Diploma Thesis

Retrospective assessment of LVSI in stage I endometrioid adenocarcinoma of the uterus using digital pathology

submitted by

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I hereby confirm that the present diploma thesis is the result of my own independent scholarly work. I also confirm that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University's regulations and procedures concerning plagiarism.

Graz, 22.04.2024 *Saskia Weber m.p.*

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Zusammenfassung

Hintergrund: im Vergleich zu nicht-endometrioiden histologischen Typen, sind endometrioide Adenokarzinome des Uterus meistens mit einer guten Prognose assoziiert und werden vorwiegend chirurgisch therapiert. Jedoch beeinflussen Stadium, Grading und verschiedene andere unabhängige Prognosefaktoren das Outcome. Einer dieser Faktoren ist die Lymphovaskuläreinvasion (LVSI), welche ein bekannter negativer Prognosefaktor in endometrioiden Endometriumkarzinomen ist und die Therapie beeinflusst. Aufgrund der prognostischen und therapeutischen Wichtigkeit legen die Weltgesundheitsorganisation (WHO) und die ESGO/ESTRO/ESP Leitlinien Kriterien für die Evaluierung und Interpretation von LVSI dar. Unter Verwendung eines dreistufigen Klassifikationssystems (keine LVSI, fokale LVSI, substantielle LVSI), geben sie an, dass ausschließlich substantielle LVSI (definiert als 5 oder mehr LVSI) eine prognostische Signifikanz hat, wohingegen fokale LVSI (definiert als <5 LVSI) keine hat. Erst vor kurzem wurde die substantielle LVSI in die aktuelle Version der FIGO-Stadien (2023) aufgenommen. Die ESGO/ESTRO/ESP Leitlinien sind die einzigen die auch zwischen unifokaler und multifokaler LVSI differenzieren, wobei die letztere automatisch als substantiell gewertet wird. Wir untersuchten in einer retrospektiven Studie die vielen Aspekte der LVSI, evaluierten die Anzahl, räumliche Verteilung und Tiefe der LVSI und setzten sie in Bezug auf das klinische Outcome.

Methodik: alle zwischen den Jahren 2004 und 2018 diagnostizierten endometrioiden Endometriumkarzinome mit N0, L1, FIGO-Stadium I und G1-G3 wurden eingeschlossen. Weiters wurden die klinischen Daten bezüglich Rezidivs und Todesfall dokumentiert. In Summe wurden 25 geeignete Fälle ausgehoben und analysiert. Alle Tumorschnitte wurden eingescannt und mit Hilfe von QuPath wurden alle LVSI markiert und konnten für weitere Berechnungen verwendet werden. Die Gesamtzahl an LVSI wurde für jeden einzelnen Fall gezählt. Weiters wurde untersucht, ob eine Tendenz zur Anordnung in Clustern (Fokus) vorliegt. Verschiedene Clusterdurchmesser (0,5mm, 1mm, 2mm, 3mm, 4mm,) wurden getestet und die Anzahl von Clustern pro Fall wurde dokumentiert. Die Beziehung zwischen der Anzahl von Clustern und dem Rezidiv und den Todesfällen wurde untersucht. Ebenso wurde die Beziehung zwischen einer tiefen Lokalisation von LVSI und dem Outcome untersucht.

Resultate: In dieser Studie konnte beobachtet werden, dass substantielle und diffuse LVSI ein höheres Risiko für Rezidiv und eine erhöhte Mortalität haben, im Vergleich zu fokaler

LVSI. Ebenso konnte die Grenze von 5 LVSI als geeignet verifiziert werden. Weiters fanden wir, dass Unifokalität, definiert als das Vorhandensein eines einzelnen Fokus mit <5 LVSI, nicht mit einem schlechterem Outcome assoziiert ist, da Rezidive und Todesfälle nur in Fällen mit Multifokalität beobachtet wurden, wobei der Großteil der Fälle auch mindestens 5 LVSI in Summe hat. Es war nicht möglich, aufgrund der niedrigen Anzahl an Grenzfällen mit Bifokalität und geringer Anzahl an LVSI in Summe, in unserer Kohorte zu beweisen, dass eine multifokale Verteilung von LVSI automatisch mit einem erhöhten Risiko zusammenhängt. Weiters konnten wir einen starken Zusammenhang zwischen tiefer Lokalisation von LVSI im Myometrium und negativem Outcome nachweisen.

Conclusio: in dieser Arbeit konnte bewiesen werden, dass nur substantielle LVSI eine wichtige Rolle in der Prognose besitzt und die in den internationalen Leitlinien gebräuchliche Grenze passend ist, um zwischen fokaler und substantieller LVSI zu differenzieren. Der Einsatz von digitaler Pathologie konnte sich als besonders nützlich beweisen, um LVSI zu untersuchen und sollte auch in zukünftigen Studien mit einer größeren Fallzahl eingesetzt werden, um Multifokalität und den prognostischen Wert zu untersuchen, da es aufgrund der kleinen Kohorte nicht möglich war den Einfluss zu bestimmen. Die tiefe Lokalisation von LVSI im Myometrium war ein starker Indikator für ein herabgesetztes Überleben in unserer Studie und sollte in weiteren Studien untersucht werden.

Abstract

Background: in contrast to non-endometrioid histotypes, endometrioid adenocarcinoma of the uterine corpus are mostly associated with an overall favourable prognosis and get mostly treated with just surgery. However, staging, grading and along with different other independent prognostic factors influence the outcome. One of these factors is lymph-vascular space invasion (LVSI), which is a known negative prognostic factor in endometrioid endometrial cancer and influences treatment options. Due to the prognostic and therapeutical importance the World Health Organisation (WHO) and the ESGO/ESTRO/ESP guidelines provide criteria for LVSI evaluation and interpretation. By using a three-tiered scoring system (no LVSI, focal LVSI, substantial LVSI), they state that only substantial LVSI (defined as 5 or more LVSI) is of prognostic significance, whereas focal LVSI (defined as <5 LVSI) is not. Most recently, substantial LVSI has been incorporated in the current version of FIGO staging (2023). The ESGO/ESTRO/ESP guidelines are the only ones which also differentiate between unifocal and multifocal LVSI, considering the latter automatically as substantial. In a retrospective study we investigated the many aspects of LVSI, evaluated number, spatial distribution and depth of LVSI and correlated them with clinical outcome.

Method: all diagnosed endometrioid endometrial cancer cases between the year 2004 and 2018, with N0, L1, FIGO Stage I, G1-G3 have been included. Further clinical data regarding cancer recurrence or death has been noted. In total 25 eligible cases were retrieved and analysed. All tumour slides were scanned and with the help of QuPath all LVSI have been marked and could be used for further calculations. The total number of LVSI for each case was counted. Further, it was investigated if they had the tendency to aggregate in clusters (foci). Different cluster diameters (0,5mm, 1mm, 2mm, 3mm, 4mm) have been tested and the number of clusters per case was documented. The relation between number of cluster and cancer recurrence and death was assessed. An association between deep location of LVSI and outcome was also investigated.

Results: In this study it could be observed that substantial and diffuse LVSI have a higher risk for cancer recurrence and are associated with a higher mortality, compared to focal LVSI. Further, it could be verified that the cut-off of 5 LVSI is adequate. What is more, we found that unifocality, defined as presence of a single focus with <5 LVSI, was not associated with worse outcome, as recurrence and death were only seen in cases with multifocality, the vast majority of which had also at least 5 LVSI in total. Considering the

low number of borderline cases with bifocality and small number of total LVSI in our cohort, it was not possible to collect evidence that a multifocal distribution of LVSI automatically defines a higher risk. Further, we observed a strong association between deep location of LVSI in the myometrium and adverse outcome.

Conclusion: In this work it could be proven that only substantial LVSI plays a key role in the prognosis and that the used cut-off according to international guidelines is adequate to differentiate between focal and substantial LVSI. The use of digital pathology proved itself to be a very useful tool in examining LVSI and should be used for further investigations regarding multifocality and to determine the prognostic value in future studies with a larger number of patients, as it was not possible due to our small cohort to prove its impact. The deep location of LVSI in the myometrium was in our study a strong indicator of decreased survival and should be observed in future studies.

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List of abbreviations

AGO	Arbeitsgemeinschaft für gynäkologische Onkologie
ASR	Age standardized rate
BMI	Body Mass Index
CTNNB1	Catenin Beta 1
DNA	Desoxyribonucleic acid
EAH	Atypical endometrial hyperplasia
EBRT	External beam radiotherapy
EC	Endometrial carcinoma
EEC	Endometrioid endometrial cancer
EDM	Exonuclease domain mutation
EH	Endometrial hyperplasia without atypia
ESGO	The European Society of Gynaecological Oncology
ESP	The European Society of Pathology
ESTRO	The European Society for Radiotherapy and Oncology
FIGO	The International Federation of Gynaecology and Obstetrics
H&E	Haematoxylin and Eosin
HER2/neu	Human epidermal growth factor receptor 2
HNPCC	Hereditary non-polyposis colorectal cancer
HRd	Homologous recombination deficiencies
K-RAS	Kristen Rat Sarcoma Viral oncogene homolog
L1CAM	L1 cell adhesion molecule
LPP	Lower cervical pathway
LVSI	Lymph vascular space invasion
MELF	Microcystic elongated and fragmented
MMRd	Dysfunction in mismatch repair
MRI	Magnetic resonance imagining
MSI	Microsatellite instability
NEC	Non endometrioid cancer
NSMP	No specific molecular profile subtype
PET	Positron emission tomography
PIK3CA	Phosphoinositide 3 Kinase

POLE	DNA polymerase epsilon
PORTEC	Postoperative radiation therapy in endometrial cancer
PTEN	Phosphatase and tensin homolog
RD	Recurrence and/or death
SEIC	Serous intraepithelial carcinoma
SHBG	Sex hormone binding globulin
SNL	Sentinel lymph node
TCGA	The Cancer genome atlas
TVUS	Transvaginal ultrasound
UICC-TNM	The Union for International Cancer Control – TNM
UPP	Upper cervical pathway
WHO	World Health Organisation

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1 Introduction

1.1 Endometrial carcinoma

1.1.1 Epidemiology

The endometrial carcinoma (EC) is the most frequent gynaecological malignancy in the western world, with around 121.578 newly diagnosed women in Europe in 2018 stated by the ESGO/ESTRO/ESP (2020) guidelines (2). According to the AGO (Arbeitsgemeinschaft für gynäkologische Onkologie) the incidence of EC is 12/100.000 and approximately 940 new cases of EC occur every year in Austria. The incidence correlates with a higher age, which can be seen as 90% of EC appear in women over the age of 50, and only 5% of all EC appear below the age of 40 (3).

