

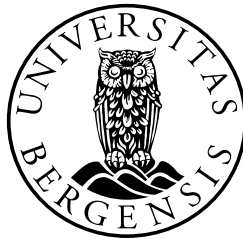
**Diagnostic, Prognostic and Therapeutic Aspects
of Endometrial Stromal Sarcomas, Low Grade
and
Undifferentiated Endometrial Sarcoma**

Weiwei Feng

Dissertation for the degree of Philosophiae Doctor (PhD)

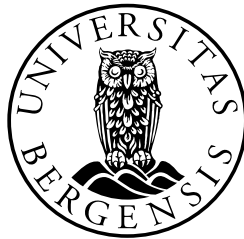
University of Bergen, Norway

2013



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WeiweiFeng



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Dissertation date: June 24, 2013

Scientific environment

The studies constituting this research project have been performed at
the Department of Pathology, Stavanger University Hospital, Stavanger and
Clinical Institute-1, Medical-Odontologic Faculty, University in Bergen, Norway,
the Departments of Gynaecology and Pathology, Obstetrics and Gynaecology Hospital
of Fudan University, Shanghai, China,
the Departments of Pathology and Gynecologic Oncology and
the Department of Experimental Therapeutics, MD Anderson Cancer Center, Houston,
Texas,
the Departments of Pathology and Obstetrics and Gynecology, Duke University
Medical Center, Durham, NC, USA



In affiliation with the Clinical Institute-1, Medical-Odontologic Faculty, University in Bergen, Bergen, Norway.



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This thesis is dedicated to my parents and husband Lizing for their support through times of distress and long working hours, and to our daughter Zianchen, my great pride and joy

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Preface

Uterine endometrial stromal sarcomas are very rare; world-wide, we have estimated that 1500-2500 new cases occur annually. The World Health Organization (WHO) further divides them as Endometrium Stromal Sarcomas, Low Grade (ESS), and Undifferentiated Endometrial Sarcomas (UES). The median age of patients is 43 (19-63) years. More than 90% of patients with ESS survive without recurrence, while UES patients have a very poor prognosis.

Due to the extreme rarity, diagnosis and treatment are hardly standardized and often less optimal (apart from in highly specialized very large centers). In 2003, the WHO has changed the definition and diagnostic criteria as the pre-2003 criteria were not well reproducible. However, even after 2003 most publications have used the pre-2003 criteria. Moreover, the WHO-2003 description of the essential criteria (different nuclear atypia degrees and necrosis) is not very exact or quantitative and does not at all define the diagnostic and prognostic implications of moderate nuclear atypia. The scientific basis of the WHO-2003 therefore is weak and knowledge how reliable the criteria are for diagnosis, prediction of disease outcome and treatment is virtually non-existent.

Before 2003, 20% to 35% low-grade ESS recurred, but WHO 2003-defined low-grade ESS has 10 years' recurrence rates of less than 10%. With so few recurrences, the balance between treatment guaranteeing cure and overtreatment ("not too little" or "too much") becomes increasingly important. However, primary treatment practices range from limited surgery only to extensive surgery combined with adjuvant chemotherapy and radiotherapy.

In view of the above mentioned it is therefore of critical importance to validate the diagnostic and prognostic value of the WHO-2003 criteria and definitions of ESS-

Low grade and Undifferentiated Endometrial Sarcoma, to develop additional accurate and reproducible diagnostic criteria and to evaluate how much treatment is needed in ESS-low grade.

List of publications

Paper I

Prognostic Value of the Diagnostic Criteria Distinguishing Endometrial Stromal Sarcoma from Undifferentiated Stromal Sarcoma, Two Entities within the Invasive Endometrial Stromal Neoplasia Family. Weiwei Feng, Anais Malpica, Stanley J. Robboy, Einar Gudlaugsson, Keqin Hua, Xianrong Zhou, and Jan P. A. Baak. Int J Gynecol Pathol. 2013 May; 32(3): 299-306.

Paper II

Prognostic Indicators in WHO2003 Low Grade Endometrial Stromal Sarcoma. Weiwei Feng, Keqin Hua, Einar Gudlaugsson, Yinhua Yu, Xianrong Zhou, Jan P.A. Baak. Histopathology. 2013 Apr;62(5):675-87.

Paper III

Diagnostic and prognostic morphometric features in WHO2003 invasive Endometrial Stromal Tumours. Weiwei Feng, Anais Malpica, Yu Yinhua, Emiel Janssen, Einar Gudlaugsson, Xianrong Zhou, Jan P.A. Baak. Histopathology. 2013 Apr;62(5):688-94.

Paper IV

Proliferation biomarkers reliably predict recurrence in World Health Organization 2003 defined Endometrial Stromal Sarcoma, Low Grade. Weiwei Feng, Anais Malpica, Einar Gudlaugsson, Stanley Robboy, Keqin Hua, Xianrong Zhou, Jan P.A. Baak. Manuscript, Submitted February 10, 2013.

Paper V

Stages I to II WHO 2003-Defined Low-Grade Endometrial Stromal Sarcoma: How Much Primary Therapy Is Needed and How Little Is Enough? Weiwei Feng

Weiwei, Keqin Hua, Malpica A, Xianrong Zhou, Jan P. Baak. Int J Gynecol Cancer. 2013

Mar;23(3):488-93

Abbreviations

| | |
|-------------|---|
| BSO | Bilateral Salpingo-Oophorectomy |
| c-kit | The stem cell factor receptor, c-kit (CD117), acts as an inhibitor of apoptotic cell death upon binding of its ligand |
| °C | Centigrade |
| CONN1 | A syntactic structure feature describing the number of nuclei connected to one other nucleus only |
| DAB | 3,3'-Diaminobenzidine |
| DIA | Digital Image Analysis system |
| OG | Oestrogen Receptor |
| EG | Einar Gudlaugsson |
| EGFR/HER-1 | Epidermal Growth Factor Receptor |
| PR | Progesterone Receptor |
| ESS-LG | Endometrium Stromal Sarcomas, Low Grade (ESS), and Undifferentiated Endometrial Sarcomas (UES), defined as such by the WHO-2003 |
| EST | Endometrial Stromal Tumours |
| FIGO | International Federation of Gynecology and Obstetrics 2009 stages I to II |
| GnRH-a | Gonadotropin-Releasing Hormone-analogues |
| H&E | Haematoxyllin and Eosin |
| HER-2 | Human Epidermal Growth Factor Receptor-2 |
| HGESS | High grade ESS according to the pre-2003 WHO definition |
| HPF | High Power Fields of vision |
| HR | Hazard Ratio (HR) |
| JAZF1-JJAZ1 | Juxtaposed with another zinc finger protein 1 (JAZF1) also known as TAK1-interacting protein 27 (TIP27) or zinc finger protein 802 (ZNF802) is a protein that in humans is encoded by the <i>JAZF1</i> gene |
| JB | Jan Baak |
| Ki-67 | Antigen KI-67 is a nuclear protein that is associated with and may be necessary for cellular proliferation |
| LGESS | Low grade ESS according to the pre-2003 WHO definition |
| MAI | Mitotic Activity Index (MAI) |
| MI | Mitotic Index |
| MIB 1 | An antibody directed at the protein Ki-67, a product of the MKI-67 gene |

| | |
|----------|--|
| MMMCP | Multicenter Morphometric Mammary Carcinoma Project |
| Mod | <i>Moderate atypia</i> |
| MST | Minimum Spanning Tree |
| n | Number |
| NCCN | National Comprehensive Cancer Network |
| OPS | Ovary Preservation Surgery |
| OPS | ovary preservation surgery (OPS) |
| P | Probability of no difference |
| PDGFR | Platelet-derived growth factor receptors (PDGF-R) are cell surface tyrosine kinase receptors for members of the platelet-derived growth factor (PDGF) family |
| PPH3 | PhosphoHistone-3 |
| ROC | Receiver Operating Curve |
| Svr | Severe atypia |
| UES | Undifferentiated Endometrial Sarcomas |
| UK | United Kingdom |
| VEGF | Vascular Endothelial Growth Factor |
| VIS | Visiopharm digital image analysis system |
| WHO | World Health Organization |
| WHO-2003 | The 2003 definition of the WHO for ESS and UES |
| XZ | Xiarong Zhou |

Abstract

Endometrial stromal sarcoma (ESS) has been traditionally divided into low and high grade based on the mitotic activity but in 2003 the World Health Organization (WHO2003) has changed the definition. However, since then, many studies still used the old criteria and few focused on WHO2003-defined ESS.

In the first article (Weiwei Feng, Anais Malpica, Stanley J. Robboy, Einar Gudlaugsson, Keqin Hua, Xianrong Zhou, and Jan P. A. Baak . ***Prognostic Value of the Diagnostic Criteria Distinguishing Endometrial Stromal Sarcoma from Undifferentiated Stromal Sarcoma, Two Entities within the Invasive Endometrial Stromal Neoplasia Family.*** *Int J Gynecol Pathol* 2013 May; 32(3): 299-306.), the prognostic value of the diagnostic criteria distinguishing Endometrial Stromal Sarcoma from Undifferentiated Endometrial Stromal Sarcoma was compared. The World Health Organization (WHO2003) recognizes three endometrial stromal neoplasms: non-invasive endometrial stromal nodule and the two invasive neoplasms, Endometrial stromal Sarcoma (ESS) and Undifferentiated Endometrial Sarcoma (UES). Of import, the WHO2003 does not define moderate atypia (an important differentiating diagnostic criterion for ESS and UES) nor does it discuss its significance. Moreover, studies on reproducibility and additional prognostic value of other diagnostic features in large are lacking. Using strict definitions, we analyzed agreement between routine and expert-review necrosis and nuclear atypia in 91 invasive endometrial stromal neoplasias. The overall 5- and 10-years recurrence-free survival rate estimates were 82% and 75%. Necrosis was well and nuclear atypia reasonably well reproducible. The 10-year recurrence free survival rates for necrosis Absent/Inconspicuous versus Prominent were 89% and 45% ($P < 0.001$) and for review-confirmed None/Mild, moderate, severe atypia 90%, 30% and $<20\%$ ($P < 0.00001$).

Therefore, cases with Moderate/Severe atypia should be grouped together. Nuclear atypia and necrosis had independent prognostic value (Cox regression). Once these features were taken into account, no other feature had independent additional prognostic value, including the mitotic count. Using “None/Mild atypia, necrosis Absent/Inconspicuous” as ESS, versus “Moderate/Severe atypia present or necrosis present” as UES resulted in 68 ESS and 23 UES cases with disease specific overall mortality-free survival 99% versus 48%, $P < 0.00001$, $HR=45.4$). When strictly defined microscopic criteria are used, the WHO2003 diagnoses ESS and UES are well reproducible and prognostically strong.

In the second article (Weiwei Feng, Keqin Hua, Einar Gudlaugsson, Yinhua Yu, Xianrong Zhou, Jan P.A. Baak. ***Prognostic indicators in WHO 2003 Endometrial Stromal Sarcoma, Low Grade. Histopathology. 2013 Apr; 62(5): 675-87***), the prognosticators in ESS, Low Ggrade were investigated. We reviewed the WHO 2003diagnostic criteria in 91 tumours (previously classifiedas ESS low and high grade). There were 68 cases of ESS-LG and 23 of undifferentiated endometrial sarcoma (UES). In the ESS-LG cases, the prognostic value of clinicopathological variables was studied. With a median follow-up of 79 months (range: 20–474 months), the recurrence and death rates were 5/ 68 (7%) and 1/ 68 (1.5%) in the ESS-LG cases. Ovarian preservation or no ovarian preservation ($P < 0.0001$, hazard ratio (HR) 10.4) and mitotic activity index (MAI) (0–3 versus >3 , $P = 0.005$, HR 8.6) had independent prognostic value. Other frequently used MAI thresholds – age, tumour diameter, and vessel invasion – were not prognostic. Among patients without ovarian preservation ($n = 61$), none of 53 with MAI 0–3 suffered recurrence, contrasting with two of eight (25%) of those with MAI >3 ($P = 0.003$); one of these two recurrence patients died ($P = 0.02$). Among patients with ovarian preservation (n

= 7), three (43%) suffered recurrence but none died, and MAI had no additional prognostic value. Conclusions: In ESS-LG, ovarian preservation and MAI >3 are associated with increased risk of recurrence.

