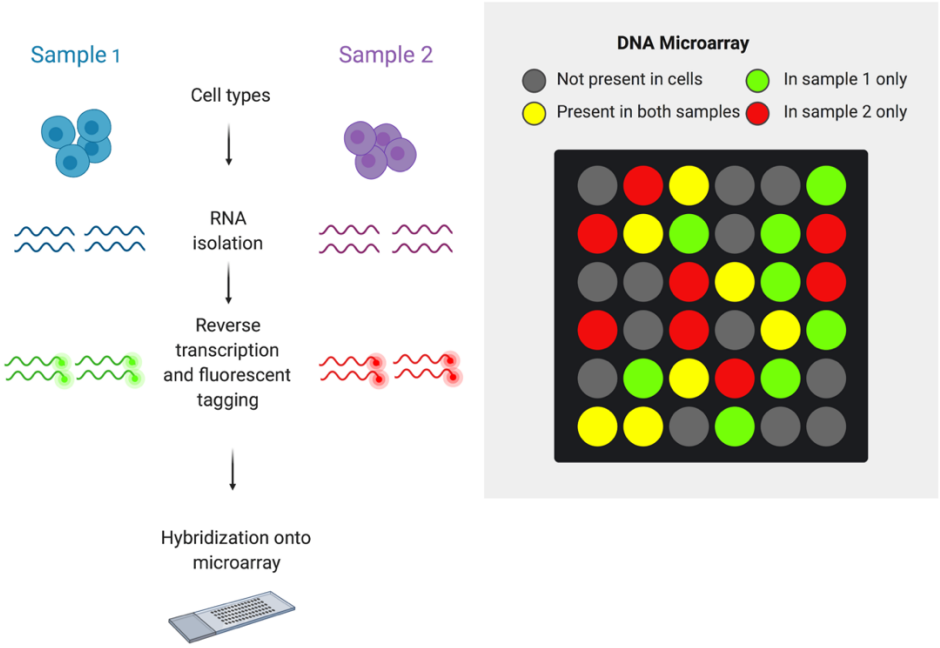

Figure 7. Simplified picture of the principles of a DNA microarray. Created with BioRender.com.

7.7 IMAGE ANALYSIS

Stained glass slides from tissue blocks or TMAs can be digitized by the use of digital slide scanners. The glass slides are thereby converted into high-resolution digital files that can be imported to a software for digital image analysis. In project III and IV the open source software QuPath[116] was used to analyze the scanned images. In our studies, we have used QuPath for cell quantification, calculation of nuclear area and quantification of immunohistochemical and immunofluorescence expression.

8 STATISTICAL ANALYSIS

Descriptive statistics has been used in all papers. The characteristics of the patient cohorts have frequently been presented in tables with mean values and standard deviations.

Different methods have been used for survival analysis. Cox proportional hazard regression models have been used for both analysis of each variable one at the time as a single explanatory variable (crude result) and also together as multiple explanatory variables (adjusted result). The result has been presented with hazard ratios, 95% confidence intervals and *p*-values.

Survival curves for different groups has been plotted according to the Kaplan-Meier method. To compare survival between groups, the log-rank test has been used and presented with *p*-value.

The t test was used for testing differences in nuclear area, cells per area and immunohistochemical expression between the RNA groups in paper III. An empirical Bayes moderated t test was used to test for differentially expressed genes between the RNA groups. To organize the genes into ontologies and summarize them, DAVID (Database for Annotation, Visualization and Integrated Discovery)[117,118] and REVIGO (Reduce and Visualize Gene Ontology)[119] were used.

9 RESULTS

Paper I

In the first paper, we showed that when dividing the cases of undifferentiated uterine sarcoma into two groups based on mitotic index (over or under 25 mitoses/10 HPF (2.24 mm^2)), there was a statistically significant difference in prognosis between the groups. The expression of either ER, PR or the presence of the YWHAE-FAM22 translocation was associated with a low mitotic index. Nuclear atypia, stage, presence of tumor necrosis or expression of p53, P16, Ki67, Cyclin-D1, or ANLN did not have a statistically significant impact on survival.

Paper II

In the second paper, we showed that in the crude Cox Proportional Hazard model, mitotic index group, patient age, stage, and the presence of tumor necrosis were prognostic variables. In the adjusted model, mitotic index group and stage had a statistically significant impact on overall survival. Nuclear atypia was not prognostic. None of the cases had the YWHAE-FAM22 or JAZF1-JJAZ1 translocation.