Endometrial carcinomas are accountable for 42.000 deaths worldwide, and out of all cancer associated deaths in females 1,9% are due to EC, stated by the S3-Guidelines. In Europe the 5-year survival-rate is around 72% (4).

Figures 1 and 2 are showing the age standardized rate of incidence and mortality from selected countries from 1980 to 2018, indicating an increased mortality rate and incidence in females.

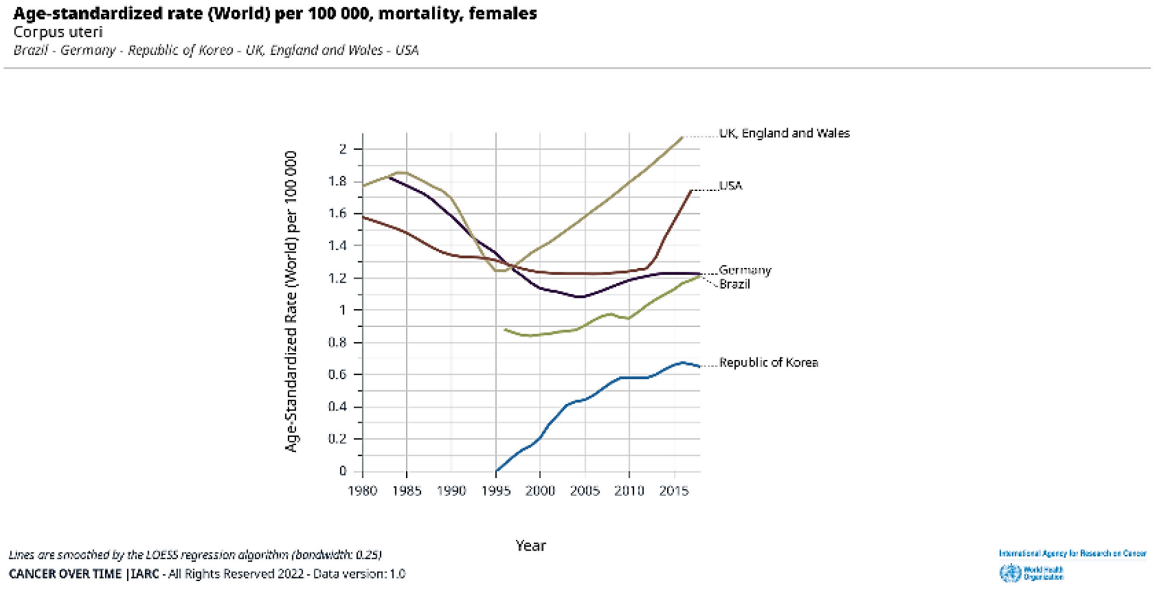

Figure 1: ASR (WORLD) per 100 000, mortality, females - corpus uteri from IARC (1)

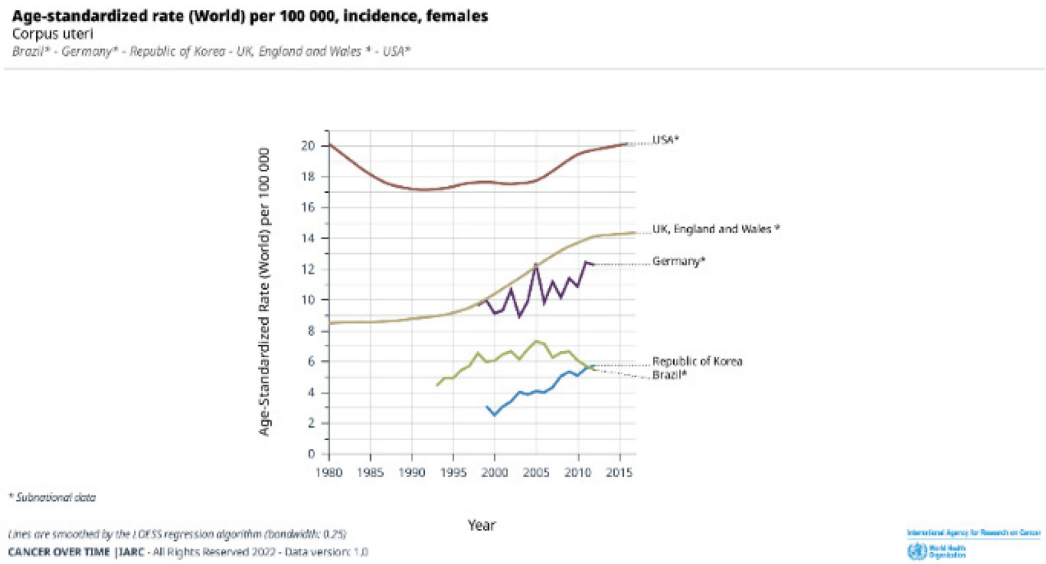

Figure 2: ASR (World) per 100 000, incidence, females - corpus uteri from IARC (1)

Looking at the world chart, geographic differences can be observed. Compared to developing countries, EC are more common in industrial countries. This presumably correlates with achieving an older age and higher rates of obesity, which can be observed more frequently in high-income countries (5). The mentioned north-south gradient is shown in figure 3.

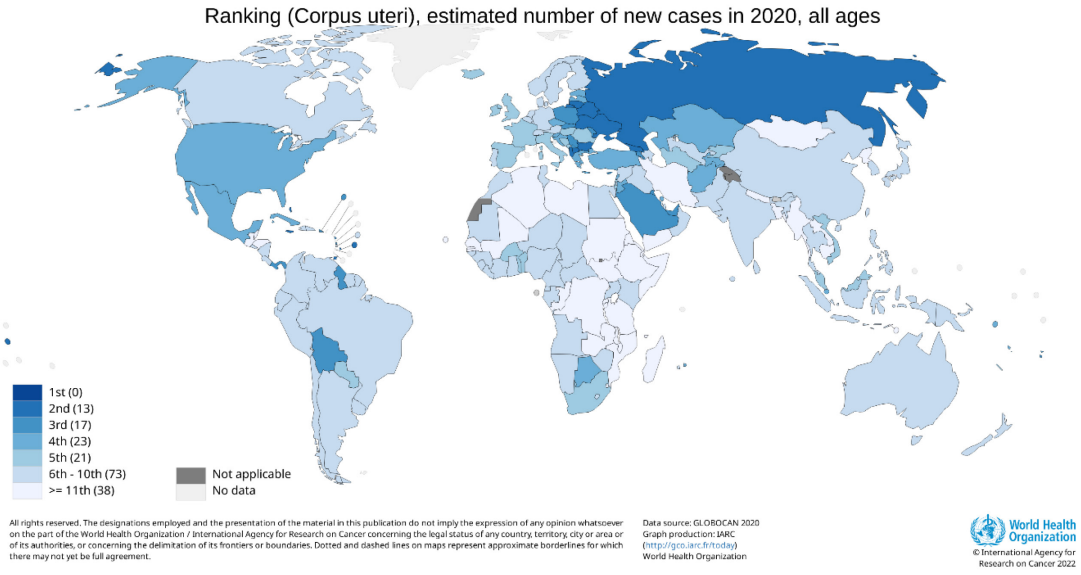

Figure 3: Ranking (Corpus uteri), estimated number of new cases in 2020, all ages from IARC (6)

In the US the lifetime risk of EC is higher in white females, compared to African American females, but several observations have shown that there is an increased rate in black women of EC with a more aggressive histology. Analysis found out that black women below 50 years present endometrial cancers with a higher grade, a more aggressive

histology and later tumour stage compared to an equal cohort of white females, leading to higher mortality rates in black females (7, 8).

1.1.2 Risk factors

As already stated, higher age is associated with endometrial carcinoma, as EC mostly affects postmenopausal women. Furthermore, obesity favours the development of endometrial cancer, due to an increased transformation from androgens to oestrogens. Elevated oestrogen levels are well-known risk factors for EC, either endogenous – as seen in polycystic ovarian syndrome, or in women with early menarches and late menopause – or through exogen exposure. Especially postmenopausal patients with breast cancer have a 4-fold risk for EC due to therapies with Tamoxifen, because of its antioestrogen mechanism. Additionally, unopposed hormone replacement therapy with sole oestrogen increases the risk by a 4 to 8-fold for endometrial cancer and should therefore be combined with progesterone to decrease the risk of EC development (3).

On the other hand protective factors such as parity and oral contraceptive decrease the risk of EC, due to their influence on oestrogen (8).

Familial cancer syndromes such as Lynch syndrome and Cowden syndrome are known predispositions for EC, both inherited autosomal dominant. The hallmark of the Lynch syndrome is a germline mutation in the DNA mismatch repair genes, and it not only leads to an increased lifetime risk of EC, and a higher risk for EC at a younger age, but multiple cancer entities as well, such as colorectal cancer. Same applies for females with Cowden syndrome as it leads to an increased risk of being diagnosed with endometrial cancer by the age of 70 up to 28% (9).