In the third study (Weiwei Feng, Anais Malpica, Yu Yinhua, Emiel Janssen, Einar Gudlaugsson, Xianrong Zhou, Jan P.A. Baak. ***Diagnostic and prognostic morphometric features in WHO2003 invasive Endometrial Stromal Tumours.*** Histopathology. 2013 Apr; 62(5):688-94), the value of morphometric features has been evaluated to distinguish mild and moderate atypia in and predict recurrence of World Health Organization-2003 defined Endometrial Stromal Sarcomas-Low Grade (ESS-LG) and highly malignant undifferentiated uterine sarcomas (UES). Nuclear and cytologic size, shape and arrangement were morphometrically evaluated in 41 cases with consensus none/mild (n=38) or moderate atypia (n=3). None of the cases showed necrosis. The prognostic value of these features to predict recurrence was also assessed. Seven features differed. The mean and standard deviation of the nuclear volume and the distance between the nuclei were the best discriminators between the non/mild versus moderate atypia, with the maximum of the nuclear volume as a practically and rapid evaluable alternative. Using these features, all mild and moderate atypias were correctly classified. Seven cases recurred. The distance between the nuclei and percentage of nuclei with one neighbor (assessed with morphometric minimum spanning tree analysis) predicted recurrence. We conclude that in invasive endometrial stromal tumours, morphometric features are useful diagnostic support tools to distinguish mild from moderate atypia and predict recurrence

In the fourth study (Weiwei Feng, Anais Malpica, Iva Skaland, Einar Gudlaugsson, Stanley Robboy, Keqin Hua, Xianrong Zhou, Jan P.A. Baak. ***Proliferation biomarkers reliably predict recurrence in World Health Organization 2003 defined Endometrial Stromal Sarcoma, Low Grade.*** Manuscript, Submitted February 10, 2013), the value of proliferation biomarkers predicting recurrence in WHO-2003 defined ESS, Low Grade was studied. Using single and multivariate analysis, the prognostic value of classical mitosis counts (defined according to the Mitotic Activity Index=MAI protocol) in haematoxyllin-eosin (H&E) sections, and immunohistochemical proliferation biomarkers (Ki-67 and PhosphoHistone-3 (PPH3)) were examined in 24 invasive endometrial stromal sarcomas. Three of 24 (12.5%) ESS recurred, of which only one tumor exceeded stage II and stage was prognostically not significant. The mitotic count with H&E (MAI), PPH3 and Ki-67 were all prognostic (P=0.001, 0.002 and 0.03). MAI is prognostically the strongest proliferation biomarker, but can be tedious to reliably assess in poor quality sections. PPH3 counts can be easier to perform and closely resemble (but are higher than) the true mitoses counts. The fact that Ki-67 is the least prognostic, results from it staining not only nuclei in the M(itosis) phase of cycling cells, but also and mostly nuclei of proliferating cells in the non-mitotic (G₁, S and G₂) phases of the cell division cycle, many of which likely have genetic damage and die before becoming new daughter cells. In conclusion, in WHO2003-defined ESS Low Grade neoplasias, high levels of proliferation as measured by MAI, PPH3 and to a lesser degree also Ki-67 are predictive of tumors that will recur.

Finally, in the fifth study (Feng Weiwei, Hua Keqin, Malpica Anais, Zhou Xianrong, Jan P. Baak). ***Stages I to II WHO 2003-Defined Low-Grade Endometrial Stromal***

Sarcoma: How Much Primary Therapy Is Needed and How Little Is Enough? Int J Gynecol Cancer. 2013 Mar; 23(3): 488-93.), the effect of different treatment modalities was studied. Before 2003, 20% to 35% low-grade ESS recurred, but WHO 2003-defined ESS- low-grade has 10 years' recurrence rates of less than 10%. With so few recurrences, the balance between treatment guaranteeing cure and overtreatment ("not too little" or "too much") becomes increasingly important. However, primary treatment practices range from limited surgery only to extensive surgery combined with adjuvant chemotherapy and radiotherapy. We focused on the primary treatment of early-stage WHO 2003-defined ESS, low-grade. The effect of different therapeutic strategies was studied in 57 patients with International Federation of Gynecology and Obstetrics 2009 stages I to II expert-reviewed WHO 2003-defined ESS, low-grade treated at a single institution between 1992 and 2007. The patients' median age was 43 years (range, 19-63 years). After 68 months' median follow-up (range, 17-140 months), recurrence and mortality rates were 9% and 2%, respectively. The patients with WHO 2003-defined ESS, low grade with ovary-preserving primary surgery had a much higher recurrence rate (75%) than those without (2%; $P < 0.0001$).

Lymphadenectomy, radical abdominal hysterectomy, and omentectomy did not influence survival. Ten patients refused chemotherapy. With univariate analysis, multiple-agent chemotherapy improved the prognosis ($P = 0.02$) With multivariate analysis, only ovary preservation-or-not surgery had independent prognostic value. We concluded that in International Federation of Gynecology and Obstetrics 2009 stage I to stage II WHO 2003-defined ESS, low grade, total abdominal hysterectomy with bilateral salpingo-oophorectomy is sufficient surgery, but ovary-preserving primary surgery increases the risk of recurrence. More extensive surgical procedures than total abdominal hysterectomy with bilateral salpingo-oophorectomy do not

improve prognosis. Chemotherapy may improve progression-free survival but a large sample size is needed to confirm this.

I. Introduction

1. Endometrial stromal Sarcoma and Undifferentiated Endometrial Sarcoma

1.1 General background

Neoplastic lesions of the uterine endometrium can be of epithelial and mesenchymal origin. Cancers are the most frequent. Tumours from the endometrial stroma are less frequent and according to the World Health Organization 2003 classification are divided into three types: the non-invasive benign stromal nodules, and the invasive endometrial stromal sarcomas, low grade (ESS-LG) and undifferentiated endometrial sarcomas (UES)¹.

This thesis will study ESS-LG and UES. ESS-LG is a rare uterine neoplasm accounting for 0.2% of all uterine malignancies and 15% of all types of uterine sarcomas. However, a higher incidence of 0.7% also has been reported². UES are approximately 5 times less frequent. Based on the numbers in Abeler's classical publication², the incidence of ESS in Norway (with about 4.5 million inhabitants) can be estimated as approximately 2-3 per year. Koivisto-Korander reported that the incidence rates were about 0.3 per 100,000 for ESS in Denmark, Finland, Iceland, and Norway during the study-period (1978-2007)¹¹⁵. With 650 million inhabitants in the Western world, 1.3 billion in China and 1 million in India, 1500-3000 new ESS will annually occur worldwide.

1.2 Classification of endometrial stromal tumours

The classification of endometrial stromal tumours (EST) has changed over the past two decades. Until the 1980s, ESTs were regarded as benign if the borders were smooth, pushy and non-infiltrating and mitoses were few. After the publication of Norris et al, in 1966³, endometrial stromal tumours started to be classified according to the number of mitotic figures, with < 10 per 10 high power fields as low grade and ≥ 10 as high grade. However, one study⁴ showed that the reproducibility between pathologists in assessing mitosis counts was poor, casting doubt on the value of the number of mitotic figures as a single diagnostic criterion.

In 1982 Evans⁵ at MD Anderson Cancer Center in Houston proposed a change of the paradigm used to approach this disease. Briefly, the paradigm hypothesized the existence of two different entities arising from the endometrial stroma: one, characterized by the presence of uniform cells resembling the cells of the normal proliferative endometrium and a vascular-rich pattern (endometrial stromal sarcoma) and the other characterized by markedly atypical, pleomorphic cells lacking the distinct vascular pattern seen in ESS (undifferentiated stromal sarcoma). This approach was endorsed by other investigators and ultimately accepted in 2003 by the World Health Organization classification (WHO2003)¹. Benign endometrial stromal nodules have a non-invasive pushing border. ESS-LG and Undifferentiated Endometrial Sarcomas have invasive borders.

According to the World health Organization 2003 (WHO2003) classification, the term ESS, low grade is used for "*endometrial tumours composed of cells resembling to those of proliferative phase endometrial stroma. Numerous thin-walled arteriolar type (plexiform) vessels are characteristically present. It is distinguished*

from the stromal nodule on the basis of myometrial infiltration and/or vascular space invasion". Later on, in the histopathology section it says that "by definition, significant atypia and pleomorphism are absent" and also: "necrosis is typically absent or inconspicuous". Moreover, it also says: "Although most tumours are paucimitotic, mitotic rates of 10 or more than 10 per 10 higher power fields can be encountered, and such a higher mitotic index does not itself alter the diagnosis". "Macroscopically, cystic and myxoid degeneration as well as necrosis and hemorrhage are seen occasionally¹.

UES is defined as *a high grade endometrial sarcoma that lacks specific differentiation and bears no histological resemblance to endometrial stroma. Histologically, UES show marked cellular atypia and abundant mitotic activity, often including atypical forms. Macroscopically, UES often show prominent hemorrhage and necrosis¹* Using this newest WHO2003 classification, some tumours previously classified as high-grade ESS are now referred to as ESS-LG while others are termed UES. Figures 1-3 gives examples of the histopathology of ESS-LG and UES.

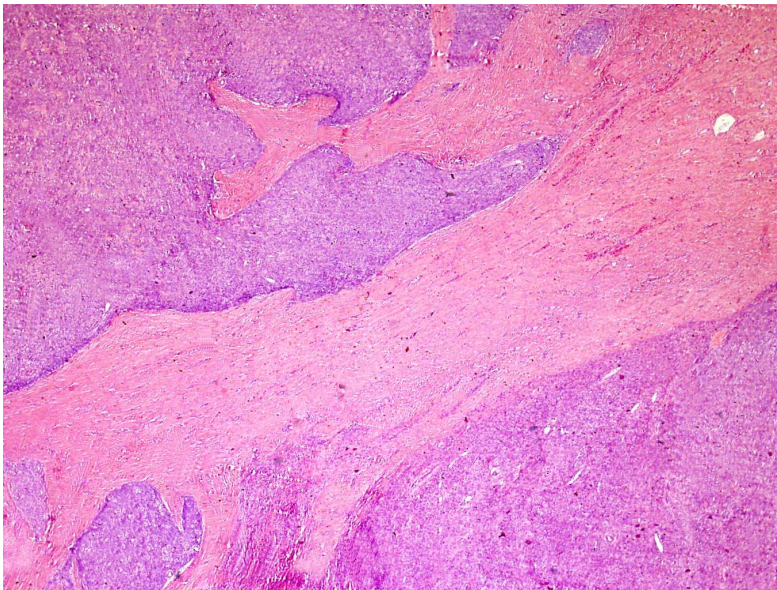


Figure 1. Endometrial stromal Sarcoma, low grade, with invasive border.