Paper III

In the third paper, we showed that the cases could be divided into four groups with different mRNA expression pattern. Gene ontology analysis showed activation of pathways related to genital tract development, extracellular matrix, muscle function and proliferation in the different groups. The result of the chromosomal copy number analysis showed a spectrum of variation, from cases that were diploid or near diploid to cases with extensive chromosomal aberrations. The adjusted Cox Proportional Hazard model showed that mitotic index group, hormone receptor expression and mRNA group had a statistically significant impact on overall survival. In the ontology analysis, the mRNA group with the worst prognosis showed overexpressed pathways related to the extracellular matrix (so called “ECM group”). When further analyzed by image analysis, the ECM group was characterized by reduced cell density and increased nuclear size compared to the other groups. The ECM group also showed higher expression of the four ECM related proteins, matrix metalloproteinase 14, collagen 1, collagen 6 and fibronectin, when evaluated with immunohistochemistry.

Paper IV

In the fourth paper, we showed that all included cases of LGESS had infiltration of CD8+ lymphocytes. Based on the mean expression of each marker as a cut-off, high and low expression groups were defined. We demonstrated that the group with a high number of CD8+ lymphocytes had a better prognosis than the group with a low number. However, the difference in survival was not statistically significant. We also demonstrated that high numbers of FOXP3+ was associated with a favorable prognosis. Calculating the ratio of CD8+/FOXP3+ cells showed a statistically significant ($p=0.0087$) better survival for the patients with a low ratio. No expression of PD-L1 was seen, however most cases expressed B7-H4. The quantity of CD68+ macrophages, CD68+CD163+ M2-type macrophages and IDO1 was not prognostic.

10 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Undifferentiated uterine sarcomas are highly malignant, aggressive tumors with a poor prognosis. It is a rare tumor type, and the knowledge of prognostic markers, biology and optimal treatment is limited.

The first three papers in this thesis focus on the biology and identifying prognostic subgroups within undifferentiated uterine sarcomas. Many tumor types are heterogeneous, and the prognosis differs between patients with the same tumor type. Different prognostic markers are identified in other tumors, for example tumor thickness in melanomas[120].

In the first paper, we demonstrated that a mitotic index cutoff can be used for prognostic classification of UUS. Earlier studies had been limited by the change of diagnostic criteria over the years as well as rareness of these tumors. We could also see that 7/12 patients in the low mitotic index group survived for more than five years, which was an interesting finding considering that UUS previously has been regarded a group of tumors with uniformly very poor prognosis. The expression of hormone receptors (ER or PR) correlated with a low mitotic index, but is also an interesting finding for a possible target therapy.

In the second paper, we validated our previous finding of mitotic index as a prognostic marker. Mitotic counting is a quite simple method to implement in the clinical routine as it requires no extra equipment, and is already used in other tumors. One drawback with mitotic counting is the varying reproducibility. However, in our two studies most cases did not have a mitotic index close to the cut-off. Based on our results, we proposed that mitotic index could be used as prognostic marker in UUS. We also proposed that UUS tumors should be divided into “mitogenic” and “not otherwise specified” types based on the mitotic index, to reflect the difference in prognosis between the two types.

In the third paper, we identified four subgroups of UUS based on gene-expression analysis. The four groups had different clinical and pathological characteristics, and gene ontology showed different activated pathways. The group with overexpression of pathways related to the extracellular matrix (ECM) had the worst prognosis, and also had specific immunohistochemical characteristics related to extracellular proteins. The overexpression of matrix metalloproteinases in the ECM group is a possible therapeutic target in the future. These findings also raise interesting questions about how these tumors interact with the tumor microenvironment and the importance of the interaction for the tumor

aggressiveness. Another interesting finding was that about 50% percent of the cases were diploid or near diploid, which might indicate that there are unknown translocations in these tumors.

In the fourth paper, we characterized the immune microenvironment in low-grade endometrial stromal sarcomas. The immune microenvironment has been recognized in other tumor types to be of great importance for both tumor progression and treatment response. We could demonstrate a statistically significant better survival for LGESS patients with a low ratio of CD8+/FOXP3+ cells compared to patients with a high ratio. In many tumor types, high numbers of FOXP3+ cells are associated with a poor prognosis. However, in some tumors, high amounts of FOXP3+ cells has been associated with a better prognosis. The mechanism for this is unknown. No expression of PD-L1 was seen, but expression of another check-point inhibitor, B7-H4, was seen in most cases. This is interesting since blockade of check-point inhibitors is an expanding field with new treatments under development.