1.1.3 Morphology

The uterus is a muscular organ which can be subdivided into the corpus uteri, the fundus uteri and the cervix uteri. Furthermore, the corpus is made up of three layers: the endometrium on the inside, the myometrium in between and the perimetrium on the outside. The endometrium itself is built up from glandular cells and further has a vascularized stroma which is rich of cells (10). Therefore, the endometrial carcinoma originates from the epithelial cells of the cavum uteri, and firstly expands into the myometrium (5).

1.1.3.1 Histopathology

A dualistic model to classify endometrial carcinoma into two subtypes, Type I EC and Type II EC, on the basis of clinical, epidemiologic and prognostic features has been introduced by Bokhman in 1983 (11). Table 1 describes the two subtypes of EC.

Table 1: Dualistic model by Bokham (1983) of the endometrial carcinoma from the S3-guidelines (4)

	Type I	Type II
Oestrogen association	Yes	None
Origin	Endometrial hyperplasia	Atrophic endometrium
Age	55-65	65-75
Prognosis	More favourable	Unfavourable
Stage	FIGO I	FIGO II-IV
Histological subtype	Endometrioid, variations	Serous, clear cell

The dualistic model has played an important role in the diagnosis of EC for many decades, nonetheless it lacks discrimination of various features. Therefore, a classification on histological parameters is not an ideal guide to determine endometrial cancer outcomes. As a result, the ESGO/ESTRO/ESP guidelines recommend a risk stratification using molecular classification, because molecular subtyping is a more objective and reproducible approach compared to histopathological classification (2, 11).

Overall, two histological subtypes of EC can be described: endometrioid EC (EEC) and non-endometrioid EC (NEC) (11).

Type I EC describes endometrioid adenocarcinoma (EEC) (3). Endometrioid adenocarcinoma mostly develop from a hyperplastic endometrium, due to unopposed hyperoestrogenism. In comparison, these types of EC have a better outcome than type II EC, as they present a less aggressive histology and EEC are more likely to be detected at an early stage of the disease (12).

Histological presentation of endometrioid adenocarcinoma are glandular cells with complex structures, presenting simple or pseudostratified cylindrical cells, along with elongated nuclei. With tumour progression the glandular differentiation turns into solid areas and cell nodes (13). Overall type I EC keep their intraepithelial polarity (14).

Using immunohistochemistry methods, an expression of oestrogen and progesterone receptors can be observed in most EEC, while p16 is poorly expressed. Mutations in PTEN, K-RAS and Beta-Catenin are common in this type of endometrial carcinoma (13).

Type II EC (NEC) describe serous or clear cell carcinoma, which make up to 15% of all EC types, and further also include undifferentiated EC and Carcinosarcoma (3). Type II EC develops independently of oestrogen, and since these types show a more unfavourable histology, they are associated with a decreased overall survival (12).

Serous EC appear in around 10% of all EC diagnosis and are more likely to be diagnosed in stage III-IV ECs along with locoregional lymph node metastasis. They are characterized by high-grade atypia and by papillary morphologies with atypical neoplastic cells (13). Additionally, an important feature of serous EC, in comparison to endometrioid adenocarcinoma, is the loss of intraepithelial polarity (14). Necrotic areas, as well as psammoma bodies can be observed (15).

Ki67 – a proliferative index – is a highly increased immunohistochemistry marker and together with p16 used as a diagnostic tool. As serous EC are not associated with hyperoestrogenism they lack at the expression of oestrogen and progesterone receptors in immunohistochemistry compared to endometrioid adenocarcinoma (13).

Only 2% of EC fall into the clear cell carcinoma group, which is a very heterogenous category. The characteristic morphology, and hence the name, is the clear cytoplasm due to a high level of glycogen. Nuclei atypia in its various forms can be found as well (13).

Clear cell EC are also negative for oestrogen and progesterone receptors, and also express p53, p16 and Ki-67 in immunohistochemistry observations as seen in serous EC (13).

Grading of endometrial carcinoma corresponds with the histotype. An exception is made with serous and clear cell EC as they are always considered G3 because of their histological criteria; hence the grading only applies for type I EC and is shown in Table 2 (4).

Table 2: Grading of endometrioid adenocarcinoma according to FIGO from the S3-Guidelines (4)

	Histological criteria
Grade 1 (G1)	Less than 5% non-squamous or solid growth pattern
Grade 2 (G2)	6-50% non-squamous or solid growth pattern
Grade 3 (G3)	Over 50% non-squamous or solid growth pattern

1.1.3.2 Origin

The most common precursor of endometrial cancer is the atypical endometrial hyperplasia (EAH), defined as a clonal proliferation of atypical glandular cells of the endometrium with more glands than stroma. EAH is the result of unopposed hyperoestrogenism and associated with mutations like PTEN, KRAS, PIK3CA and/or CTNNB1 (16). Atypical cells present histological features such as a prominent eosinophile cytoplasm, a round fair nucleus and a prominent nucleolus as well as coarsened chromatin (10). In comparison to the EAH an endometrial hyperplasia without atypia (EH) can be found as well, even though it is not seen as a precancerous lesion, EH is associated with a 3- to 4-fold risk of EC. Typically the EH originates from prolongated and disordered cell proliferations, with an increase in stroma and glands (17).

Unopposed hyperoestrogenism is often seen in obese females, as a result of a higher peripheral conversion rate in the adipose tissue of androgens to oestrogen, and further due to a downsize of the freely moving sex hormone binding globulin (SHBG), which as a consequence results in higher free oestrogen levels in the serum (12). Progesterone deficiency and continuous high unopposed levels of oestrogen due to increased anovulation in obese woman, makes them vulnerable to proliferation of endometrial cells, suppression of apoptosis and more errors in DNA replication. Numerous investigations have shown that obese women with EC have a worse outcome compered to normal-weighted affected (18). Unopposed oestrogen exposition can also be found in women with early menarche, late menopause and diabetes (12).

Apart from that, the second morphological precursor is the so called serous intraepithelial carcinoma (SEIC), which shows glandular cells of the endometrium lined by atypical glands, with morphological features as seen in serous EC. Nonetheless, the SEIC is not an invasive disease (16). Immunohistochemistry can be a helpful diagnostic tool as SEIC shows an expression of Cyclin E, p53 and Ki-67. In some cases, when being diagnosed, SEIC are already accompanied by metastasis in neighbouring organs and the peritoneum (17). According to the S3-Guidelines it is classified as a carcinoma of the surface and not as a preneoplasia, due to the fact that in above 50% it is accompanied by a extrauterine serous carcinoma and is barely diagnosed without an extrauterine lesion (4).

1.1.4 Molecular Pathology

1.1.4.1 Genetic

Of all endometrial cancer cases, up to 3% are inherited. The most common germline mutation is the so-called Lynch-syndrome, previously known as hereditary non polyposis colorectal cancer (HNPCC) (3).

Familial cancer syndromes are caused by a germline mutation of DNA-repair genes or tumour-suppressor genes, and moreover, since these syndromes are mainly inherited autosomal dominant, result in a 50% risk of inheriting this genetic predisposition (4).

Cancer associated with Lynch syndrome is the result of a mutation in DNA-mismatch repair genes MSH2, MSH6, MLH1 and PMS2, and affected women have a lifetime risk of EC up to 60% (12). It is recommended that females with a known or suspected familial history of Lynch syndrome should be provided with genetic counselling and consequently mutational analysis via MMR immunochemistry or MSI test should be carried out. Furthermore, medical checkup for EC is suggested at the age of 35 by annual transvaginal ultrasound (TVUS) and tissue sampling. After completion of family planning hysterectomy and bilateral salpingo-oophorectomy should be performed ideally before reaching the age of 40, to decrease the risk of EC and ovarian cancer, and in addition hormone replacement therapy is suggested in premenopausal women after bilateral salpingo-oophorectomy (2).

1.1.4.2 The Cancer Genome Atlas

Histopathological diagnoses have been the current standard for many years and still play an important role in the decision-making regarding treatment, which is why the definition of EC was predominantly based on histological criteria supported with immunohistochemical methods. Despite that, studies have found that ECs with similar histological patterns can result in a distinctive different outcome (19). In addition, difficulties regarding clinical decisions occur when histological subtyping overlaps with grading. Immunohistochemical markers such as p53, PTEN or markers for hormone receptors like progesterone and oestrogen can be a useful tool distinguishing between subtypes allowing a better characterization of EC (20). But while G1 and G2 EEC lack TP53 mutations, around 50% of G3 EEC show TP53 mutations, contributing to a poorer outcome. As a result, suggestions have been made of implementing a binary system, in which G1 and G2 are combined and further defined as low-grade and G3 defined as high-grade, leading to a better consideration of the biology of EEC. Another common genetic

alteration in EC are microsatellite instabilities, which studies show appear mostly in just EEC regardless of grading (21, 22).

A milestone in studies of EC has been the implementation of a molecular classification by The Cancer Genome Atlas (TCGA). Endometrial carcinomas are therefore classified into 4 molecular subclasses:

1. POLE Mutation (ultramutated subclass)
2. MMRd (hypermutated subclass)
3. p53abn (copy-number high subclass)
4. No specific molecular profile subtype (NSMP) (copy-number low subclass) (23, 24)

As a result, the World Health Organisation (WHO) recommends the use of more reproducible molecular classification from TCGA, instead of diagnosis based on histological types (23).

With this molecular classification most of EC can be defined as one singular molecular type. Nonetheless, around 6% of EC cannot be defined as a single molecular type as they show more than one molecular feature, such as a combination of POLE exonuclease domain mutation (EDM) and p53 mutations, or MMRd with POLE EDM, yet all three mutations can occur in one tumour as well. EC with more than one molecular type are referred to as “multiple classifier”(25).