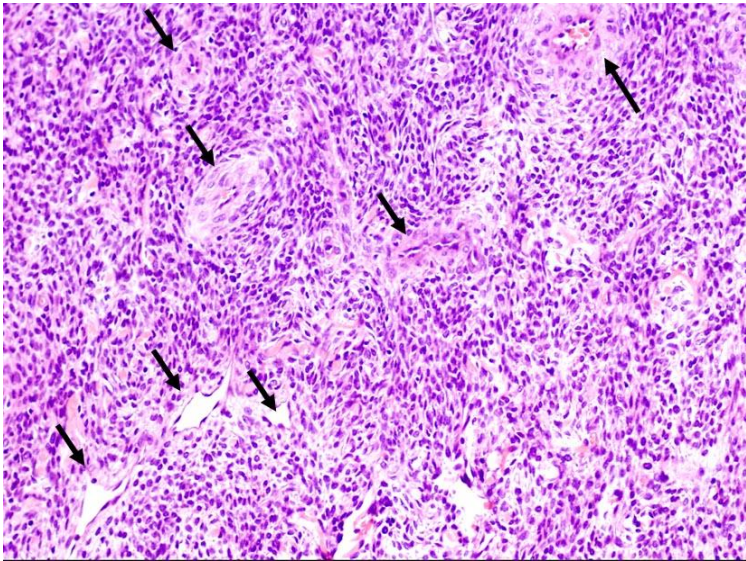


Figure 2. Typical vascular pattern of Endometrial stromal Sarcoma, low grade.

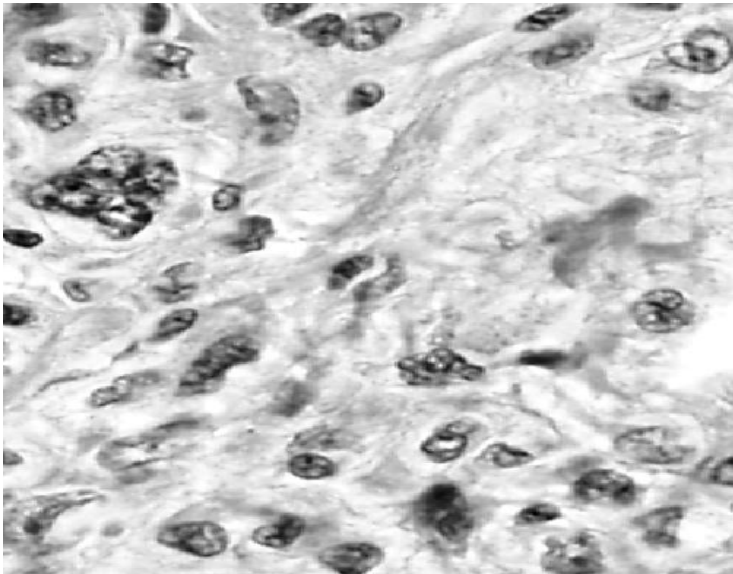


Figure 3. Severe nuclear atypia of Undifferentiated Endometrial Sarcoma

1.3 Confusion in the current WHO 2003 criteria

The text of the WHO 2003 clearly indicates that the distinction between ESS-LG and UES is no longer based on mitotic activity, but on cellular atypia and necrosis. However, the essential features to distinguish necrosis and cellular atypia are rather vaguely and not quantitatively defined. What is “*inconspicuous*” and what is “*conspicuous*” necrosis? As far as we know, a truly scientific analysis of these terms is lacking. We assume that “tumor necrosis” is meant, rather than coagulative necrosis, but this is also not clearly mentioned. In addition, the WHO2003 does not at all describe the significance of moderate atypia, nor is mild and severe atypia exactly defined.

Finally, the terms ESS-LG and UES in the WHO2003 classification are somewhat strange. The addition “*low grade*” to ESS, suggests that there is also a high grade ESS, but this is not further discussed in the WHO2003. This raises the question as to the validity of considering UES as an entity distinct from ESS-LG as opposed to being one end of the spectrum where ESS-LG is at the other. By analogy, should grade 3 endometrial adenocarcinoma be potentially considered as a different entity from grade 1 endometrial carcinoma, since both have differing prognoses, even though both seem to arise by the same mechanism? In contrast, molecular evidence now points to why borderline serous tumor/grade 1 serous carcinoma of ovary is a completely different entity from high grade serous carcinoma as both seem to arise by different pathogenetic mechanisms, even though they share many histomorphologic features. For example, the former is KRAS & BRAF positive and the latter KRAS & BRAF negative. Another issue is whether the WHO2003, in eliminating the category high grade ESS, has simply substituted UES in its place (*Old dog, new name*). Or is there a hitherto not described third invasive entity, high grade ESS, in between

ESS-LG and UES? Should the category of ESS-high grade be reintroduced if it is shown that certain ESS have identifiable criteria associated with a worse prognosis than usual for WHO2003 defined ESS-LG, but still differs in some fundamental way so that it not can be a subset of the current UES cases? This is one of the issues we will try to answer in the first study described in this thesis.

In summary, knowledge about reproducibility and validity of diagnostic and prognostic criteria for the WHO2003 classification is rather limited and uncertain, as the WHO 2003 also acknowledges: *“a small minority of cases share features of low grade ESS and undifferentiated sarcoma, and their classification is controversial”*. We therefore set out to study in a large number of WHO2003 defined cases with long follow-up the reproducibility and prognostic value of the essential diagnostic features.

1.4 Differential diagnosis

The distinction of benign endometrial stromal nodule (which is not invasive) versus the invasive ESS-LG and UES has been described above in general. The WHO2003 microscopic features also have been described. Here follow some more specific differential diagnostic remarks. ESS should be distinguished from:

1. Cellular leiomyoma,
2. Adenomyosis with stromal predominance and
3. Adenomyosis with sparse glands.

Especially important is the differential diagnosis of ESS-LG and common highly cellular leiomyoma. We used the following criteria favouring cellular myoma (Table 1).

Table 1. Criteria favouring cellular myoma in the differential diagnosis of Endometrial stromal Sarcoma

1. A fascicular growth pattern,
2. Large tick-walled vessels,
3. Merging or slight interdigitation with the adjacent myometrial fascicles,
4. Presence of cleft-like spaces and
5. Absence of foamy histiocytes (which are often present in endometrial stromal tumours).
6. CD10 immuno-staining shows strong positivity in ESS vessels but rarely and if positive, less intense in cellular myomas.

ESS-LG should be also distinguished from adenomyosis with stromal predominance or adenomyosis with sparse glands may be misdiagnosed as ESS-LG. Careful sampling usually demonstrates areas of more typical adenomyosis with glands. Additionally, in adenomyosis the constituent cells generally appear atrophic without mitotic activity, in contrast to the proliferative expansive appearance of the stromal cells in ESS-LG where mitotic figures are usually identified⁶.

1.5 Prognostic factors in ESS-LG and UES

ESS-LG generally has a good prognosis, with 5- and 10-year actuarial survival for patients with stage 1 tumours of 98% and 89%, respectively. However, it has been stated by Chang KL that recurrence rates can be as high as 20% of stage 1 tumours recur and even more of the stage 2-4 neoplasms⁷. However, as mentioned before, the number of cases studied since the WHO2003 classification was introduced is very

limited and we will further study the recurrence rate in our own material in this PhD thesis.

UES has a much worse prognosis, but again no reliable data are available because even most of the more recent series of the invasive endometrial stromal tumours do not make a distinction between ESS and UES. As the definitions of ESS before 2003 and after 2003 are essentially different, studies on prognostic factors before 2003 cannot be applied to ESS-LG. A PubMed analysis for ESS articles since 2003 on the key words “endometrial stromal sarcoma” resulted in nearly 479 hits but many of these are case reports which cannot be used for evaluation of prognostic factors. Prognostic studies on ESS after 2003 are very rare. Unfortunately, some of these studies included various types of uterine sarcomas, but the breakdown of results into histological subtypes was either not provided or endometrial stromal sarcomas accounted for a small minority of cases and were not separately discussed⁸⁻¹⁵. In others, the old classification as low and high grade endometrial stromal sarcomas was still used¹⁶⁻²⁵.

In previous studies, many features were prognostic: tumour diameter, histologic grade, involvement of surgical margins by tumour, deep myometrial invasion, menopausal status, and age^{19,20,7,26,27}. However, most of these studies were published before 2003 and included “ESS high grade” cases which now often (but not always) would have been regarded as UES. Only seven other studies evaluated prognostic factors in ESS-LG and UES according to the WHO-2003, but the numbers of patients studied were small^{2,28-33}. Some studies stated that bilateral salpingo-oophorectomy does not seem to improve the outcome^{21,22,32,34}, but the old classification was used in those studies. One study used WHO-2003 criteria showed ovary preservation was a risk factor for recurrence³³.

Although WHO 2003 does not use mitotic counts as essential diagnostic criteria, four studies showed mitotic activity index (MAI) still were prognostic in studies from after 2003^{2, 28, 116, 117}. In Abeler's study², the total area at specimen level of the 10 high power fields used for MAI was 2.8 mm², much higher than the area widely used (1.59 mm²) (see above). The other study showed that low mitotic index (MI<5) was associated with long disease free survival in ESS, but the clinical significance should be further addressed since only 14 cases were included²⁸. The third study reported that survival was significantly longer in the in 21 localized cases (stage I and II) with a low mitotic index ($P < 0.0001$)¹¹⁶. The fourth study showed that there was a significant difference in the recurrence rate between cases with different mitotic index ($\geq 10/10$ HPF and $< 10/10$ HPF, $P = 0.009$), especially in LGESS group including 39 cases¹¹⁷.

Moreover, many studies only did univariate but not multivariate analysis. Thus, the knowledge of prognostic factors in WHO2003 defined ESS-LG is limited and their value uncertain. The objective of the present study is to further evaluate the prognostic value of clinico-pathological variables in a large number of WHO-2003 defined ESS-LG cases with long follow-up.

1.6 Biomarkers in ESS-LG and UES

To date, several biomarkers have been studied in endometrial stromal tumors, such as Beta-catenin, P53, WT-1, C-kit, MIB-1, PDGF etc. Since ESS-LG and UES are rare tumors, the sample size of those studies is small. But those studies suggest UES and ESS-LG have distinct immunohistochemical and cytogenic profiles. Table 2 gives an overview about biomarkers study in Endometrial tumors.