The fourth paper also required a setup of new collaborations and development of methods. Hopefully, this will work as a fundament for new studies, including both validation of the results in paper four in a new independent cohort, as well as investigating the immune microenvironment in other types of uterine sarcomas.

Uterine stromal sarcomas are a heterogenous group of tumors, ranging from low-grade endometrial stromal sarcomas with a general good prognosis but with risk of recurrence, to undifferentiated uterine sarcomas with a much poorer prognosis. The rarity of uterine sarcomas, especially stromal sarcomas, hampers their inclusion in large randomized clinical trials for evaluation of new treatments. In the developing era of personalized medicine and immune therapy, it becomes of increasing importance to learn more about the biology and tumor microenvironment of different malignancies. The findings in this thesis provide additional insights in the biology of uterine stromal sarcomas, and will hopefully lead to new, more effective and more precise therapeutic options for uterine sarcomas in the future.

11 ACKNOWLEDGEMENTS

I owe gratitude to a large number of people who have helped me during the work. Thank you all! I would particularly want to mention:

My supervisor **Joseph Carlson** and my co-supervisors **Anders Isaksson** and **Hanna Dahlstrand** for support and making this work possible**.**

All collaborators in my projects. **Anders Isaksson's group** at Uppsala University and **Kaisa Lehti's group** at Karolinska Institutet, especially **Okan Gültekin**. Also **Anna Måsbäck** at Skåne University Hospital and all our international collaborators **Ben Davidson, John K Schoolmeester, Tjalling Bosse, Marisa R Nucci** and **Cheng-Han Lee**.

There are a lot of people in the department of pathology and cytology at Karolinska University Hospital that has inspired me, supported me and given me a lot of opportunities to discuss both research and clinical work over the years. Especially I would like to thank **Cristian Ortiz-Villalón, Peter Zickert, Anders Herder, Britta Krynitz, Ismini Vassilaki** and **Anders Höög**.

I would also like to thank **Caroline Rönnlund** and **Sandra Wessman** for participation and research discussions in our own journal club.

A special thanks to my college and running partner **Stephanie Robertson** for proofreading and all our discussions about research, work, training and life. I know your thesis will be great.

Finally, I also wish to thank **my family**. Especially my father **Lennart Hardell** for inspiration and never stop believing in research, and my mother **Catharina Wahlstedt. Johan, Ebba and Olof** for being patient and letting me have the time I needed for finishing the thesis.

12 REFERENCES

1. Abeler, V. M. *et al.* Uterine sarcomas in Norway. A histopathological and prognostic survey of a total population from 1970 to 2000 including 419 patients. *Histopathology* **54**, 355–364 (2009).

2. World Health Organization Classification of Tumours. *Tumours of the breast and female genital organs*. (IARCPress, 2002).

3. Kurihara, S. *et al.* Endometrial stromal sarcomas and related high-grade sarcomas: Immunohistochemical and molecular genetic study of 31 cases. *Am. J. Surg. Pathol.* **32**, 1228–1238 (2008).

4. Lee, C.-H. *et al.* 14-3-3 Fusion Oncogenes in High-Grade Endometrial Stromal Sarcoma. *Proc. Natl. Acad. Sci.* **109**, 929–934 (2012).

5. Lee, C.-H. H. *et al.* The clinicopathologic features of YWHAE-FAM22 endometrial stromal sarcomas: a histologically high-grade and clinically aggressive tumor. *Am. J. Surg. Pathol.* **36**, 641–53 (2012).

6. World Health Organization Classification of Tumours. *WHO Classification of Tumours of Female Reproductive Organs*. (International Agency for Research on Cancer(IARC), 2014).

7. Busca, A., Gulavita, P., Parra-Herran, C. & Islam, S. IFITM1 Outperforms CD10 in Differentiating Low-grade Endometrial Stromal Sarcomas from Smooth Muscle Neoplasms of the Uterus. *Int. J. Gynecol. Pathol.* **37**, 372–378 (2018).