POLE mutation

A mutation in the exonuclease domain of the DNA polymerase epsilon (POLE) is the underlying cause of this subgroup. Consequently, POLE mutations cause a disturbance of proofreading function in DNA replication, resulting in increased DNA mutations. Besides that, CD8+ lymphocytes infiltrate the EC as the result of excessive antitumour response, as the mutated DNA acts as neo-antigen and induces immune response (24). POLE mutations are often found in endometrioid EC, additionally heterogeneity and ambiguity in morphology, combining endometrioid and serous histopathology, within the same tumour can be observed (23).

This molecular subtype of EC is mostly found in younger, normal-weighted women and despite it being associated with high grade and expanded lymph-vascular space invasion (LVSI) has a more favourable outcome (16).

MMRd

This subgroup is the result of a mismatch repair deficiency (MMRd). The consequence of MMRd is the loss of expression of MMR proteins MLH1, MSH2, MSH6 or PMS2, and therefore increasing the appearance of mismatches, deletions and microsatellite instability (MSI) (24). Mutations can develop via three pathways: firstly a germline mutation in MMR proteins MLH1, MSH2, MSH6 and PMS2, also known as Lynch Syndrome; secondly a somatic MMR; and lastly sporadic mutation (26).

This molecular subtype appears in older women as well as in younger females independent of the BMI, but when found in young women they are often associated with Lynch syndrome compared to older women who are associated with sporadic mutations (16).

MMRd mutated EC display a likewise pattern to POLE mutated EC. This subtype is also characterized by higher tumour grade, but mostly endometrioide histotype, and lack of invasion of lymphocytes, as seen in POLE mutated EC (19). MMRd EC have intermediate outcome, as they present higher grade, LVSI and advanced tumour stage (24).

p53abn

TP53, a tumoursuppressorgene, plays an important role in the cell cycle and codes for the transcriptionfactor p53. When damage in the DNA occurs, p53 activates target genes halting the cell cycle, further activates repair mechanisms and if damage is non-correctable induces apoptosis; hence it also earned the name “guardian of the genome”. Considering all of this, mutations in the TP53 gene lead to loss of function in p53 and therefore cell cycle control fails, and genetic damage accumulates, due to the loss of DNA-repair mechanisms (10).

Increased somatic copy-number alterations are the characteristics of this subtype, as a result of somatic TP53 mutations. Common findings in this subtype are molecular alterations such as enlargement of human epidermal growth factor receptor 2 (Her-2/Neu) and homologous recombination deficiencies (HRd) (24).

P53abn is most commonly found in older females, females with low body weight and a more advanced stage, resulting in a more unfavourable prognosis making up 50-70% of EC mortality (26).

Serous carcinoma and carcinosarcoma originate often on the ground of this molecular mutation, nonetheless mixed histology and high grade EEC and clear cell carcinoma can also present p53abn mutation (23).

NSMP group

In this subgroup, also named as copy-number low subclass, the specific molecular mutation is unknown. Mostly low to intermediate EEC fall into this subgroup and overall result in a moderate outcome (24). Demographically speaking they are often found in women with a higher BMI (23).

EC with positive oestrogen and progesterone receptors fall into this subtype. With the aim to improve categorization of this subtype, associations with CTNNB1 and L1 cell adhesion molecule (L1CAM) mutations have been made, resulting in a poorer outcome. Because, L1CAM, a membrane glycoprotein, taking part in tumour cell migration, is associated with adverse prognostic factors such as deep myometrial invasion, LVSI and lymph node involvement (26).

1.1.5 Staging

Table 3 on the following page shows the current TNM classification in comparison to the FIGO staging classification.

Over the years some changes have been made regarding the TNM-classification of endometrial carcinoma. As seen in table 3, showing the current classification of EC, T1 is subdivided into T1a and T1b according to the depth of myometrial invasion. However, this was not always the case as in the 6th edition of the TNM-classification endometrial carcinoma have been subdivided into T1a, T1b and T1c. Category T1a was given when EC was restricted to the endometrium, T1b was defined as tumour invasion less than 50% of myometrium and lastly T1c meant that 50% or more of myometrium was invaded by the tumour (27).

Table 3: 8th edition of TNM-/FIGO classification of the endometrium carcinoma (28)

TNM	FIGO	Definition
TX		Primary tumour cannot be assessed
T0		No evidence of primary tumour
T1	I	Tumour confined to corpus uteri
T1a	IA	Tumour limited to endometrium or infiltrating less than 50% of the myometrium
T1b	IB	Tumour infiltrating 50% or more of the myometrium
T2	II	Tumour infiltrates the cervical stroma, but no extension beyond the uterus
T3	III	Local and/or regional extension
T3a	IIIA	Tumour infiltrates serosa and/or adnexa
T3b	IIIB	Infiltration of the vagina or parametrium
N1	IIIC	Metastases in pelvic and/or paraaortic lymph nodes
	IIIC1	Metastases in pelvic nodes
	IIIC2	Metastases in paraaortic nodes
T4	IVA	Tumour infiltrates bladder or rectal mucosa
M1	IVB	Distant metastases, including intraabdominal metastases

Changes have been also made in the 2023 FIGO classification. Compared to the old FIGO classification (seen in table 3 above) new definitions of the substages have been implemented. Stage IAI describes non-aggressive EC types which are limited to the endometrium or grow as a polyp, Stage IA2 describes ECs of non-aggressive type with less than 50% myometrial invasion and no or just focal lymph vascular space invasion and low-grade EC which are confined to the uterus with accompanying low-grade EEC ovarian involvement are defined as Stage IA3. In Stage IB ECs with non-aggressive histology and more than 50% of myometrial invasion and no or just focal lymph vascular space invasion are included. ECs with an aggressive histotype are defined as Stage IC. In Stage II subclasses have also been implemented with IIA describing non-aggressive ECs with invasion of the cervical stroma, IIB defining ECs with non-aggressive histology and substantial lymph vascular space invasion and IIC includes aggressive types of EC with any myometrial invasion. In Stage IIIA ECs with adnexal and uterine serosa invasion are included, IIIB describes EC with invasion of the vagina or parametrium as well as pelvic

metastasis and IIIC includes EC with pelvic and paraaortic lymph nodes. Lastly ECs with invasion to the bladder or rectum are defined as IVA, while IVB includes ECs with extra pelvic metastasis in the peritoneum and IVC describes ECs with distant metastasis (29).

When the endometrial cancer expands from the inner lining of the uterus to the surrounding myometrium the tumour becomes invasive. As the border between endometrium and myometrium is quite diffuse, measuring the depth of invasion can be difficult, hence it is measured from normal or hyperplastic endometrium and starting from this point measuring to the deepest point of invasion (4).

Perineural invasion is inconsequential in the prognosis of endometrial cancer. Venous invasion appears as macroscopic and microscopic invasion, although the macroscopic invasion is neglectable regarding the outcome (4).

A very important factor for the overall survival of patients with EC is lymph vascular space invasion (LVSI), since it is associated with a poorer outcome, notably in a higher number of LVSI (5).

ESGO/ESTRO/ESP guidelines recommend that the histotype of EC, grading, myometrial invasion and lymph-vascular space invasion should be recorded, as they are important prognostic risk factors. Additional information to established pathological features is provided with molecular classification, and consequently should be mentioned in the report. Table 4 shows the definition of prognostic risk groups (2).

Table 4: Definition of prognostic risk factors according to the ESGO/ESTRO/ESP guidelines (2)

Risk group	Unknown molecular classification	Known molecular classification
Low	• Stage IA EEC, low grade and LVSI negative or focal LVSI	• Stage I-II POLE-mutation, without residuals • Stage IA MMRd/NSMP EEC, low grade, LVSI negative/focal
Intermediate	• Stage IB EEC, low grade and LVSI negative or focal LVSI	• Stage IB MMRd/NSMP EEC, low grade, LVSI negative/focal

	• Stage IA EEC, high grade and LVSI negative or focal LVSI • Stage IA, non-endometroid EC, no myometrial invasion	• Stage IA MMRd/NSMP EEC, high grade, LVSI negative/focal • Stage IA p53abn, non-endometroid EC, no myometrial invasion
High-intermediate	• Stage I EEC, substantial LVSI, regardless of grading or depth of invasion • Stage IB EEC, high grade, regardless of LVSI • Stage II	• Stage I MMRd/NSMP EEC, substantial LVSI, regardless of grading or depth of invasion • Stage IB MMRd/NSMP EEC, high grade, regardless LVSI • Stage II MMRd/NSMP EEC
High	• Stage III-IVA, without residual disease • Stage I-IVA non-endometroid EC, including myometrial invasion, without residuals	• Stage III-IVA MMRd/NSMP EEC, without residuals • Stage I-IVA EEC, including myometrial invasion, without residuals • Stage I-IVA MMRd/NSMP, serous, undifferentiated carcinoma, carcinosarcoma including myometrial invasion, without residuals
Advanced metastatic	• Stage III-IVA with residuals • Stage IVB	• Stage III-IVA with residuals, any type of molecular subtype • Stage IVB of any molecular subtype

1.1.6 Diagnostic methods

The key symptoms of endometrial carcinoma are abnormal pre-, peri- or postmenopausal bleedings, which occur in almost all Stage I ECs. As the tumour grows additional symptoms like gastrointestinal or urological disorders, appear due to the invasive growth (3). Since vaginal bleeding is an early symptom, the majority of postmenopausal women can be diagnosed at an early point of the disease and therefore have a higher chance of effective treatment (30).

Preventive medical examination in asymptomatic females is not recommended, whereas in symptomatic perimenopausal women, especially with postmenopausal bleeding, a tissue sample of the endometrium should be taken for further diagnostics. It is recommended that women with an increased risk of EC, as in patients with Lynch syndrome, undergo an annual preventive medical examination at the year of 35, where transvaginal sonography and tissue sampling of the endometrium is performed (3).