Table 2. Overview of biomarkers studies in Endometrial tumors.

| Marker | Year | Author | Case number | positivity | Significance |
|--------------|------|--------------------------|---|--|---|
| Beta-catenin | 2009 | Kidal ³⁵ | 82ESS, 18UES | 68% ESS | Nuclear expression related to spread of tumor, but not related to survival. |
| | 2008 | Kurihara S ³⁶ | 18 ESS 7 UES-U* 6 UES-P* | 47% LGEES 85% UES-U 33% UES-P | |
| | 2010 | Kurihara S ³⁷ | 8 ESN, 16 ESS, 13 UES | 6/13 UES | UES, coincident expression of beta-catenin and cyclin D1 |
| | 2008 | Jung CK ³⁸ | 2 ESN 12 ESS 8 UES | 92% ESS, 75% UES Strong expressed in 67% ESS 40% UES | |
| | 2004 | Hrzenjak A ³⁹ | 10 ESS 4 UES | Increased in UES | |
| | 2005 | Nq TL ⁴⁰ | | 40% ESS | |
| SFRP4 | 2004 | Hrzenjak A ³⁹ | 10 ESS, 4UES | decreased in 10 ESS, more lower in 4 UES | SFRP4 is a putative suppressor |
| Cyclin D1 | 2010 | Kurihara S ³⁷ | 8 ESN, 16 ESS, 13 UES | 2/8 ESN; 1/17 LGEES, 6/13 UES | UES, coincident expression of beta-catenin and cyclin D1 |
| | 2012 | Lee CH ¹¹⁸ | 12 YWHAE- FAM22 ESS 34 JAZF1 ESS 21 ESS-LG with no genetic changes | 100% in YWHAE- FAM22 ESS, not in other ESS | Cyclin D1 is an indicator of YWHAE-FAM22 ESS |
| ER, PR | 2013 | Jakate K ¹¹⁹ | 23 ESS-LG 10 UES-U 7 UES-P | 83% ESS-LG 10% UES-U 0% UES-P | |
| | 2008 | Kurihara ³⁶ | 18 ESS | 94% ESS, | |

| | | | | | |
|----------------------------------|------|-----------------------------------|--|--|---|
| | | | 7 UES-U 6 UES-P | 57% UES-U, 0 UES-P | |
| | 2000 | Reich O ⁴⁴ | 21 LGESS | ER+ 71% PR+ 95% | Heterogenous expressin for ER PR |
| | 2004 | Balleine RL ⁴¹ | 9 ESS | PRA, PRB 100%. PRA dominant | |
| | 2003 | Popiolek D ⁴⁵ | 11LGESS | 100% | |
| Aromatase | 2004 | Reich O ⁴² | 23 LGESS | 83%, high expressed in 22% | Aromatase inhibitor treatment |
| Estrogen sulfotransfe rase | 2007 | Reich O ⁴³ | 29 ESS | 5/29 | |
| | 2013 | Jakate K ¹¹⁹ | 23 ESS-LG 10 UES-U 7 UES-P | 0% ESS-LG 10% UES-U 57% UES-P | |
| | 2012 | Park JY ¹²⁰ | 39 LGESS | 10.3% | P53 associated with poorer DFS and OS |
| P53 | 2008 | Kurihara ³⁶ | 18 ESS 7 UES-U 6 UES-P | 0 ESS and UES-U, 100% UES-P | |
| | 2003 | Popiolek D ⁴⁵ | 11LGESS | 1/11 | |
| YWHAE- FAM22 | 2012 | Isphording A ¹²¹ | 6 YWHAE- FAM22 ESS 7 JAZF1- SUZ12 ESS 3 JAF1- PHF1 ESS, 6 UES | RT-PCR 100% in 6 YWHAE- FAM22 ESS | Associate to higher grade and aggressive feature |
| MEAF6- PHF1 | 2012 | Panagopoul os I ¹²² | 1 ESS with recurrence | | |
| JAZF1 | 2013 | Jakate K ¹¹⁹ | 23 ESS-LG 10 UES-U 7 UES-P | 32% in ESS- LG, 0% in UES | |
| JAZF1-JJAZ1 | 2008 | Kurihara S ³⁶ | 18 LGESS, 7UES-U, 6UES-P | 50% LGESS 1/3 UES-U, 0/6 UES-P | UES-p is dffere from LGESS |
| | 2011 | Amador- Ortiz C ⁴⁶ | 6 primary extra uterine ESS | 1/6 | |
| | 2007 | Oliva E ⁴⁷ | 9 ESN, 1 | 3/6 | |

| | | | | | |
|-------|------|-----------------------------|---|-------------------------|---|
| | | | LGESS | assessable EST+ | |
| | 2007 | Sato K ⁴⁸ | 1 extrauterine ESS | 1 / 1 | |
| | 2007 | Nucci MR ⁴⁹ | 4 ESN, 24 LGESS | 4/4 ESN 8/16 ESS | |
| | 2005 | Hrzenjiak A ⁵⁰ | 20 ESS 2 UES | 80% ESS 0% UES | |
| | 2006 | Micci F ⁵¹ | 3 ESS | | |
| | 2004 | Huang Y ⁵² | 8 ESS 4 metastatic ESS 1 extra-uterine ESS 2 recurrent ESS | 33% (5/15) | |
| | 2001 | Koontz JI ⁵³ | 4 ESS | 4/4 | |
| PDGFR | 2007 | Liegl B ⁵⁴ | 37 c-kit-ESS | PDGFa 65%, PDGFβ 0% | PDGFa + ESS may benefit with tyrosine kinase inhibitors |
| | 2011 | Cheng X ⁵⁵ | 13 ESS | PDGFa 33% PDGFβ36% | |
| | 2007 | Adams SF ⁵⁸ | 42 Uterine sarcomas | PDGFRβ 0%, PDGFRα70% | PDGF-α is a potential target |
| c-kit | 2011 | Cheng X ⁵⁵ | 13 ESS | 8% | |
| | 2008 | Zafrakas ⁵⁶ | 12 uterine sarcomas | Very weak | |
| | 2007 | Mitsuhashi T ⁵⁷ | 1 UES | 0/1 | |
| | 2007 | Adams SF ⁵⁸ | 42 uterine sarcomas | 10% | |
| | 2006 | Salvatierra A ⁵⁹ | 1 HGEES | 1/1 | Responding to Imatinib Mesylate(gleevec) |
| | 2004 | Leath ¹⁹ | 3 ESS | 3/3 | |
| | 2006 | Nakayama M ⁶⁰ | 5 ESS | 0/5 | |
| | 2004 | Geller ²⁰ | 16 LGESS and HGEES | 2/9 LGESS 5/7 HGEES | |
| | 2003 | Wang L ⁶¹ | 11 LGESS | 3/11 LGESS | |

| | | | | | |
|--------------|------|----------------------------|----------------------------------|--|---|
| | | | 3 HGEES | 2/3 HGEES | |
| | 2003 | Rushing RS ⁶² | 2 ESS, 2UES | 2/2 ESS 2/2 UES | |
| | 2003 | Winter WE ⁶³ | 1 ESS | 0/1 | |
| | 2003 | Klein WM ⁶⁴ | 10 LGEES 2 HGEES | 1/10 LGEES 0/2 HGEES | |
| MIB 1(Ki-67) | 2003 | Popiolek D ⁴⁵ | 11LGEES | 2/11 | MIB1 predict LGEES recurrence |
| | 2005 | Kir G ⁶⁵ | 8 ESS, 7ESN | ESS express MIB1 more frequently than in ESN | |
| | 2013 | Park JY ¹²⁰ | 39 LGEES | 53.8% | Ki-67 associated with poorer DFS and OS |
| | 2013 | Jakate K ¹¹⁹ | 23 ESS-LG 10 UES-U 7 UES-P | More frequent in UES than ESS-LG | |
| EGFR/ HER-1 | 2012 | Capobiano G ¹²³ | 7 ESS-LG 3 UES | 90% in all 10 cases | |
| | 2011 | Cheng X ⁵⁵ | 13 ESS | 0 | |
| | 2007 | Mitsubishi T ⁵⁷ | 1 UES | 1/1 | |
| | 2005 | Monifar F ⁶⁶ | 20 ESS, 3 UES | 74%LGEES, | Gefitinib therapy |
| HER-2 | 2011 | Cheng X ⁵⁵ | 13 ESS | 0 | |
| | 2004 | Amant F ⁶⁷ | 21 ESS 4 UES | 0 in ESS 1/4 UES | |
| | 2005 | Monifar F ⁶⁶ | 20 ESS, 3 UES | 0 | |
| GnRH I,II | 2005 | Reich O ⁶⁸ | 30 ESS | Expressed in most ESS, Recurrence stain stronger | |
| VEGF | 2011 | Cheng X ⁵⁵ | 13 ESS | 54% | |
| WT-1 | 2007 | Coosemans ⁶⁹ | 15 ESS | 7/15 | |
| P16 | 2013 | Iwasaki SI ¹²² | 4 ESS-LG 12 UES | 0% in ESS-LG 83% in UES | |

Note: * UES-U: undifferentiated endometrial sarcoma with nuclear uniformity; UES-P:

undifferentiated endometrial sarcoma with nuclear pleomorphism.

1.7 Cytogenetics and Proliferation in ESS

Among biomarkers listed above, cytogenetics and proliferation markers are promising. A variety of cytogenetic abnormalities involving chromosome 7 have been reported in endometrial stromal sarcomas. Two zinc finger genes, *JAZF1* and *JJAZ1*, at the sites of the 7p15 and 17q21 breakpoints have been identified. The presence of *JAZF1-JJAZ1* markedly inhibited apoptosis and induced proliferation rates (the latter only when normal *JJAZ1* was suppressed)^{53, 70-72}. It has been suggested that increased cell survival and accelerated cellular proliferation occur upon allelic exclusion of the un-rearranged copy of that gene⁷⁰. In addition, recently studies suggest a subset of endometrial stromal sarcoma harbors t(10;17)(q23;p13), which results in the genetic fusion between *YWHAE* and 1 of 2 highly homologous *FAM22* family members - *FAM22A* or *FAM22B*. In contrast to classic low-grade ESS with *JAZF1-SUZ12* fusions, *YWHAE-FAM22* ESS displays high-grade histologic features and is associated with more aggressive disease course^{124, 125}, and cyclin D1 can be included in the immunohistochemical panel as an indicator of *YWHAE-FAM22* ESS¹¹⁸. A novel fusion of *MYS/Es1-Associated factor 6* and *PHF1* was found in Endometrial Stromal Sarcoma¹²⁶. As most pathology laboratories currently do not have access to translocation analysis, and FISH probes for the assessment of *JAZF1/JJAZ1* and other fusions are not commercially available, it is important to have easy and widely available methods allowing pathologists to assess which patients with an ESS are at high recurrence risk. Proliferation markers are amongst the most promising.

1.8 Proliferation markers

Measurement of proliferation by the Mitotic Activity Index is one of the oldest, yet still always very useful proliferation biomarkers⁷³⁻⁷⁶. However, as we will see in the

study on proliferation markers, the methodology in the studies since 2003 has raised certain questions, especially reproducibility, which we will try to solve. Others have described that c-kit and Ki-67 may have additional prognostic value in ESS, but the number of cases studied was small and sometimes before 2003^{19, 20, 45, 55-65}.

Phosphohistone 3, a useful mitosis marker⁷⁷ showed high inter-observer reproducibility and was found prognostically very strong in node-negative breast cancer less than 71 years^{78, 79}. The significance of PPH3 has not been investigated in ESS patients. We therefore have analysed in review confirmed WHO2003 ESS, long follow-up and known outcome the prognostic value of c-kit, Ki-67 and Phosphohistone-3 next to ovary preserving surgery and Mitotic Activity Index.

1.9 Behaviour and treatment of ESS-LG and UES

The behavior of ESS-LG is relatively indolent but late recurrence and distal metastasis may occur. In contrast, UES is associated with a much worse prognosis. Cases with transition of ESS into high-grade sarcoma have been reported⁸⁰. Due to its rarity, the literature about treatment of ESS-LG and UES is limited both in number of articles and number of patients described.

At the present time, total hysterectomy and bilateral salpingo-oophorectomy, with/without lymph-node dissection is recommended by the National Comprehensive Cancer Network (NCCN 2011)⁸¹, however, the role of pelvic lymphadenectomy is still matter of debate^{8, 16-20, 31, 32, 82-88}. This is due to the limited number of cases of uterine ESS who have received lymphadenectomy as part of their treatment in spite of an incidence of nodal metastases that ranges from 0 to 33%^{1, 17, 19, 80, 82-86}. In addition, whether bilateral salpingo-oorphorectomy affects outcome in early stage ESS is still controversial^{21, 22, 32-34}. Different outcomes of fertility-sparing

surgery was revealed by two case reports^{89,90}.