8. D'Angelo, E. & Prat, J. Uterine sarcomas: A review. *Gynecol. Oncol.* **116**, 131–139 (2010).

9. Glenn McCluggage, W. & Lee, C. H. YWHAE-NUTM2A/B Translocated High-grade Endometrial Stromal Sarcoma Commonly Expresses CD56 and CD99. *Int. J. Gynecol. Pathol.* **38**, 528–532 (2019).

10. Lee, C.-H. & Nucci, M. R. Endometrial stromal sarcoma - the new genetic paradigm. *Histopathology* **67**, 1–19 (2015).

11. Nucci, M. R. Practical issues related to uterine pathology: endometrial stromal tumors. *Mod. Pathol.* **29**, S92–S103 (2016).

12. Hoang, L., Chiang, S. & Lee, C. H. Endometrial stromal sarcomas and related neoplasms: new developments and diagnostic considerations. *Pathology* **50**, 162–177 (2018).

13. Prat, J. FIGO staging for uterine sarcomas. *Int. J. Gynecol. Obstet.* **104**, 177–178 (2009).

14. Brierley, J., Gospodarowicz, M. & Wittekind, C. *TNM classification of malignant tumours*. (John Wiley & Sons, Ltd, 2017).

15. Prat, J. & Mbatani, N. Uterine sarcomas. *Int. J. Gynecol. Obstet.* **131**, 105–110 (2015).

16. American Joint Committee of Cancer. *AJCC Cancer Staging Manual*. (Springer International Publishing, 2017).

17. Amant, F. *et al.* Gynecologic cancer intergroup (GCIG) Consensus review for Endometrial Stromal Sarcoma. *Int. J. Gynecol. Cancer* **24**, S67–S72 (2014).

18. Dangoor, A. *et al.* UK guidelines for the management of soft tissue sarcomas. *Clin. Sarcoma Res.* (2016). doi:10.1186/s13569-016-0060-4

19. Amant, F., Coosemans, A., Debiec-Rychter, M., Timmerman, D. & Vergote, I. Clinical management of uterine sarcomas. *Lancet Oncol.* **10**, 1188–1198 (2009).

20. Thanopoulou, E. & Judson, I. Hormonal therapy in gynecological sarcomas. *Expert Rev. Anticancer Ther.* **12**, 885–894 (2012).

21. Hrzenjak, A., Dieber-Rotheneder, M., Moinfar, F., Petru, E. & Zatloukal, K. Molecular mechanisms of endometrial stromal sarcoma and undifferentiated endometrial sarcoma as premises for new therapeutic strategies. *Cancer Lett.* **354**, 21–27 (2014).

22. Reich, O. & Regauer, S. Estrogen replacement therapy and tamoxifen are contraindicated in patients with endometrial stromal sarcoma. *Gynecol. Oncol.* **102**, 412–413 (2006).

23. Altman, A. D., Nelson, G. S., Chu, P., Nation, J. & Ghatage, P. Uterine Sarcoma and Aromatase Inhibitors. *International Journal of Gynecological Cancer* **22**, 1006–1012 (2012).

24. Reich, O. & Regauer, S. Hormonal therapy of endometrial stromal sarcoma. *Curr. Opin. Oncol.* **19**, 347–352 (2007).

25. Pautier, P. *et al.* Gynecologic cancer intergroup (GCIG) consensus review for high-grade undifferentiated sarcomas of the uterus. *Int. J. Gynecol. Cancer* **24**, S73–S77 (2014).

26. Pautier, P. *et al.* A randomized clinical trial of adjuvant chemotherapy with doxorubicin, ifosfamide, and cisplatin followed by radiotherapy versus radiotherapy alone in patients with localized uterine sarcomas (SARCGYN study). A study of the French sarcoma group. *Ann. Oncol.* **24**, 1099–1104 (2013).

27. Pautier, P. *et al.* A randomized clinical trial of adjuvant chemotherapy with doxorubicin, ifosfamide and cisplatin (API), followed by radiotherapy versus radiotherapy alone in patients with localized uterine sarcomas (SARCGYN study). Update at 10 years. *Ann. Oncol.* **28**, 521–522 (2017).

28. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: The next generation. *Cell* **144**, 646–674 (2011).

29. Pietras, K. & Östman, A. Hallmarks of cancer: Interactions with the tumor stroma. *Experimental Cell Research* **316**, 1324–1331 (2010).