Physical examination of the uterus and adnexa in search for pathologies should be done and to exclude other sources of bleeding. Apart from that, transvaginal ultrasound is one of the first examination tools and can be used to measure the endometrium diameter. Another common tool to find the cause of abnormal bleeding is the hysteroscopy, in which the uterine cavum can be visualized. Another advantage of this method is the possibility to take a tissue sampling during the screening. Nonetheless, the final diagnosis is made through histology. With a Pipelle a endometrium sample can be taken and used for further screening through pathologists (30). WHO-classification and FIGO-stages should be used as guidelines to sort EC into risk groups, and therapy should be guided on these. The pathologic report should include histopathologic type and grading of EC (2).

Sentinel lymph node mapping is a staging method with the aim to reduce the side effects of lymphadenectomy and to gain important information of lymph node status (5). A sentinel lymph node (SNL) is the first node which drains the anatomical area, meaning that if the SNL is negative the following nodes are most likely to be negative as well. Lymph node involvement is an important prognostic parameter as the recurrence-free survival rate in females with positive lymph nodes decreases (31). The ESGO/ESTRO/ESP guidelines suggest considering SNL biopsy for staging in low- and intermediate-risk EC, while a lymphadenectomy is recommended in high-intermediate- and high-risk EC (2).

MRI (Magnetic resonance imaging) is a great tool for diagnosis and staging of EC as they are very accurate in describing the depth of myometrial invasion, cervical stroma involvement and lymph node metastasis. Another great radiological tool are PET- (Positron Emission Tomography) Scans as they are highly specific in the assessment of lymph node metastases and should therefore be performed during pre-operative work up (2).

1.1.7 Therapy

In early-stage diseases the recommended approach is hysterectomy including bilateral salpingo-oophorectomy as a minimal invasive surgery, it is important to watch out on intraperitoneal tumour spilling, tumour rupture or morcellation and to avoid these at all cost (2).

Adjuvant treatment depends on the risk group and the knowledge of molecular classification. No adjuvant therapy is advised in females with low-risk EC, however if POLE-mutation is found in low-risk EC adjuvant treatment should be evaluated. Adjuvant brachytherapy is the primary approach in intermediate risk EC, as well as in high-intermediate risk EC, with the aim to diminish vaginal recurrence. In high-intermediate risk EC with substantial LVSI external beam radiotherapy (EBRT) can be advised, besides that adjuvant chemotherapy is an option particularly for high-grade EC and in cases with substantial LVSI. In high-grade LVSI negative females and stage II G1 EEC adjuvant brachytherapy can be considered as sole treatment. Furthermore, in high-risk EC EBRT and adjuvant chemotherapy is suggested simultaneously, aside from this approach another option is sequential chemotherapy and radiotherapy (2).

In Stage III and IV EC surgical approach with maximal tumour reduction including resection of enlarged lymph nodes is the recommended approach, while systematic lymphadenectomy should not be carried out. If surgery is not possible systemic therapy should be performed, surgery at a later point might be carried out in cases with good response to systemic therapy. In patients with unresectable EC a multi-disciplinary approach should be considered, options include radiotherapy along with EBRT and intrauterine brachytherapy or neoadjuvant chemotherapy before surgery or radiotherapy (2).

In early-stage EC patients benefit form adjuvant therapy like pelvic radiotherapy and vaginal brachytherapy as these approaches improve local control, but overall survival is

not increasing. In the PORTEC-1 (postoperative radiation therapy in endometrial cancer) trial, local recurrence rates decrease with whole pelvic radiotherapy, but on the other hand 5-year survival did not improve (32, 33).

1.2 Lymph vascular space invasion in endometrial carcinoma

1.2.1 General aspects of local and metastatic spread

Endometrioid adenocarcinomas of the uterine corpus infiltrate the uterine wall, the cervical stroma, the uterine adnexa, the parametrial tissue and the serosa of the uterus, as they progress locally. The infiltration of the above mentioned structures defines the T-category of the UICC-TNM-classification und contributes to determine the FIGO-stage. Stage IV or pT4 defines advanced tumours with local extrauterine growth with or without direct infiltration of adjacent organs or anatomical structures. Although endometrioid carcinoma can also show a haematogenous spread with distant metastasis in various organs (mainly lung, brain, bones and liver), a metastatic spread occurs mainly via the lymphatic system (lymph nodes metastasis), as typical for carcinomas in general (28, 34, 35).

1.2.2 Lymph node metastasis

Locoregional lymph nodes of endometrial carcinoma are the pelvic and the paraaortic nodes. Anatomical studies described mainly two pelvic lymphatic paths: one is called the upper paracervical pathway (UPP) and on the other hand a lower paracervical pathway (LPP). The UPP drains into the medial external lymph nodes as well as the obturator nodes, afterwards draining to the lateral precaval and paraaortic areas. The LPP runs into the presacral region draining into the internal iliac and presacral lymph nodes and further to the medial paraaortic and precaval region. Even if in most cases the pelvic lymph nodes are the first to be involved, a non-pelvic pathway can be described, draining directly into the paraaortic nodes. A detailed comprehension of the lymphatic drainage is especially important to define and recognize sentinel lymph nodes, for the analysis of the latter has currently und vastly replaced a complete lymphadenectomy, thus minimizing post-surgical complications. Noteworthy, inguinal lymph nodes are not included in regional metastasis but are classified as distance metastasis (pM1) (35, 36).

In up to 50% of EC pelvic lymph nodes can be involved while paraaortic metastases are found in just 7-8% of total EC and additional in 30% of EC with pelvic metastases. However, the risk of lymph node metastasis depends on tumour type, grading (in the endometrioid histotype) and local extent as defined by the “T”-category of TNM. In low-

grade endometrioid adenocarcinomas the rate of metastatic involvement of regional lymph nodes is far lower. Positive paraaortic lymph nodes are rarer in a low-grade setting and accordingly, are mostly seen in non-endometrioid or high-grade endometrioid adenocarcinoma and direct extension in the outer half of the myometrium or cervix (35).

1.2.3 Lymph-vascular space invasion

Lymph-vascular space invasion (LVSI) in EC is defined as tumour cells within a space lined by endothelial-cells located in the uterus wall and located beyond area of the primary tumour and can be normally recognized by conventional histology (H&E). If required, the use of immunohistochemical markers of lymphatic endothelial cells (such as Podoplanin – D240) can assist in the diagnosis in difficult cases (37-40).

Real LVSI should be differentiated from mimics. Common mimics are artefacts such as tumour- and non-tumour cell displacement inside the myometrial gaps or vessels, which especially occur after surgical manipulation. Other common issues are poor fixation, autolysis or tumour necrosis. Microcystic elongated and fragmented invasion types (MELF), can also be mistaken for neoplastic lymphatic emboli. This growth patter though is often associated with real LVSI (40, 41).

LVSI can lead to metastatic spread to the locoregional lymph nodes, making it a very important prognostic factor. As a result, LVSI is associated with cancer recurrence and decreased survival, especially in early-stage EC (40, 42).

Accordingly, LVSI has been considered in all guidelines, as well as in the current 5th edition of the WHO classification of female genital tumours (2020), as stage-independent adverse prognostic risk factor and it has now been included in the most recent update of the FIGO (2023) staging system (23, 29, 43).

Even if older studies used an oversimplified definition of LVSI, which is there merely defined as present or not present, it is now broadly accepted that not all cases, but only those with a certain extension and number of LVSI have a prognostic impact. Hence, in order to differentiate between relevant and not relevant LVSI, approaches have been made by building various scoring systems using quantitative analysis of affected lymphatic vessels (44, 45).

The use of a three- or even four-tiered system allows a more detailed characterization of lymph-vascular space invasion (45). Various scoring systems are displayed in table 5 and table 6.

Despite the acknowledgment of the prognostic importance of such distinction, there is still unfortunately no broad consensus on the definition of focal and substantial LVSI in diverse official guidelines and in the literature in general.

Table 5: three-tiered scoring system for LVSI according the ESGO/ESTRO/ESP guidelines and WHO (2, 43).

Three-tiered scoring system	
Absent	- No LVSI found
Focal	- A singular LVSI-focus around the tumour (ESGO/ESTRO/ESP) - <5 vessels (ESGO/ESTRO/ESP and WHO 2020)
Substantial	- Diffuse or multifocal LVSI (ESGO/ESTRO/ESP) - 5 or more vessels (ESGO/ESTRO/ESP and WHO 2020)

Table 6: scoring system of LVSI by Hachisuga et al. (46).

Four-tiered scoring system	
No LVSI	No LVSI found
Mild LVSI	A focus of LVSI
Severe LVSI	Diffuse or multifocal LVSI or massive LVSI in the myometrium

1.2.3.1 Influence of LVSI on the outcome of EC patients

The importance of LVSI becomes clear, as it can be used to predict lymph node metastasis, due to its association with tumour spreading and disease recurrence. This is supported by studies, that show that LVSI correlates with lymph node involvement and disease

recurrence, due to the fact that LVSI-positive cases had higher recurrence rates and increased lymph node metastases compared to the groups without LVSI (47). Further studies like Jorge et al. support this as well, as they show that LVSI in not locally advance endometrioid adenocarcinomas (pT1) is associated with a 3-16-fold higher risk of lymph node involvement, and additionally LVSI should be used as an independent prognostic variable in predicting overall survival. LVSI is a very important prognostic factor especially in N0 patients, as it outlines a distinct association with overall survival, when compared to FIGO-stage I cases without substantial LVSI (48). A Danish group study found out that 5-year recurrence was remarkably increased in LVSI-positive cases for all EEC FIGO-Stages, but especially in FIGO-Stage I EC the probability of distant metastasis was higher in LVSI positive cases. On the other hand, their study showed that LVSI not only predicts local recurrence, but also non-locoregional recurrence in abdominal or distant nodes, remarkably the risk was 4-fold higher even in stage I low- to intermediate risk EC in LVSI positive cases (49).