With regard to adjuvant therapy, several studies have concluded that radiation therapy may control local recurrence but does not improve overall survival^{16, 19, 20, 80, 82, 85} while the role of chemotherapy is still unclear^{18, 20, 21, 91}. Hormone therapy is recommended for the treatment of FIGO stages II, III and IV ESS by NCCN, since it was reported that LGESS showed 71% positivity for ER and 95% positivity for PR⁹². Polymorphism 1558 C > T in the aromatase gene may represent a high-risk allele with increased local estrogen levels¹²⁷.

The use of tamoxifen and HRT is discouraged in ESS⁹³, whereas progestins, aromatase inhibitor letrozole, gonadotropin-releasing hormone-analogues (GnRH-a) and mifepristone (RU-486) have been reported to be effective or in clinical trial.^{32, 94-96, 128}. Among these, the usage of progestin could either decrease recurrence rate or effectively treat the recurrent ESS-LG patients^{129, 130}.

As a result, therapies used vary widely from observation without additional therapy after limited surgery, to hormonal therapy, chemotherapy and radiation therapy, either alone or in varying combinations. Therefore, the roles of extensive surgical- and chemotherapy in ESS deserve further attention.

I.2. Patients

Hospital records of patients diagnosed as endometrial stromal sarcoma between 1954 and 2007 were retrieved from the Gynecology and Pathology departments of Obstetrics and Gynecology Hospital of Fudan University, Shanghai, China. Patient records were reviewed for clinico-pathologic variables, surgical management and adjuvant therapy, recurrence and patient outcome. Stage was determined according to 2009 International Federation of Gynecology and Obstetrics (FIGO) criteria for endometrial stromal sarcomas (ESS) and adenosarcomas⁹⁷. All tissue samples were obtained after receiving informed consent, according to institutional rules. Ninety-eight cases were diagnosed in that time period in our hospital as endometrial stromal sarcomas, low or high grade (LGESS, HGESS) according to the old classification. With independent review of the original diagnoses by experienced gynaecological pathologists, special attention was paid to the differential diagnosis of ESS and common highly cellular leiomyoma, as follows. First, we used the following criteria favouring cellular myoma; a fascicular growth pattern, large tick-walled vessels, merging or slight interdigitation with the adjacent myometrial fascicles, presence of cleft-like spaces and absence of foamy histiocytes (which are often present in endometrial stromal tumours). Moreover, CD10 immuno-staining was used (showing strong positivity in ESS but rarely and if positive, less intense in cellular myomas). Doing so, we could not be confirmed at review in 7 consultation cases, leaving 91 cases. Using the 2003 World Health Organization (WHO2003) classification of tumours 68 ESS cases and 23 UES cases were diagnosed.

I.3. Methods used

3.1 Stage assessment

Stage was determined using the 2009 FIGO rules for staging of endometrial and leiomyosarcomas^{93,94} (Table 3).

Table 3. The FIGO criteria for staging endometrial stromal tumours.

| FIGO stage | Criterion |
|-------------------|--|
| 1 | <i>Tumor limited to uterus</i> |
| 2 | <i>Tumor extends to the pelvis</i> |
| 3 | <i>Tumor invades abdominal tissues</i> |
| 4 | <i>Tumor invades rectum or bladder, or distal metastasis</i> |

3.2 Definition of the microscopic criteria

Diagnostic criteria per WHO2003 assessed absence or presence of prominent necrosis, and the degree of nuclear atypia, which we assessed using the features proposed by Kempson and Hendrickson⁹⁸. Tumor cell necrosis, as opposed to other forms of necrosis, shows a relatively abrupt transition between necrotic and preserved cells. The nuclear ghost outlines of the necrotic cells can often be seen throughout the necrotic area, and inflammatory cells are uncommon. Necrosis is usually conspicuous in UES, the size exceeding more than half of a field of vision with a 40 times objective (about 0.4-0.6 mm in diameter).

Assigning the degree of atypia took into account the degree of nuclear pleomorphism, nuclear size, nuclear membrane irregularities, chromatin density, and nucleolar size and prominence. *Mildly (insignificantly) atypical cells* show minimal

variation in nuclear size and shape, and nucleoli are small. *Moderate atypia* (Mod) shows scattered pleomorphic nuclei. *Severe atypia* shows many and severely pleomorphic nuclei. Because only few cases showed no nuclear atypia, we grouped the atypia as None/Mild. For analysis, we thus distinguished None/Mild, and separately moderate and severe atypia (Svr). Although moderate and severe atypia were evaluated separately, whenever appropriate they are reported together as Mod/Svr.

Vessel invasion required the presence of tumor cells surrounded by a clear space lined with endothelial cells. Shrinkage artifact and true vessel invasion required a discrepancy of the shape of the space and the tumour cells. In rare cases of doubt, CD10 immuno-histochemistry was used.

3.3 Morphometric methods

In the WHO2003 classification the distinction between ESS and UES is not made on the basis of mitotic count but on nuclear pleomorphism and necrosis. Unfortunately it can be difficult to reproducibly distinguish between mild and moderate atypia. Quantitative pathology aims at obtaining an objective assessment of tumour differentiation by measuring cell and tissue characteristics rather than by just giving a subjective description. A number of quantitative methods can objectively describe the nuclear and cytoplasmic size, shape and arrangement⁹⁹. In the present study, we set out to evaluate the additional diagnostic and prognostic value of these quantitative pathological features in Endometrial stromal Sarcomas and Undifferentiated Endometrial Sarcomas.

An interactive video-overlay measuring system (QPRODIT®, Leica, Cambridge, U.K.) was used for the morphometric analysis. With this system, the microscopic

image is recorded by a video camera and displayed on the computer screen. In each representative section of the tumour a measurement area, showing the subjectively highest degree of atypia was carefully selected and demarcated. The measurements were performed in this “measurement area” which was electronically demarcated at low magnification (blind re-selection in 10 random cases appeared well reproducible). To avoid selection bias and guarantee reproducibility, the nuclear area and shape measurements within the measurement area were performed at high magnification in 50 fields of vision systematically random spread over the whole measurement area. Nuclear morphometry allows for measuring the geometric characteristics of the tumour cell nuclei. For nuclear area, size, shape and volume measurements, a point grid was superimposed over each measurement area and only nuclei hit by one of the points of the point grid were measured. The methodology for applying this method has been described in detail before, avoids selection bias and increases reproducibility¹⁰⁰⁻¹⁰². Information was obtained about the size and shape of each nucleus measured.

The arrangement of the nuclei was analysed with the minimum spanning tree (MST). This is a method in syntactic structure analysis that provides quantitative data from a microscopic image¹⁰³⁻¹⁰⁵. In brief, all nuclei (points) within a certain area of tissue are connected by lines, giving a tree figure. The sum of all lines in this tree is minimal and there are no loops in it. From this tree we can derive quantitative data, such as the average, minimum, maximum, and standard deviation of line length but also the number of nuclei connected in the tree with one, two or more neighbours. Ten random fields of vision were selected in the tumour areas in each case. The centre of each neoplastic nucleus in a field of vision was registered, the minimum spanning tree constructed and the quantitative features calculated. Different patterns

of nuclear arrangement give different trees and thus different quantitative data about the distance and arrangement of the nuclei. With Voronoy's tessellation, a quantitative impression of the cytoplasmic features of the stromal cells was obtained. Figure 4 illustrates the morphometric methods. Reproducibility of the quantitative pathological features was good, in agreement with previous studies¹⁰⁰⁻¹⁰⁵.

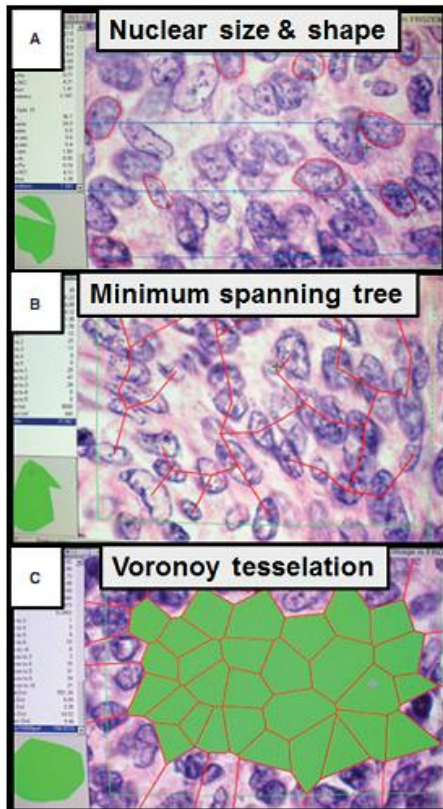


Figure 4. Examples of the different morphometric methods used. The microscopic image is displayed on the screen of the interactive video-overlay measuring system (QPRODIT). A. Morphometry of nuclear size and shape. The electronic point grid superimposed by QPRODIT hits certain nuclei. To avoid selection bias by the observer, only those nuclei can be measured which are hit by such a grid point. Tracing the

boundaries results in many quantitative size and shape related quantitative features of the nuclei. B. Minimum spanning tree analysis. A minimum spanning tree of moderate atypia. Different morphologies give different MSTs, and different quantitative data. C. Voronoy tessellation. Using the points placed by the observer in the centre of the nuclei, the program equally divides the space between the points. The resulting quantitative features of the polygons are then calculated, which are a measure of the cytoplasm of the cells.

3.4 Assessment of Mitotic Activity Index (MAI) and vascular invasion

Although the WHO2003 no longer considers mitotic activity as a diagnostic criterion for the distinction of ESS and UES, we still assessed the number of mitoses in 10 fields of vision with a 40x objective, field diameter 450 micrometer, numeric aperture 0.65, using the MMMCP protocol for counting mitoses ¹⁰⁶, which is as follows:

1. With a black marker on the glass slide, demarcate the most poorly differentiated peripheral area of the tumor. Avoid necrotic, heavily inflamed or benign areas. This area is called the measurement area of minimally 1x1 and maximally 5x5 millimeter.
2. In the measurement area, at x400 magnification (objective 40, field diameter 450 μm at specimen level) mitoses are counted in 10 consecutive neighboring fields of vision in the most cellular area (representing a total area of 1.59 mm^2 at specimen level).
3. Only certain mitoses are counted, doubtful structures and apoptotic bodies are ignored. The total resulting number of well-defined mitotic figures counted in the 10 fields of vision is the Mitotic Activity Index.

4. If $2 < \text{MAI} < 6$, the MAI was assessed once more, and the highest number of the two counts is taken as the MAI.

An accurate MAI assessment takes 3-5 minutes. Correction of the MAI for the percentage of tissue occupied by stroma or the number of tumor cells was not applied since it was previously shown that this does not substantially improve the prognostic value of the MAI and is substantially time consuming^{107,108}. Possible error sources and alternative mitosis counts methods have been discussed in detail elsewhere. The MAI is not sensitive to fixation delay^{109, 110}. The MAI was well reproducible between collaborating laboratories when strictly using the MMMCP protocol¹⁰⁶.