30. Lu, P., Weaver, V. M. & Werb, Z. The extracellular matrix: A dynamic niche in cancer progression. *Journal of Cell Biology* **196**, 395–406 (2012).

31. Cooper, G. M. & Hausman, R. E. *The cell : a molecular approach*. (Sinauer Associates, 2016).

32. Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* **100**, 57–70 (2000).

33. Giam, M. & Rancati, G. Aneuploidy and chromosomal instability in cancer: A jackpot to chaos. *Cell Div.* **10**, 1–12 (2015).

34. Gordon, D. J., Resio, B. & Pellman, D. Causes and consequences of aneuploidy in cancer. *Nat. Rev. Genet.* **13**, 189–203 (2012).

35. Taylor, B. S. *et al.* Advances in sarcoma genomics and new therapeutic targets. *Nature Reviews Cancer* **11**, 541–557 (2011).

36. Abeshouse, A. *et al.* Comprehensive and Integrated Genomic Characterization of

Adult Soft Tissue Sarcomas. *Cell* **171**, 950-965.e28 (2017).

37. Hoang, L. N. *et al.* Novel high-grade endometrial stromal sarcoma: A Morphologic Mimicker of Myxoid Leiomyosarcoma. *Am. J. Surg. Pathol.* **41**, 12–24 (2017).

38. ELSTON, C. W. & ELLIS, I. O. pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* **19**, 403–410 (1991).

39. Rakha, E. A. *et al.* Prognostic significance of nottingham histologic grade in invasive breast carcinoma. *J. Clin. Oncol.* **26**, 3153–3158 (2008).

40. Rakha, E. A. *et al.* Breast cancer prognostic classification in the molecular era: The role of histological grade. *Breast Cancer Res.* **12**, (2010).

41. Simpson, J. F., Dutt, P. L. & Page, D. L. Expression of mitoses per thousand cells and cell density in breast carcinomas: A proposal. *Hum. Pathol.* **23**, 608–611 (1992).

42. Haapasalo, H., Pesonen, E. & Collan, Y. Volume Corrected Mitotic Index (M/V-INDEX): The Standard of Mitotic Activity in Neoplasms. *Pathol. Res. Pract.* **185**, 551–554 (1989).

43. Bullough, W. S. Mitotic activity in the tissues of dead mice, and in tissues kept in physiological salt solutions. *Exp. Cell Res.* **1**, 410–420 (1950).

44. Baak, J. P. A. Mitosis Counting in Tumors. *Hum. Pathol.* **21**, 683–685 (1990).

45. Donhuijsen, K., Schmidt, U., Hirche, H., van Beuningen, D. & Budach, V. Changes in mitotic rate and cell cycle fractions caused by delayed fixation. *Hum. Pathol.* **21**, 709–714 (1990).

46. Yigit, N. *et al.* Are we counting mitoses correctly? *Ann. Diagn. Pathol.* **17**, 536–539 (2013).

47. van Diest, P. J. *et al.* Reproducibility of mitosis counting in 2,469 breast cancer specimens: Results from the Multicenter Morphometric Mammary Carcinoma Project. *Hum. Pathol.* **23**, 603–607 (1992).

48. Deroo, B. J. *et al.* Estrogen receptors and human disease. *Nature* **116**, 561–570 (2014).

49. Check, J. H. The role of progesterone and the progesterone receptor in cancer. *Expert*

Rev. Endocrinol. Metab. **12**, 187–197 (2017).

50. Reich, O., Regauer, S., Urdl, W., Lahousen, M. & Winter, R. Expression of oestrogen and progesterone receptors in low-grade endometrial stromal sarcomas. *Br. J. Cancer* **82**, 1030–4 (2000).

51. Muller, P. A. J. & Vousden, K. H. P53 Mutations in Cancer. *Nat. Cell Biol.* **15**, 2–8 (2013).

52. Duffy, M. J. *et al.* P53 as a target for the treatment of cancer. *Cancer Treat. Rev.* **40**, 1153–1160 (2014).

53. Wade, M., Li, Y. C. & Wahl, G. M. MDM2, MDMX and p53 in oncogenesis and cancer therapy. *Nat. Rev. Cancer* **13**, 83–96 (2013).