Nonetheless, the above mentioned studies did not differentiate between the number or distribution of LVSI and merely used a two-tiered scoring system, where LVSI is described as present or not. But as Bosse et al. pointed out in their study that not only the presence of LVSI is relevant but also the number, as they showed that females with substantial LVSI compared to focal LVSI had a higher risk for not only locoregional recurrence but also distant recurrence and decreased overall survival (45). This statement is also supported by Restaino et al. who also found out that EC with diffuse LVSI had decreased disease free survival and overall survival, besides that they also showed that diffuse LVSI is an independent prognostic factor for not only locoregional lymph node involvement but also distant spreading (50).

ESGO/ESTRO/ESP guidelines (2020) incorporate the influence of LVSI in their risk assessment. As a result, patients with focal LVSI under certain circumstances are sorted into different risk groups, and therefore get different therapy options, as patients with substantial LVSI. What is more, in the molecular categories of NSMP or MMRd, Stage IA EEC low grade without LVSI or focal LVSI are considered low-risk, but if substantial LVSI is found are defined as high-intermediate risk. Same applies for Stage IA EEC high grade without or with focal LVSI they are allocated in the intermediate risk group and mainly get treated with brachytherapy but are allocated in the high-intermediate risk group if they show substantial LVSI and therefore get treated with external beam radio therapy.

But substantial LVSI does not affect POLE-mutated and TP53-mutated adenocarcinomas in Stage I, as they are automatically assigned to low-risk and high-risk groups respectively. Regarding non-endometrioid EC, they are sorted into high-risk prognostic groups, also independently from LVSI (2).

As a result it is important to include LVSI in EC treatment planning, as evidences show that patients with substantial LVSI benefit under the above mentioned circumstances from adjuvant external beam radiotherapy instead of vaginal brachytherapy or no adjuvant therapy (45, 50).

1.3 Hypothesis

An observation of the clinical history of a cohort of patients with endometrioid adenocarcinoma of the uterus in FIGO-Stage I (2009) with standardly documented and thoroughly, objectively examined lymph vascular space invasion should provide insights on the evaluation of LVSI.

Particularly, it should be defined whether and in which extent the following parameters play a prognostic key role:

- Number of LVSI
    - In a second step a verification of the cut-off of 5 vessels for the distinction between relevant and not relevant LVSI should be made within our cohort
- Spatial distribution of LVSI
    - Especially regarding uni- and multifocality, clustering and diffuse extension
- Depth of LVSI in the myometrium and the prognostic impact

Digital pathology (DP) should assist in the detailed analysis of the above mentioned parameters.

2 Methods

2.1 Case selection and data collection

Patients were included in this retrospective study if they fulfilled the following criteria: endometrioid adenocarcinoma, of any grade (G1-G3), FIGO stage I (2009), with lymph vascular space invasion (L1), and available follow-up for at least 5 years (time of diagnosis: from 2004 until 2018).

78 potentially eligible cases were preliminarily selected from the databases PAS Xanthos of the Diagnostic and Research Institute of Pathology at the Medical University of Graz, with the help of the Institute for medical informatics, statistics and documentation. The cases were searched by the inclusion criteria.

Out of the 78 preselected cases 53 were discarded either because they did not fulfil the inclusion criteria after careful examination of all histological reports (i.e. not fitting histotype, involvement of locoregional lymph nodes etc.) or were not retrievable from the archive for diverse reasons.

Our cohort consists therefore of 25 patients (age-range at diagnosis: 18-85 years old).

2.1.1 Variables used for analysis

Every patient that fulfilled the inclusion criteria and has been found in PAS Xanthos was assigned to a case number and therefore anonymized. Further, age and year of diagnosis have been stated, along with the histopathological criteria including histotype, Grading, FIGO-Stage and T-, N-, L-, V-parameter. This information has been collected from the affiliated reports. As there has been a change in the TNM-classification between the 6^{th} and 7^{th} edition in the classification of EC regarding the T-stadium, all T-stadiums that have been classified according to the 6^{th} edition were reclassified according to the current TNM-classification. The mentioned variables have been documented in a chart for further analysis.

Regarding lymph-vascular space invasion variables that were considered to be used for the analysis were the number of LVSI in total, their depth (inner or outer myometrium) and the number of histological blocks with carcinoma. As for the digital workup the number of LVSI in total, the cluster of LVSI based in distance, and the number of LVSI within the clusters have been assessed.

Regarding the clinical variables local recurrence or distant recurrence (yes/no) and death due to EC (yes/no) have been used for analysis. This information has been gathered through the follow-up reports in Pas Xanthos or openMEDocs.

2.2 Histological work up

The histological samples were observed under light microscope with the help of Dr. Luca Abete. The aim of this was to first of all confirm the endometrioid histotype and the presence of LVSI. In a next step the total number of LVSI was counted under the light microscope for each sample and documented in the chart. Furthermore, it was documented if LVSI appeared in the outer or inner part of the myometrium, as well as the number of samples that included part of EEC. In all cases with more than 20 LVSI, we abstained from a precise count and labelled them automatically as cases with “diffuse LVSI” due to its obviously relevance in terms of prognostic impact.

2.3 Digital pathology

2.3.1 Digital pathology and whole slide imaging

All histological slides with endometrioid endometrial cancer and noted LVSI were scanned with the scanner Histech P1000 and therefore digitalised. All scanned slides were archived and could be used for further analysis in the software program QuPath.

2.3.2 QuPath

The software QuPath was used in order to visualize and navigate through each digital slide und also annotate manually all detected lymph vascular space invasions. The software allows an exact and objective assessment of their number and spatial distribution (position, mutual distances) in each digital slide.

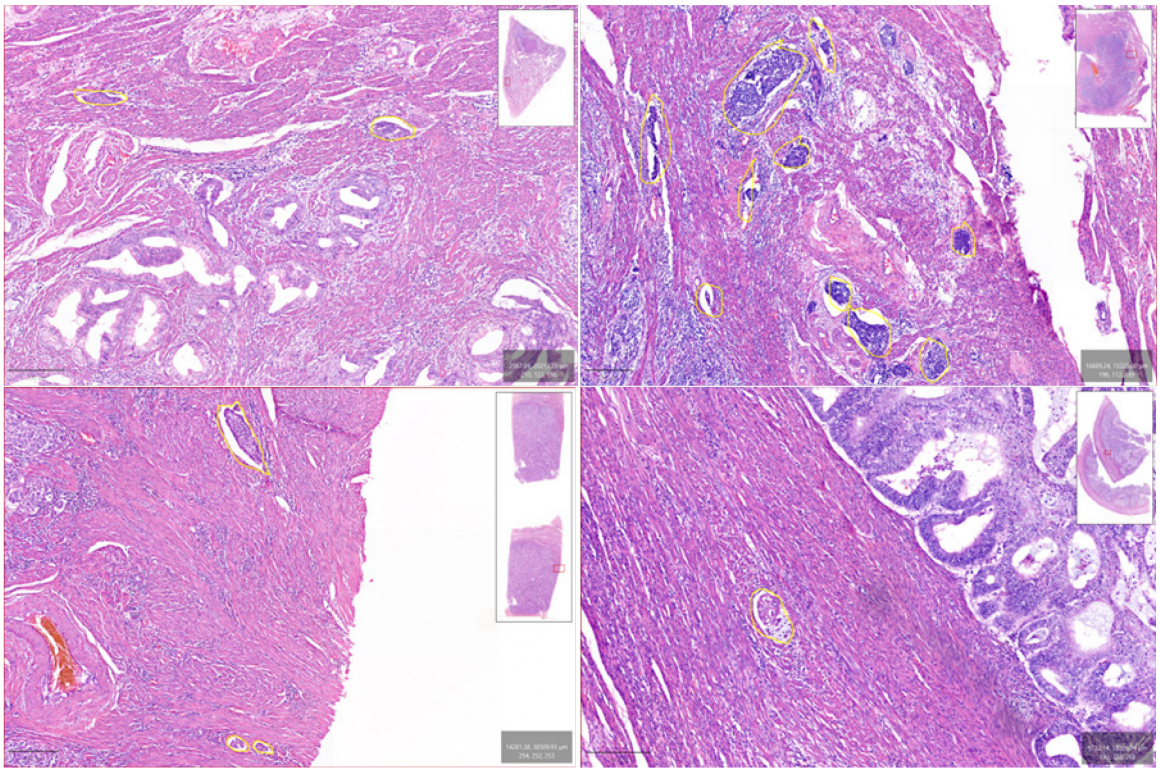

Figure 4: examples of different LVSI spatial distribution and annotation in QuPath

2.4 Data analysis

With the help of Dipl.-Ing., Dr. techn. Emilian Rudolf Jungwirth, BSc and Dipl.-Ing. Markus Plass, BSc the in QuPath collected data have been analysed. The LVSI on each slide were preliminarily counted and mapped on charts with coordinates according to their localisation on the slide. In a second step it was determined whether the LVSIs were grouped in clusters. Five different diameter groups of LVSI-clusters (0,5mm, 1mm, 2mm, 3mm, 4mm) have been tested. Regardless of the diameter, all LVSI within a cluster show a reciprocal distance which is smaller than or equal to the maximum diameter of the cluster itself. The software was programmed not to include further LVSIs, if the farthest distance between two LVSIs over-exceeded the predefined maximal cluster-diameter. These not included LVSIs would then automatically be assigned to the next cluster.

Two systems of measuring the distance between single LVSI (centre to centre and minimal distance of the digital selected LVSI-areas) were also considered and compared.