Mitotic activity index (MAI) was defined as the total number of unambiguous mitotic figures per 10 high power fields, using a field diameter of 450 micrometer at specimen level. This resulted in 1.59 mm² total section area for 10 fields of vision. The sampling and counting protocol was the same as described elsewhere in detail for breast cancer¹¹¹. The counts were made by different pathologists, and again by one of us with many years' experience in mitosis counts (JB, who was blinded to the MAI results of the routinely assessed MAI, and also to the original diagnosis, treatment and outcome). In case of discrepancy of more than 2 mitoses with the original MAI assessment, re-assessment was done by two of us (JB, XZ), using a discussion microscope and agreement was always obtained. For vessel invasion we used the classical features: tumour cells surrounded by a clear space lined with endothelial cells. In order to distinguish between shrinkage artifacts and real vessel invasion, we used discrepancy between the shape of the space and the tumour cells. Although CD10 alone does not distinguish between endometrial stromal tumors and highly cellular leiomyomas, in case of doubt CD10 immunohistochemistry was used to further support the histopathological impression.

3.5 Immunohistochemistry

Immunohistochemistry (IHC) was done in twenty-four WHO2003 defined ESS cases. Antigen retrieval and antibody dilution were optimized prior to the study onset. To ensure uniform handling of samples, all sections were processed simultaneously. Four micrometer paraffin sections adjacent to the H&E sections used for histologic assessment were mounted onto Superfrost Plus slides (Menzel, Braunschweig, Germany) and dried for one hour at 60 °C. Sections were deparaffinized in xylene and rehydrated in decreasing concentrations of alcohol. Antigen was retrieved with a highly stabilized retrieval system (ImmunoPrep, Instrumec, Oslo, Norway) using 10 mM TRIS/1 mM EDTA (pH 9.0) as the retrieval buffer. Sections were heated for 3 min at 110 °C followed by 10 min at 95°C and cooled to 20° C. All the antibodies are well characterized regarding their specificity and sensitivity. The following antibodies were used: rabbit polyclonal anti-phosphohistone H3 (ser 10) (clone JBW301, 1:3000, Upstate Cell Signaling Solutions, Lake Placid, New York, USA), Ki-67 (clone MIB-1,1:50, DAKO, Glostrup, Denmark) was used at a dilution of 1:50, c-Kit (clone CD117, dilution 1: 400, DAKO, Glostrup, Denmark).

Anti-phosphohistone H3 was incubated for 60 min at 22°C. All other antibodies were incubated for 30 min at 22°C. Dako antibody diluent (S0809) was used. The EnVision™Flex detection system (Dako, K8000) was used for visualization of anti-phosphohistone H3. For Ki-67 the EnVision™Flex+ detection system (Dako, K8002) was used. Sections were incubated for 5 min. with peroxylase-blocking reagent (SM801), 20 min with the EnVision™ FLEX+ Mouse Linker (SM804, only for Ki-67), 20 min with the EnVision™ FLEX /HRP Detection Reagent (SM802), 10 min with EnVision™ FLEX DAB+ Chromogen (DM827)/ EnVision™ FLEX Substrate Buffer

(SM803) mix and 5 min with EnVision™ FLEX Hematoxylin (K8008). The slides were dehydrated and mounted. All immunohistochemical stainings were performed using a Dako Autostainer Link 48 instrument and EnVision™ FLEX Wash Buffer (DM831).

3.6 Evaluation and reproducibility of Ki-67 and PPH3

Ki-67 and PPH3 were counted in the same measurement area as described above for the MAI (in 10 High Power Fields of vision=HPF, in total 1.59 mm²). Ki-67 and PPH3 expressions were defined in two ways:

1. *The total number* of positive nuclei and mitoses in 10 HPF, and
2. *The percentage* of the positive versus the total number of positive plus negative nuclei and mitoses.

As the results were comparable, only the total number of positive nuclei and mitoses in 10 HPF will be further presented.

To assess inter-observer reliability, the counts were done by two independent pathologists (JB, EG) who were unaware of both the clinical outcome and each other's counts results. One pathologist (EG) performed the counts twice, to also assess intra-observer reproducibility. In order to do an objective quality control as well, Ki-67 was also performed using the VIS digital image analysis (DIA) system (Visiopharm, Hørsholm, Denmark) with the same image processing principles described before (figure 5).

The reproducibility of the Ki-67 counts by digital image analysis on different days by different observers was close to perfect. The Ki-67 and PPH3 counts of the two pathologists correlated reasonably well with each other. Correlation with the digital image analysis results ($0.61 < R < 0.69$, $P < 0.003$) was fair, though with a wide variation in several cases.

The Ki-67 and PPH3 were counted in the same measurement area (10 fields of vision, in total 1.56 mm²) described above for the MAI. Ki-67 was defined as *the total number* of Ki-67 positive nuclei and mitoses, whereas PPH3 was defined as *the percentage* of the PPH3 positive nuclei and mitoses.

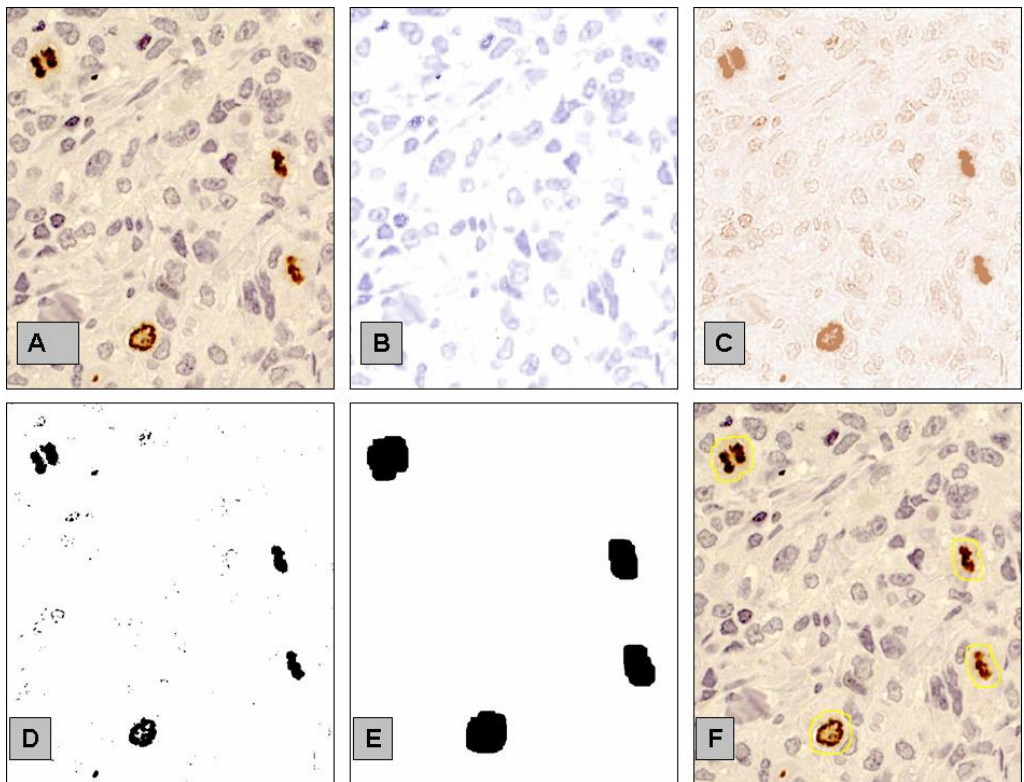


Figure 5. Example of PPH3 counting by digital image analysis. (A) Original image. (B) Classified image, brown DAB-stained objects are labeled yellow, hematoxylin stained objects blue, and background red. (C) Displayed image with counted objects surrounded by a blue line. (D-F) details of image processing, metaphase chromosomes are fused into one object and small objects are removed. Courtesy: Skaland et al, 2008.

3.7 Statistics analysis

SPSS version 15 (SPSS; Chicago, Illinois, USA) was used for the statistical analyses. To evaluate the prognostic significance of the variables, univariate (Kaplan-Meier) and multivariate (Cox model) survival analysis were performed, using recurrence free survival and overall disease related survival as endpoints. MAI and other continuous variables such as age were stratified using quartiles and receiver operating curve (ROC) analysis (MedCalc, Mariakerke, Belgium) and then tested for their prognostic significance. When neighbouring value classes of a variable appeared to have the same prognostic value, they were combined to one class. The threshold values for the different variables with this method were similar as the ones obtained with ROC analysis and only the most important are shown here. $P < 0.05$ was used as the threshold for significant or not. To analyse differences between the different features, Student's t-test or Mann-Witney test was used whichever applicable. With binary multiple regression, the independent value of the features was evaluated to distinguish the different grades.

II Aims of the thesis

Question to be answered

The aims of this thesis were to answer the following questions:

1. What is the reproducibility and prognostic value of the essential diagnostic criteria in WHO2003-defined invasive Endometrial Stromal tumours?
2. What is the value of clinical, therapeutic and histopathological prognostic indicators in WHO2003 Endometrial Stromal Sarcoma?

3. Do morphometric features have diagnostic and prognostic value in WHO2003 invasive Endometrial Stromal Tumours?
4. What is the value of *proliferation biomarkers in ESS*?
5. *Which Therapeutic Aspects* of Endometrial Stromal Sarcoma and Undifferentiated Endometrial Sarcoma have prognostic value?

III. Results

Paper 1: Prognostic Value of the Diagnostic Criteria Distinguishing Endometrial Stromal Sarcoma from Undifferentiated Stromal Sarcoma, Two Entities within the Invasive Endometrial Stromal Neoplasia Family. *Int. J Gynecology Pathology.* 2013 May; 32(3): 299-306.

The World Health Organization (WHO2003) recognizes three endometrial stromal neoplasms: non-invasive endometrial stromal nodule and the two invasive neoplasms, Endometrial stromal Sarcoma (ESS) and Undifferentiated Endometrial Sarcoma (UES). Of import, the WHO2003 does not define moderate atypia (an important differentiating diagnostic criterion for ESS and UES) nor does it discuss its significance. Moreover, studies on reproducibility and additional prognostic value of other diagnostic features in large are lacking. Using strict definitions, we analyzed agreement between routine and expert-review necrosis and nuclear atypia in 91 invasive endometrial stromal neoplasias. The overall 5- and 10-years recurrence-free survival rate estimates were 82% and 75%. Necrosis was well and nuclear atypia reasonably well reproducible. The 10-year recurrence free survival rates for necrosis Absent/Inconspicuous versus Prominent were 89% and 45% ($P < 0.001$) and for review-confirmed None/Mild, moderate, severe atypia 90%, 30% and $<20\%$ ($P < 0.00001$). Therefore, cases with Moderate/Severe atypia should be grouped

together. Nuclear atypia and necrosis had independent prognostic value (Cox regression). Once these features were taken into account, no other feature had independent additional prognostic value, including the mitotic count. Using “None/Mild atypia, necrosis Absent/Inconspicuous” as ESS, versus “Moderate/Severe atypia present or necrosis present” as UES resulted in 68 ESS and 23 UES cases with disease specific overall mortality-free survival 99% versus 48%, $P < 0.00001$, $HR=45.4$). When strictly defined microscopic criteria are used, the WHO2003 diagnoses ESS and UES are well reproducible and prognostically strong.

Paper 2. Prognostic Indicators in WHO2003 Low Grade Endometrial Stromal Sarcoma. *Histopathology*, 2013 Apr; 62(5): 675-87.