54. Scholzen, T. & Gerdes, J. The Ki-67 protein: From the known and the unknown. *J. Cell. Physiol.* **182**, 311–322 (2000).

55. Cuylen, S. *et al.* Ki-67 acts as a biological surfactant to disperse mitotic chromosomes. *Nature* **535**, 308–312 (2016).

56. Penault-Llorca, F. & Radosevic-Robin, N. Ki67 assessment in breast cancer: an update. *Pathology* **49**, 166–171 (2017).

57. Yerushalmi, R., Woods, R., Ravdin, P. M., Hayes, M. M. & Gelmon, K. A. Ki67 in breast cancer: prognostic and predictive potential. *The Lancet Oncology* **11**, 174–183 (2010).

58. Qie, S. & Diehl, J. A. Cyclin D1, cancer progression, and opportunities in cancer treatment. *J. Mol. Med.* **94**, 1313–1326 (2016).

59. Sherr, C. J., Beach, D. & Shapiro, G. I. Targeting CDK4 and CDK6: From discovery to therapy. *Cancer Discov.* **6**, 353–367 (2016).

60. Lee, C. *et al.* Cyclin D1 as a Diagnostic Immunomarker for Endometrial Stromal Sarcoma With YWHAE-FAM22 Rearrangement. *The American Journal of Surgical Pathology* **36**, 1562–1570 (2012).

61. Li, J., Poi, M. J. & Tsai, M. D. Regulatory mechanisms of tumor suppressor P16INK4A and their relevance to cancer. *Biochemistry* **50**, 5566–5582 (2011).

62. He, S. & Sharpless, N. E. Senescence in Health and Disease. *Cell* **169**, 1000–1011

(2017).

63. LaPak, K. M. & Burd, C. E. The Molecular Balancing Act of p16INK4a in Cancer and Aging. *Mol. Cancer Res.* **12**, 167–183 (2014).

64. Zhang, L. & Maddox, A. S. Anillins. *Curr. Biol.* **20**, 135–136 (2010).

65. Piekny, A. J. & Maddox, A. S. The myriad roles of Anillin during cytokinesis. *Semin. Cell Dev. Biol.* **21**, 881–891 (2010).

66. Bremnes, R. M. *et al.* The role of tumor-infiltrating lymphocytes in development, progression, and prognosis of non-small cell lung cancer. *J. Thorac. Oncol.* **11**, 789–800 (2016).

67. Zou, W. Regulatory T cells, tumour immunity and immunotherapy. *Nat. Rev. Immunol.* **6**, 295–307 (2006).

68. Munn, D. H. & Bronte, V. Immune suppressive mechanisms in the tumor microenvironment. *Current Opinion in Immunology* **39**, 1–6 (2016).

69. Pardoll, D. Cancer and Immune System: Basic Concepts and Targets for Intervention. *Semin. Oncol.* **42**, 523–538 (2015).

70. Fridman, W. H., Pagès, F., Sautès-Fridman, C. & Galon, J. The immune contexture in human tumours: Impact on clinical outcome. *Nat. Rev. Cancer* **12**, 298–306 (2012).

71. Ostroumov, D., Fekete-Drimusz, N., Saborowski, M., Kühnel, F. & Woller, N. CD4 and CD8 T lymphocyte interplay in controlling tumor growth. *Cellular and Molecular Life Sciences* **75**, 689–713 (2018).

72. Durgeau, A., Virk, Y., Corgnac, S. & Mami-Chouaib, F. Recent advances in targeting CD8 T-cell immunity for more effective cancer immunotherapy. *Front. Immunol.* **9**, (2018).

73. Melief, C. J. M., Kastenmüller, W., Ahrends, T., Bąbała, N. & Borst, J. CD4+ T cell help in cancer immunology and immunotherapy. *Nat. Rev. Immunol.* **18**, 635–647 (2018).

74. Nathenson, M. J., Conley, A. P. & Sausville, E. Immunotherapy: A New (and Old) Approach to Treatment of Soft Tissue and Bone Sarcomas. *Oncologist* **23**, 71–83 (2018).

75. Shitara, K. & Nishikawa, H. Regulatory T cells: A potential target in cancer immunotherapy. *Ann. N. Y. Acad. Sci.* **1417**, 104–115 (2016).

76. Deng, G. Tumor-infiltrating regulatory T cells: origins and features. *Am. J. Clin. Exp. Immunol.* **7**, 81–87 (2018).