For each tested cluster diameter (0,5mm, 1mm, 2mm, 3mm, 4mm) and distance measurement between clusters (centre to centre or minimal distance) the total number of clusters per case, as well as the number of included LVSI per cluster (minimum, average,

maximum) were stated and documented in a chart. The comparison between different cluster-diameters and different criteria of measuring the cluster-cluster distance shows homogeneous and not considerably different values, hence a practical approach was decided. We chose for the discussion the 2 mm measurement for the definition of the diameter of a cluster, as it is in our opinion reasonable, visually understandable und highly reproducible under the microscope, which can not be stated with this certainty in extreme values such as 0,5 mm and 4 mm.

The same applies for the definition of the measurement criteria of cluster-cluster distances, whereas we chose the minimum distance between two consecutive clusters, which seems easier to be assessed, and therefore avoiding the need to define the exact centre of each cluster.

2.4.1 Statistic workup

A correlation analysis in SPSS has been performed for each cluster diameter (0,5mm, 1mm, 2mm, 3mm, 4mm) with the clinical variables. First the different diameters measured from the centre-centre distance were tested with the recurrence of EC (yes or no). In a second analysis the different diameters measured from the minimum distance were tested with the recurrence of EC (yes or no). Further the different diameters once measured from the centre-centre distance and second from the minimum distance were tested with the clinical variable death by EC (yes or no). The p-value was stated for all analysis. As for the graphic interpretation a box plot was made for alle tested variables.

2.4.2 Defining focal and diffuse LVSI

An important aspect for further discussion and prognostic assessment is the definition of the categories “focal” and “substantial” LVSI, which in our study was based upon international recommendations by the WHO and ESGO/ESTRO/ESP guidelines. Accordingly, focal LVSI was defined as less than 5 LVSI, and substantial LVSI was defined as 5 or more LVSI (2, 43). We assigned all cases with > 20 LVSI to a third category labelled “diffuse” LVSI.

3 Results

Out of the 25 cases 5 cases (20%) had documented cancer recurrence. And further in 5 cases (20%) death has been reported out of the total number of 25 cases. In total 9 (36%) cases showed either cancer recurrence or death. The figure below displays these results.

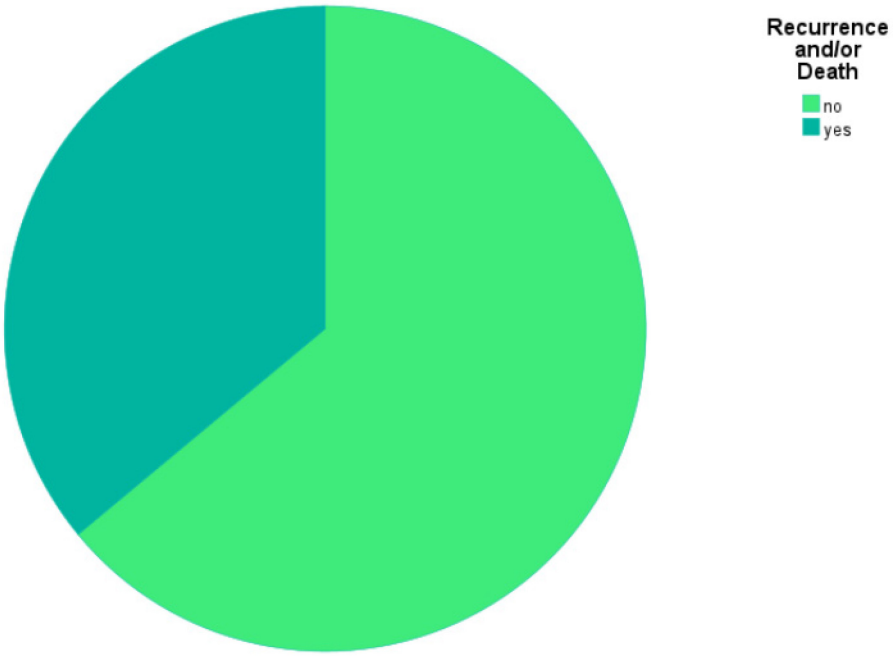

Figure 5: pie chart displaying the number of cases with and without either disease recurrence or death

3.1 Focal and diffuse LVSI

After sorting the LVSI in <5 LVSI, ≥5 <20 LVSI and ≥20 LVSI, 4 cases fell into the category of 20 or more LVSI and 21 cases had less than 20 LVSI in total. Out of these 21 cases 7 cases had less than 5 LVSI, and according to ESGO/ESTRO/ESP-guidelines fell into the focal group. Table 7 displays these findings.

Table 7: Number of LVSI

		Nr. of cases	Percent
LVSI number	<5	7	28,0%
	≥5 <20	14	56,0%
	≥20	4	16,0%

3.1.1 Recurrence and death group

Of all the cases (n= 25) that have been included 9 cases (36%) showed either death or recurrence (RD), and 16 cases (64%) showed neither.

Taking a closer look at RD-group only one case presented focal LVSI, 6 cases had more than 5 LVSI but less than 20 and 2 cases had more than 20 LVSI. These results and their equivalent percentage are displayed in the table below.

Table 8: number of LVSI (per case) in the group with recurrence and/or death

		Nr. of cases	Percent
LVSI number	<5	1	11,1%
	≥5 <20	6	66,7%
	≥20	2	22,2%

Compared to this in the group with neither death or recurrence 6 cases presented focal LVSI, 8 cases had more than 5 but less than 20 LVSI and 2 cases as well had more than 20 LVSI, which can be seen in table 9.

Table 9: number of LVSI (per case) in the group of patients alive and free of disease

		Nr. of cases	Percent
LVSI number	<5	6	37,5%
	≥5 <20	8	50,0%
	≥20	2	12,5%

When taking a look at just the cases with ≥20 LVSI respectively 2 cases (22,2%) fell into RD-group (n=9) and 2 cases (12,5%) did not show any RD (n=16). 7 cases (77,8%) out of the RD-group (n=9) had < 20 LVSI, and 14 cases (87,5%) had <20 LVSI and no RD (n=16).

Comparing the group with <5 LVSI and ≥5 LVSI, only one case (11,1%) fell into the RD-group (n=9) and had <5 LVSI and 8 cases in this group presented more than 5 LVSI (88,9%). Contrary in the group with no RD (n=16) 6 cases (37,5%) had focal LVSI and 10 cases (62,5%) had more than 5 LVSI.

Observing the groups of focal and substantial LVSI the following results can be seen. Out of all focal LVSI cases (n=7) death and/or recurrence appeared in just one case (14,3%) and in 8 (44,4%) out of 18 cases with substantial/diffuse LVSI.

3.1.2 Recurrence group

Looking at just the group with cancer recurrence out of 25 cases 5 cases had noted cancer recurrence (20%), while on the other hand 20 cases (80%) had no cancer recurrence.

In the group with cancer recurrence (n=5) zero cases had less than 5 LVSI, 3 cases presented more than 5 but less than 20 LVSI and 2 cases had more than 20 LVSI. Table 10 displays these findings and the corresponding percentage.

Table 10: number of LVSI (per case) in the group with cancer recurrence

		Nr. of cases	Percent
LVSI number	<5	0	0,0%
	≥5 <20	3	60,0%
	≥20	2	40,0%

On the contrary in the group with no cancer recurrence (n=20) 7 cases had less than 5 LVSI, 11 cases between 5 and 20 LVSI and 2 cases more than 20 LVSI. These results are shown in the table below.

Table 11: number of LVSI (per case) in the group without cancer recurrence

		Nr. of cases	Percent
LVSI number	<5	7	35,0%
	≥5 <20	11	55,0%
	≥20	2	10,0%

Examining the ≥20 LVSI category 4 cases had been noted in total. Out of all cases with cancer recurrence (n=5) 2 cases had ≥20 LVSI which equals to 40% within this group. On the other hand, from the 20 cases with no cancer recurrence (n= 20) 2 cases also had ≥20 LVSI correlating to only 10% and 18 cases presented with less than 20 LVSI which equals to 90% in this group.

Observing the difference between focal and substantial/diffuse LVSI, no focal LVSI (<5 LVSI) displayed a cancer recurrence. Out of all 18 cases with substantial/diffuse LVSI 5 cases (28,7%) presented cancer recurrence.

3.1.3 Death group

Taking a closer look at just the cases with noted death 5 cases (20%) fell into this group and 20 cases (80%) had no noted death.

When observing the 5 cases with noted death according to the LVSI distribution one case had less than 5 LVSI, 3 cases displayed more than 5 but less than 20 LVSI and one case had more than 20 LVSI, which is shown in the table below.

Table 12: number of LVSI (per case) in the group with documented death

		Nr. of cases	Percent
LVSI number	<5	1	20,0%
	≥5 <20	3	60,0%
	≥20	1	20,0%

Table 13 displays the distribution in the group with no noted death. 6 cases have been found with focal LVSI, 11 cases with more than 5 but less than 20 LVSI and 3 cases with more than 20 LVSI.

Table 13: number of LVSI (per case) in the group without documented death

		Nr. of cases	Percent
LVSI number	<5	6	30,0%
	≥5 <20	11	55,0%
	≥20	3	15,0%

In the category of ≥20 LVSI only one case was found which equals to 20% of all cases with noted death (n=5). Furthermore, within the no noted death category (n=20) 3 cases have been observed with ≥20 LVSI, which corresponds to 15%.

Examining the LVSI distribution between focal and substantial/diffuse category, in the group with noted death (n=5) 4 cases (80%) had more than 5 LVSI and only 1 case (20%) fell into the focal group. Apart from that in the no noted death group (n=20) 14 cases had substantial/diffuse LVSI and 6 cases (30%) fulfilled the criteria for focal LVSI. Observing just the cases with focal LVSI (n=7) one case (14,3%) within this group had a documented death, whereas in the group with substantial/diffuse LVSI (n=18) 8 cases (22,2%) had a documented death.