Endometrial stromal sarcoma (ESS) has been traditionally divided into low/high grade but the World Health Organization (WHO2003) has changed the definition. Since 2003, many studies still used the old criteria and few focused on WHO2003-defined ESS low grade (ESS-LG). The prognosticators in ESS-LG were investigated. We reviewed the diagnostic WHO2003 criteria in 91 tumours (previously classified as ESS low and high grade). There were 68 ESS-LG and 23 UES. In the ESS-LG, the prognostic value of clinico-pathological variables was studied. With median follow-up of 79 (range: 20-474) months, the recurrence and death rates were 5/68 (7%) and 1/68 (1.5%) in the ESS-LG. Ovarian preservation or not ($P < 0.0001$, $HR=10.4$) and mitotic activity index (MAI, 0-3 versus >3 , $P=0.005$, $HR=8.6$) had independent prognostic value. Other frequently used MAI thresholds, age, tumour diameter and vessel invasion were not prognostic. In patients without ovarian preservation ($n=61$), 0/53 with MAI 0-3 recurred, contrasting 2/8 (25%) with MAI >3 ($P=0.003$) and 1 of these 2 recurrence patients died ($P=0.02$). In patients with ovarian preservation

(n=7), 3 (43%) recurred but none died and MAI had no additional prognostic value. We conclude that in ESS-LG, ovarian preservation and MAI>3 are associated with increased recurrence risk.

Paper 3. Diagnostic and prognostic morphometric features in WHO2003

invasive Endometrial Stromal Tumours. *Histopathology, 2013 Apr; 62(5):688-94.*

The value of morphometric features has been evaluated to distinguish mild and moderate atypia in and predict recurrence of World Health Organization-2003 defined Endometrial Stromal Sarcomas-Low Grade (ESS-LG) and highly malignant undifferentiated uterine sarcomas (UES). Nuclear and cytological size, shape and arrangement were morphometrically evaluated in 41 cases with consensus none/mild (n=38) or moderate atypia (n=3). None of the cases showed necrosis. The prognostic value of these features to predict recurrence was also assessed. Seven features differed. The mean and standard deviation of the nuclear volume and the distance between the nuclei were the best discriminators between the non/mild versus moderate atypia, with the maximum of the nuclear volume as a practically and rapid evaluable alternative. Using these features, all mild and moderate atypias were correctly classified. Seven cases recurred. The distance between the nuclei and percentage of nuclei with one neighbor (assessed with morphometric minimum spanning tree analysis) predicted recurrence. In invasive endometrial stromal tumours, morphometric features are useful diagnostic support tools to distinguish mild from moderate atypia and predict recurrence.

Paper 4. Proliferation biomarkers reliably predict recurrence in World Health Organization 2003 defined Endometrial Stromal Sarcoma, Low Grade.

Manuscript, Submitted February 10, 2013

The value of proliferation biomarkers to predict recurrences in Endometrial stromal Sarcoma, low grade, was studied. This rare uterine sarcoma has a relatively indolent behaviour but recurrences and distal metastases may occur. Literature about the prognostic factors in ESS using the updated definition of ESS is limited. A variety of cytogenetic abnormalities involving chromosome 7 have been reported in endometrial stromal sarcomas. Two zinc finger genes, *JAZF1* and *JJAZ1*, at the sites of the 7p15 and 17q21 breakpoints have been identified. The presence of *JAZF1*-*JJAZ1* markedly inhibited apoptosis, increased cell survival and accelerated cellular proliferation. As most pathology laboratories currently do not have access to translocation analysis, and FISH probes for the assessment of *JAZF1*/*JJAZ1* fusion are not commercially available, it is important to have easy and widely available methods allowing pathologists to assess which patients with an ESS are at high recurrence risk. Measurement of proliferation could perhaps be a useful biomarker for this purpose. Using single and multivariate analysis, the prognostic value of classical mitosis counts (defined according to the Mitotic Activity Index=MAI protocol) in haematoxyllin-eosin (H&E) sections, and immuno-histochemical proliferation biomarkers (Ki-67 and PhosphoHistone-3 (PPH3)) were examined in 24 invasive endometrial stromal sarcomas. Three of 24 (12.5%) ESS recurred, of which only one tumor exceeded stage II and stage was prognostically not significant. The mitotic count with H&E (MAI), PPH3 and Ki-67 were all prognostic ($P=0.001$, 0.002 and 0.03). MAI is prognostically the strongest proliferation biomarker, but can be tedious to reliably assess in poor quality sections. PPH3 counts can be easier to perform and closely resemble (but are higher than) the true mitoses counts. The fact that Ki-67 is the least prognostic, results from it staining not only nuclei in the M(itosis) phase of cycling cells, but also and mostly nuclei of proliferating cells in the non-mitotic (G_1 , S and G_2) phases of the

cell division cycle, many of which likely have genetic damage and die before becoming new daughter cells. In conclusion, in WHO2003-defined ESS Low Grade neoplasias, high levels of proliferation as measured by MAI, PPH3 and to a lesser degree also Ki-67 are predictive of tumors that will recur.

Paper 5. Stages I to II WHO 2003-Defined Low-Grade Endometrial Stromal

Sarcoma: How Much Primary Therapy Is Needed and How Little Is Enough? *Int J*

Gynecol Cancer. 2013 Mar;23(3):488-93. the effect of different treatment modalities was studied. Before 2003, 20% to 35% low-grade ESS recurred, but WHO 2003-defined ESS- low-grade has 10 years' recurrence rates of less than 10%. With so few recurrences, the balance between treatment guaranteeing cure and overtreatment ("not too little" or "too much") becomes increasingly important. However, primary treatment practices range from limited surgery only to extensive surgery combined with adjuvant chemotherapy and radiotherapy. We focused on the primary treatment of early-stage WHO 2003-defined ESS, low-grade. The effect of different therapeutic strategies was studied in 57 patients with International Federation of Gynecology and Obstetrics 2009 stages I to II expert-reviewed WHO 2003-defined ESS, low-grade treated at a single institution between 1992 and 2007. The patients' median age was 43 years (range, 19-63 years). After 68 months' median follow-up (range, 17-140 months), recurrence and mortality rates were 9% and 2%, respectively. The patients with WHO 2003-defined ESS, low grade with ovary-preserving primary surgery had a much higher recurrence rate (75%) than those without (2%; $P < 0.0001$).

Lymphadenectomy, radical abdominal hysterectomy, and omentectomy did not influence survival. Ten patients refused chemotherapy. With univariate analysis, multiple-agent chemotherapy improved the prognosis ($P = 0.02$) With multivariate

analysis, only ovary preservation-or-not surgery had independent prognostic value. We concluded that in International Federation of Gynecology and Obstetrics 2009 stage I to stage II WHO 2003-defined ESS, low grade, total abdominal hysterectomy with bilateral salpingo-oophorectomy is sufficient surgery, but ovary-preserving primary surgery increases the risk of recurrence. More extensive surgical procedures than total abdominal hysterectomy with bilateral salpingo-oophorectomy do not improve prognosis. Chemotherapy may improve progression-free survival but a large sample size is needed to confirm this.

IV. General Discussion and future directions

IV.1. General Discussion

Study 1. Prognostic Value of the Diagnostic Criteria Distinguishing Endometrial Stromal Sarcoma from Undifferentiated Stromal Sarcoma, Two Entities within the Invasive Endometrial Stromal Neoplasia Family.

The objective of the first study was to evaluate the criteria used by WHO2003 to define and distinguish the various groups of malignant endometrial stromal neoplasms by evaluating a large cohort of patients with a median follow-up of 5.8 years (up to 39 years). It was found that the WHO2003 criteria of atypia and necrosis, when strictly defined, allow for reliable differentiation of malignant endometrial stromal tumours in ESS-LG versus UES as prognostically strong categories. Comparison of the original Low/High grade ESS and the WHO2003 diagnoses shows that there is moderate agreement only between the two classifications. The WHO 2003 therefore is not just a name change but results in essential differences in diagnosis, prognosis and therefore potentially also treatment.

In the WHO2003 description, the definition of ESS is somewhat ambiguous (“*tumours without significant cellular atypia and pleomorphism*”). Usually, most pathologists grade nuclear atypia as none, mild, moderate and severe, or as low and high. In the WHO2003 description, even though moderate atypia is not discussed, we felt it important to determine if this middle category had important prognostic value, and what the reproducibility is of these various nuclear atypia grades as well as necrosis. Mild (insignificant) atypia showed minimal variation in nuclear size and shape, and nucleoli were small. Quite commonly mild and no atypia were difficult to distinguish, and so were combined into a single category. Moderate atypia was defined as cases showing scattered pleomorphic nuclei and severe atypia as having many and severely pleomorphic nuclei. The absence or presence of prominent necrosis also is easily reproducible. Cases with severe atypia are easy to diagnose and in our study, half of these cases also had necrosis. Thus, both atypia and necrosis proved to be strong independent prognostic indices, but with the incorporation of moderate atypia with severe atypia, the prognostic value increased, and when tied with prominent necrosis, became the strongest prognostic feature. Of concern is still the potential inter-observer variation in the category of moderate atypia that might occur if viewed without strict rules of classification. We have shown in study 3 and study 4 of this thesis, that morphometric features and immuno-histochemical proliferation biomarkers seem helpful. Until recently, morphometric assessments were tedious, but the advent and rapid spread of digital pathology probably will drastically change this.

In conclusion, the WHO2003 criteria for classifying endometrial stromal tumours as ESS versus UES are somewhat ambiguous, but the use of the definitions for atypia grades and necrosis result in two prognostically strong and separate

categories. The major distinguishing feature is atypia that is moderate or severe on the one hand versus none or mild on the other. The presence of necrosis is also an important independently distinguishing feature.

Study 2. Prognostic Indicators in WHO2003 Low Grade Endometrial Stromal Sarcoma.

The objective of the second study was to evaluate the prognostic value of clinico-pathological variables in a large number of WHO-2003 defined ESS-LG cases with long follow-up. Our study on 68 WHO-2003 defined ESS is the second largest ever done (Abeler et al² described 85 cases) and also has long-term follow-up. ESS patients have good prognosis in general with 2-, 5-, and 10 year disease free survival rates of 97%, 93% and 93%. However, still a few patients recurred and one died of disease. With a generally very favourable outcome in ESS patients, the identification of the small number of patients with recurrence is important. Thus, suitable treatments could be considered to those identified as high risk patients. We found that ovarian preservation was the most important risk factor for recurrence, followed by mitotic activity index >3.

In previous studies, many more features were prognostic: tumour diameter, histologic grade, involvement of surgical margins by tumour, deep myometrial invasion, menopausal status, and age^{19,20, 7, 26, 27}. However, these results were either based on small number of patients or on univariate analysis only (we used multivariate analysis). Moreover, most of these studies were published before 2003 and included "ESS high grade" cases which now often would have been regarded as UES.

Special attention should be paid here to the classical large clinico-pathological study of Chang et al (1990)⁷, who studied 109 patients with endometrial stromal

sarcoma and eight patients with endometrial stromal nodule. Of the 109 patients with endometrial stromal sarcoma, follow-up was obtained on 93 (85%). They found that 36% of the Stage I patients experienced one or more relapses. Of these, 23% died of disease from with median follow-up of 79 (range: 11-360) months. Of the 17 stage III or IV patients, 13 had one or more relapses and of these, 9 died of disease. The outcome differences between Stages I, III, and IV are statistically significant ($p < 0.01$), but neither the mitotic index (number of mitoses/10 high-power fields) and cytological atypia were predictive of tumour recurrence for patients with Stage I tumours. These results may seem to contradict our results, but this is not supported by a closer analysis of the results. The study obviously was many years before the WHO 2003 classification was introduced and their definition of ESS was as follows: endometrial stromal neoplasms with myoinvasion or intravascular growth. Tumours with significant pleomorphism were excluded from the study. As they explicitly describe, they found cases with no atypia, mild, moderate and severe atypia, while their major break was between no+mild versus moderate+severe atypia. This makes clear that the ESS cases they studied included cases that currently would have been regarded as undifferentiated endometrial sarcoma. This is further supported by the much higher recurrence rates in their stage 1 cases (36%) than in our material. Their percentage of stage 3 and 4 cases also was much higher than in our study.