77. Sato, E. *et al.* Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 18538–18543 (2005).

78. Salama, P. *et al.* Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. *J. Clin. Oncol.* **27**, 186–192 (2009).

79. Carreras, J. *et al.* High numbers of tumor-infiltrating FOXP3-positive regulatory T cells are associated with improved overall survival in follicular lymphoma. *Blood* **108**, 2957–2964 (2006).

80. Biswas, S. K., Allavena, P. & Mantovani, A. Tumor-associated macrophages: Functional diversity, clinical significance, and open questions. *Semin. Immunopathol.* **35**, 585–600 (2013).

81. Mantovani, A., Marchesi, F., Malesci, A., Laghi, L. & Allavena, P. Tumour-associated macrophages as treatment targets in oncology. *Nature Reviews Clinical Oncology* **14**, 399–416 (2017).

82. Noy, R. & Pollard, J. W. Tumor-Associated Macrophages: From Mechanisms to Therapy. *Immunity* **41**, 49–61 (2014).

83. Bingle, L., Brown, N. J. & Lewis, C. E. The role of tumour-associated macrophages in tumour progression: Implications for new anticancer therapies. *J. Pathol.* **196**, 254–265 (2002).

84. Zhang, Q. wen *et al.* Prognostic Significance of Tumor-Associated Macrophages in Solid Tumor: A Meta-Analysis of the Literature. *PLoS One* **7**, (2012).

85. Pardoll, D. M. The blockade of immune checkpoints in cancer immunotherapy. *Nature Reviews Cancer* **12**, 252–264 (2012).

86. Marcucci, F., Rumio, C. & Corti, A. Tumor cell-associated immune checkpoint molecules – Drivers of malignancy and stemness. *Biochim. Biophys. Acta - Rev. Cancer* **1868**, 571–583 (2017).

87. Podojil, J. R. & Miller, S. D. Potential targeting of B7-H4 for the treatment of cancer. *Immunol. Rev.* **276**, 40–51 (2017).

88. Zhao, L. wei *et al.* B7-H1 and B7-H4 expression in colorectal carcinoma: Correlation with tumor FOXP3 + regulatory T-cell infiltration. *Acta Histochem.* **116**, 1163–1168 (2014).

89. Fauci, J. M., Straughn, J. M., Ferrone, S. & Buchsbaum, D. J. A review of B7-H3 and B7-H4 immune molecules and their role in ovarian cancer. *Gynecol. Oncol.* **127**, 420–425 (2012).

90. Jiang, J. *et al.* Tumor expression of B7-H4 predicts poor survival of patients suffering from gastric cancer. *Cancer Immunol. Immunother.* **59**, 1707–1714 (2010).

91. Tsiaousidoua, A. *et al.* B7H4, HSP27 and DJ-1 molecular markers as prognostic factors in pancreatic cancer. *Pancreatology* **13**, 564–569 (2013).

92. Quandt, D., Fiedler, E., Boettcher, D., Marsch, W. C. & Seliger, B. B7-H4 expression in human melanoma: Its association with patients' survival and antitumor immune response. *Clin. Cancer Res.* **17**, 3100–3111 (2011).

93. Postow, M. A., Callahan, M. K. & Wolchok, J. D. Immune checkpoint blockade in cancer therapy. *Journal of Clinical Oncology* **33**, 1974–1982 (2015).

94. Boutros, C. *et al.* Safety profiles of anti-CTLA-4 and anti-PD-1 antibodies alone and in combination. *Nature Reviews Clinical Oncology* **13**, 473–486 (2016).

95. Munn, D. H. & Mellor, A. L. Indoleamine 2,3 dioxygenase and metabolic control of immune responses. *Trends Immunol.* **34**, 137–143 (2013).

96. R. Selvan, S., P. Dowling, J., K. Kelly, W. & Lin, J. Indoleamine 2,3-dioxygenase (IDO): Biology and Target in Cancer Immunotherapies. *Curr. Cancer Drug Targets* **16**, 755–764 (2016).

97. Sun, L., Chen, L. & Li, H. Checkpoint-modulating immunotherapies in tumor treatment: Targets, drugs, and mechanisms. *Int. Immunopharmacol.* **67**, 160–175 (2019).

98. Farkona, S., Diamandis, E. P. & Blasutig, I. M. Cancer immunotherapy: The beginning of the end of cancer? *BMC Medicine* **14**, (2016).

99. Lee, S. & Margolin, K. Cytokines in cancer immunotherapy. *Cancers (Basel).* **3**, 3856–3893 (2011).

100. Berraondo, P. *et al.* Cytokines in clinical cancer immunotherapy. *Br. J. Cancer* **120**, 6–15 (2019).

101. Kumar, S., Saini, R. V. & Mahindroo, N. Recent advances in cancer immunology and immunology-based anticancer therapies. *Biomed. Pharmacother.* **96**, 1491–1500 (2017).

102. Khalil, D. N., Smith, E. L., Brentjens, R. J. & Wolchok, J. D. The future of cancer treatment: immunomodulation, CARs and combination immunotherapy. *Nat. Rev. Clin. Oncol.* **13**, 394–394 (2016).

103. Fesnak, A. D., June, C. H. & Levine, B. L. Engineered T cells: The promise and challenges of cancer immunotherapy. *Nat. Rev. Cancer* **16**, 566–581 (2016).

104. Weiner, L. M., Surana, R. & Wang, S. Monoclonal antibodies: Versatile platforms for cancer immunotherapy. *Nat. Rev. Immunol.* **10**, 317–327 (2010).

105. Kimiz-Gebologlu, I., Gulce-Iz, S. & Biray-Avci, C. Monoclonal antibodies in cancer immunotherapy. *Mol. Biol. Rep.* **45**, 2935–2940 (2018).

106. Torabi, A., Amaya, C. N., Wians, F. H. & Bryan, B. A. PD-1 and PD-L1 expression in bone and soft tissue sarcomas. *Pathology* **49**, 506–513 (2017).

107. Vanderstraeten, A., Luyten, C., Verbist, G., Tuyaerts, S. & Amant, F. Mapping the immunosuppressive environment in uterine tumors: Implications for immunotherapy. *Cancer Immunol. Immunother.* **63**, 545–557 (2014).

108. D'Angelo, S. P. *et al.* Prevalence of tumor-infiltrating lymphocytes and PD-L1 expression in the soft tissue sarcoma microenvironment. *Hum. Pathol.* **46**, 357–365 (2015).

109. Toulmonde, M. *et al.* Use of PD-1 targeting, macrophage infiltration, and IDO pathway activation in sarcomas a phase 2 clinical trial. *JAMA Oncol.* **4**, 93–97 (2018).

110. Tawbi, H. A. *et al.* Pembrolizumab in advanced soft-tissue sarcoma and bone sarcoma (SARC028): a multicentre, two-cohort, single-arm, open-label, phase 2 trial. *Lancet Oncol.* **18**, 1493–1501 (2017).

111. D'Angelo, S. P. *et al.* Nivolumab with or without ipilimumab treatment for metastatic sarcoma (Alliance A091401): two open-label, non-comparative, randomised, phase 2 trials. *Lancet Oncol.* **19**, 416–426 (2018).

112. Pender, A., Jones, R. L. & Pollack, S. Optimising cancer vaccine design in sarcoma. *Cancers (Basel)*. **11**, 1–14 (2019).

113. Bustin, S. A. Absolute quantification of mrna using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* **25**, 169–193 (2000).

114. ThermoFisher Scientific GeneChip Human Gene 2.1 ST Array Plate. Available at: https://www.thermofisher.com/order/catalog/product/902136#/902136. (Accessed: 1st April 2020)

115. Affymetrix OncoScan. Available at: http://www.affymetrix.com/support/technical/byproduct.affx?product=oncoscan_assay_kits. (Accessed: 28th April 2020)

116. Bankhead, P. *et al.* QuPath: Open source software for digital pathology image analysis. *Sci. Rep.* **7**, 1–7 (2017).

117. Huang, D. W., Sherman, B. T. & Lempicki, R. A. Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* **37**, 1–13 (2009).

118. Huang, D. W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **4**, 44–57 (2009).

119. Supek, F., Bošnjak, M., Škunca, N. & Šmuc, T. Revigo summarizes and visualizes long lists of gene ontology terms. *PLoS One* **6**, (2011).

120. Balch, C. M. *et al.* Prognostic Factors Analysis of 17 , 600 Melanoma Patients : Melanoma Staging System. *J. Clin. Oncol.* **19**, 3622–3634 (2001).