Total hysterectomy and bilateral salpingo-oophorectomy (BSO) is considered as the standard surgical treatment for ESS patients. Several studies challenge the inclusion of BSO as standard surgical therapy in patients with disease limited to the uterus. Amant et al ³² reported that in stages I–II premenopausal LG-ESS women undergoing hysterectomy with or without BSO, relapses occurred in 3 out of 12 (25%) and 1 out of 6 (17%), respectively. They stated that BSO does not seem to improve

the outcome, but their patient numbers were very small. Kim et al ²¹ also reported that BSO was not correlated with recurrent disease in ESS, but again this study evaluated only 22 patients. One case control study by Li ²² carefully compared 12 LGEES without BSO with 24 LGEES with BSO and found that retention of ovarian function (in the absence of metastasis) does not appear to impact risk of recurrence or overall survival in stage I LGEES. However, they used a mitotic index <10/10HPF as diagnostic criteria which we have shown may have seriously influenced the recurrence rate. Moreover, they did not match the tumour size and mitotic count between the two treatment groups studied.

Before 2009, the 1988 FIGO surgical staging system for endometrial cancer was recommended for ESS. In 2009, the FIGO committee on Gynecologic Oncology introduced a new FIGO staging systems for uterine sarcomas. In the new system, stage I and II of the 1988 FIGO staging system have been combined to represent stage I ESS. In the current study, we showed that there is no difference in the recurrence rates of ESS patients with FIGO-2009 stage I and II (95% and 92%). So, even if the disease has spread outside the uterus but still is confined to pelvic cavity, the prognosis is good. There was only one stage IV patient in this study, and she died of disease, in agreement with the single high stage patient of Abeler et al (2009)². Therefore, distant metastasis in ESS is a very rare event but when it occurs should be regarded as an unfavorable prognostic factor.

We found that the recurrence rate was significantly higher in 7 ESS patients with ovary preservation than in 61 patients with ovary castration (43% versus 3%). The seven patients with ovary preservation were all stage I and II and relatively young with a median age of 35.4 years, whereas the patients without recurrence had

a median age of 43 years. Interestingly, the median age in the study of Li et al mentioned above was 46 years in both study subgroups²³. The variation of recurrence rates in women with retained ovarian function may therefore perhaps reflect differences in age and different hormonal conditions of the patients with ESS. In patients approaching or in the menopause, retention of ovarian function may be of less concern for recurrence. Interestingly, Berchuck et al³⁴ study from 1990 on endometrial stromal tumours found that all 6 patients with ESS treated without BSO had recurrence, while only 6 of 13 patients treated by TAH–BSO recurred (100% versus 43%). Although this finding should be interpreted with care, it thus seems that ovarian preservation also increases the recurrence risk in pre- or peri-menopausal women with endometrial stromal tumours.

It is tempting to try to explain this, as follows. Due to their endometrial stromal origin, ESSs are oestrogen receptor positive. Ovarian preservation in pre- and to a certain degree also in peri-menopausal women results in persistent or occasionally high oestrogen levels, which therefore may promote small ER-positive ESS metastases. The fact that all ESS patients with recurrence in the present study were relatively young (age between 28~43 years) and premenopausal supports this hypothesis. The recurrent patients still had an excellent prognosis after resection of the metastases and oophorectomy during second surgery. Moreover, there is evidence that adjuvant hormonal treatment is beneficial to ESS patients³² and progestin therapy is currently the most effective treatment for curing and preventing local recurrence³⁰. These facts further support the hypothesis of hormone dependency of ESS tumours and their recurrence.

Before 2003, mitotic activity index had a central role in the diagnostic decision scheme³ of low grade and high grade neoplasms, but in the WHO2003 it is regarded

as unimportant in defining ESS and UES. The current study shows that most (53/68=78%) of the ESS patients without ovarian preservation combined with low mitotic activity index (0-3) can be identified as “excellent prognosis”, i.e. unlikely to get recurrence (none in our study). On the other hand, those with either ovarian preservation, or ovary castration but with MAI>3, have a high risk of 25% to recur. What is more, one study showed a trend that low mitotic index (MI<5) was associated with long disease free survival in ESS, although only 14 ESS cases were investigated ²⁸. These data may give mitotic activity a somewhat important role in the assessment of the prognosis of ESS patients.

Assessment proliferation by MAI can be well reproducible if pathologists strictly follow the assessment protocol¹¹². Computerized automated image analysis may be of further help to get objective quantifiable support data for the daily work of pathologists, as has been shown for other neoplastic organ deviations¹¹³. Alternative proliferation markers such as MIB-1⁴⁵, c-kit⁵², and phosphohistone-3^{78,79} may also be important as alternative prognostic markers, as they can ease the work of pathologists, be largely automated analysis with excellent reproducibility ¹¹⁴.

The major strength of our study is that all the ESS cases have been carefully reviewed by two experienced pathologists, the number of cases is large and UES cases were excluded. The results therefore provide detailed information which may be valuable when counselling patients with ESS. On the other hand, the limitation of this study must also be acknowledged. Only 7 cases received ovary preservation and 5 cases recurred. Multicentre studies are required to obtain statistically more sound results.

In conclusion, in ESS, ovarian preservation and high mitotic activity should be regarded as high risk factors for recurrence.

Study 3. Diagnostic and prognostic morphometric features in WHO2003 invasive Endometrial Stromal Tumours.

Study 3 has shown that many features of consensus mild, moderate and severe atypias were different with computerized morphometric analysis. Nuclei in moderate and severe atypia are generally larger than in mild atypias, but with some overlap. An even better discriminator than median nuclear area is the variation in (standard deviation of) nuclear area. This makes clear that there are more very large nuclei in the moderate/severe atypias, possibly a special cell clone. Moreover, the feature CONN1 (describing the number of nuclei connected to one other nucleus only) is much higher in mild atypias (figure 2). Combination of the two features gives good discrimination. However, in a set for testing, nearly 14% and 17% of the cases is misclassified. Therefore the practical value of computerized morphometric analysis to support the degree of atypia is limited.

We conclude that in endometrial stromal sarcomas, nuclear morphometric analysis has limited practical value to support the degree of nuclear atypia.

Study 4. Proliferation Biomarkers Predict Recurrences in Endometrial stromal Sarcoma

A variety of cytogenetic abnormalities involving chromosome 7 have been reported in endometrial stromal sarcomas. More specifically, two zinc finger genes, *JAZF1* and *JJAZ1*, at the sites of the 7p15 and 17q21 breakpoints have been identified. The presence of *JAZF1-JJAZ1* markedly inhibited apoptosis, increased cell survival and accelerated cellular proliferation. However, most pathology laboratories currently do

not have access to translocation analysis, and FISH probes for the assessment of *JAZF1/JAZ1* fusion are not commercially available. It is therefore important to have easy and widely available methods allowing pathologists to assess which patients with an ESS are at high recurrence risk. Measurement of proliferation could perhaps be a useful biomarker for this purpose. We evaluated in 24 review-confirmed WHO2003 ESS cases with long follow-up, whether Mitotic Activity Index (MAI), Ki-67, PhosphoHistone-3 (PPH3) and c-kit have prognostic value. Three cases recurred, 2 of these had ovary preservation surgery (OPS) contrasting 1/22 without OPS ($P < 0.0001$). C-kit was negative in all cases. MAI, Ki-67 and PPH3 had prognostic value ($P = 0.001$, 0.03 and 0.002 , respectively). Two of the 3 recurrent cases had a MAI of 4 (which were amongst the highest three MAI values found in our series) and also had ovary preservation surgery. The third recurrent patient did not have ovary preserving therapy but had by far the highest MAI and PPH3 of all ESS tumors. With multivariate analysis, OPS was the strongest prognosticator and MAI had independent prognostic value. PPH3 also had additional prognostic value to OPS but PPH3 was prognostically slightly less strong than the MAI. Therefore, in doubtful cases PPH3 can be used as an alternative for the MAI. We conclude that ovary preserving primary surgery and proliferation measured by either MAI or PPH3 have independent prognostic value in ESS.

5. Quality Of Life of Patients with an Endometrial Stromal Sarcoma, Low Grade: how much primary therapy is needed and how little is enough?

The impact of surgical and adjuvant therapies of uterine Endometrial Stromal Sarcomas, Low Grade (ESS-LG) defined according to the 2003 World Health

Organization classification is controversial. We present our experience with 61 WHO2003-defined ESS from a consensus expert review of 25 years (1982-2007) at a single institution. Seven more cases presented in previous studies were not included as they received single agent chemotherapy which is regarded as less effective. We recorded each patient the age, stage of disease according to the FIGO staging system, type of surgical treatment, lymph node status, type of chemotherapy regimen if applicable and follow-up from the patients' and pathology charts. The influence of different therapeutic approaches on recurrence and mortality was determined. The patients' median age was 43 (range: 19-63) years. Sixty cases were FIGO stages I-II while 1 case was stage IV. After a median follow-up of 72 (range: 20-209) months, recurrence and mortality rates were 8% and 2%. ESS patients with ovary preservation surgery had a much higher recurrence rate (50%) than those without (4%), $p < 0.0001$. Lymphadenectomy, nodal status, radical abdominal hysterectomy and omentectomy did not influence survival. Multiple agent chemotherapy improved the prognosis ($p = 0.03$) but only ovary preservation-or-not surgery had independent multivariate prognostic value. With the exception of the single stage IV patient, all other four patients who experienced recurrent disease were alive and well after cytoreductive surgery with/without chemotherapy. We conclude that in FIGO stages I-II uterine ESS, total hysterectomy with bilateral salpingo-oophorectomy is satisfactory therapy. Lymphadenectomy and extensive surgery do not improve survival. Ovary preservation therapy should be avoided. Recurrent disease can be treated effectively with cytoreductive surgery and systemic therapy.

IV.2. Future research

Several issues have not been addressed and beyond the scope of this PhD thesis to resolve and remain the subject of future research.

One is the validity of considering UES as an entity distinct from as opposed to being one end of the spectrum where ESS is at the other. By analogy, should grade 3 endometrial adenocarcinoma be potentially considered as a different entity from grade 1 endometrial carcinoma, since both have differing prognoses, even though both seem to arise by the same mechanism. In contrast, evidence now points to why borderline serous tumor/grade 1 serous carcinoma of ovary is a completely different entity from high grade serous carcinoma as both seem to arise by different pathogenetic mechanisms, even though they share many histo-morphologic features. For example, the former is KRAS & BRAF positive and the latter KRAS & BRAF negative. Issue two is whether the WHO2003, in eliminating the category high grade ESS, has simply substituted UES in its place (*"Old dog, new name"*). Finally, issue 3: should the category of ESS-high grade be reintroduced if it is shown to differ in some fundamental way with a subset of the current UES cases? Future studies based on immuno-histochemical, molecular pathology and even whole genome sequencing may resolve these conundrums.

As mentioned before, a major strength of our study is that all the ESS and UES cases have been carefully reviewed by two experienced pathologists, all patients had been diagnosed and treated in one and the same institution and the number of cases is relatively large.

On the other hand, the limitation of this study must also be acknowledged. The time between the first and the last diagnosis year (1953-2007) spans more than half a century. Only 7 cases received ovary preservation and 5 cases recurred. A second

important aspect for future research therefore is to obtain many cases from a limited number of years. Multicentre studies are required to obtain statistically more sound results.

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