3.2 Number of clusters and recurrence

The table below shows the different tested cluster diameters measured from the centre distance with the recurrence. It can be seen in all different tested cluster diameters that the p-value was <0,05 and hence there is a significance between the number of clusters (centre distance) and the recurrence rate. Even so in the 3mm and 4mm cluster diameters a high significance can be seen.

Table 14: cluster diameters (centre-centre distance) and recurrence

Cluster diameters	p-value
0,5mm	0,029
1mm	0,033
2mm	0,015
3mm	0,010
4mm	0,005

In the boxplots below it can also be observed that there are differences in the groups with recurrence and without recurrence. It can be seen that the median in the recurrence group is higher compared to the no recurrence group, especially in the tested diameters 2mm and above. Furthermore, the mean is bigger in all cancer recurrence groups as in the no cancer recurrence groups. An outliner in the no recurrence group can be observed in all tested diameters.

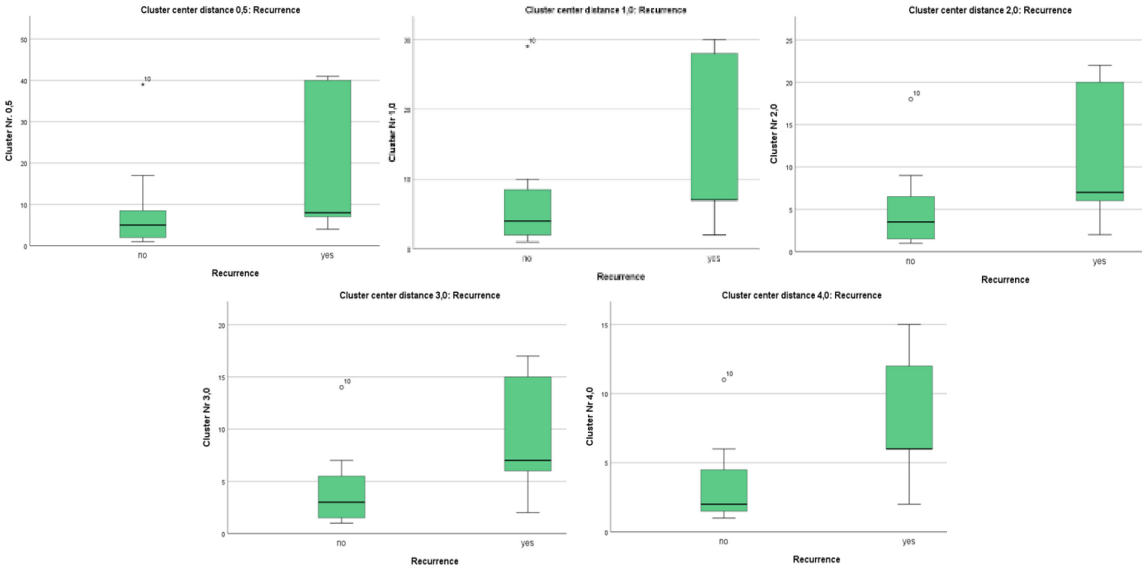

Figure 6: Number of LVSI-Clusters (centre-centre distance) and recurrence with variously defined diameters (0,5mm, 1mm, 2mm, 3mm, 4mm)

As seen in table 15 below, there has been also a significance in all tested cluster diameters and the recurrence measured from the minimum distance, as the p-value is <0,05. Moreover, a high significance in the 3mm and 4mm cluster diameter can be observed in this category as well.

Table 15: cluster diameters (minimum distance) and recurrence

Cluster diameters	p-value
0,5mm	0,022
1mm	0,033
2mm	0,012
3mm	0,010
4mm	0,008

In the tested diameters measured from the minimum distance it can also be observed that the median is higher in the cancer recurrence group compared to the no cancer recurrence group, especially in the diameters bigger than 2mm. The mean in the minimum distance is also larger in the cancer recurrence group compared to the no cancer recurrence group. Here as well an outliner can be found in all tested diameters in the no cancer recurrence group.

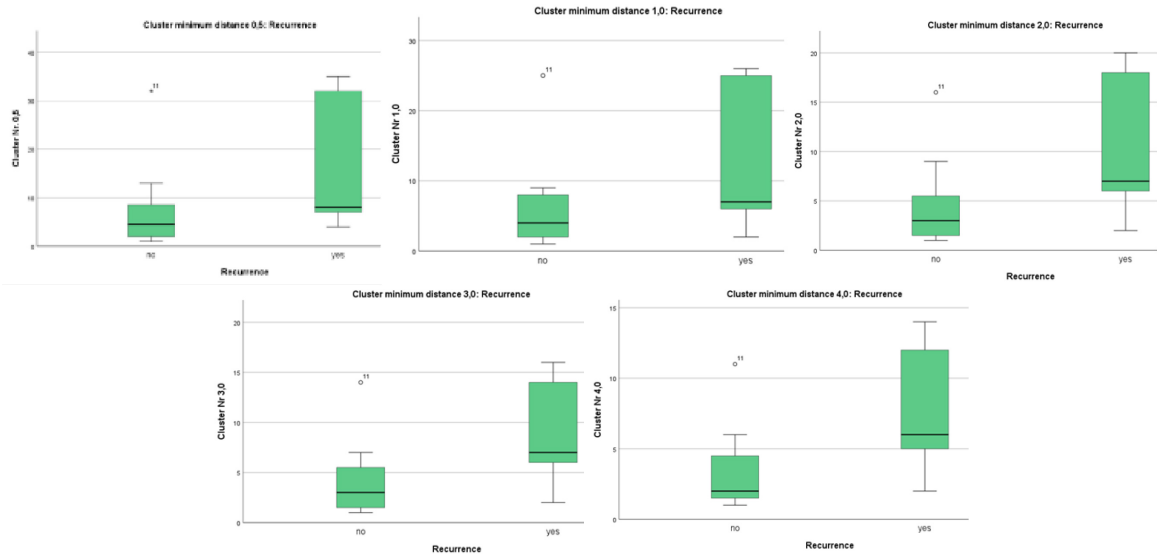

Figure 7:Number of LVSI-Clusters (minimum distance) and recurrence with variously defined diameters (0,5mm, 1mm, 2mm, 3mm, 4mm)

3.3 Number of clusters and death

Further the number of clusters was tested with the variable death. In this analysis no significance has been found, as in all tested diameters the p-value was >0,05, for the number of clusters measured from the centre distance. This can be seen in the table below.

Table 16: cluster diameters (centre-centre distance) and death

Cluster diameters	p- value
0,5mm	0,617
1mm	0,611
2mm	0,474
3mm	0,370
4mm	0,302

In the boxplots below it can also be seen that the median is nearly the same in both groups, as well as the mean, which does not vary too much in both groups. Two outliners can be observed in the 0,5mm, 1mm and 2mm tested diameters in the no death group, while only one outliner can be seen in the 3mm tested diameter. In the noted death group one outliner in each tested diameter can be found.

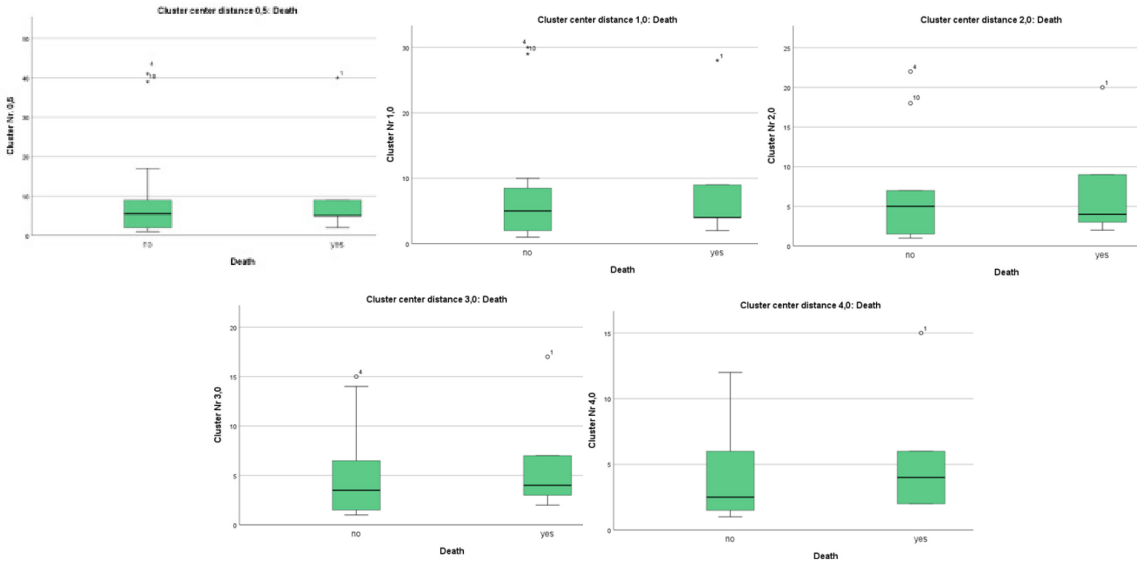

Figure 8:Number of LVSI-Clusters (centre-centre distance) and death with variously defined diameters (0,5mm, 1mm, 2mm, 3mm, 4mm)

There has also been no significance between the number of clusters and death when measured from the minimum distance, as the p-value was in all cluster diameters >0,05, seen in table 17.

Table 17: cluster diameters (minimum distance) and death

Cluster diameters	p-value
0,5mm	0,628
1mm	0,555
2mm	0,326
3mm	0,420
4mm	0,323

Again, also in the graphic display below, the boxplots show no distinct differences in both groups as the median LVSI cluster number is in both groups in all tested diameters measured from the minimum distance approximately the same. In these groups outliners can be observed as well, likewise to the tested groups with the centre diameters, except for one outliner in the 4mm diameter in the no noted death group.

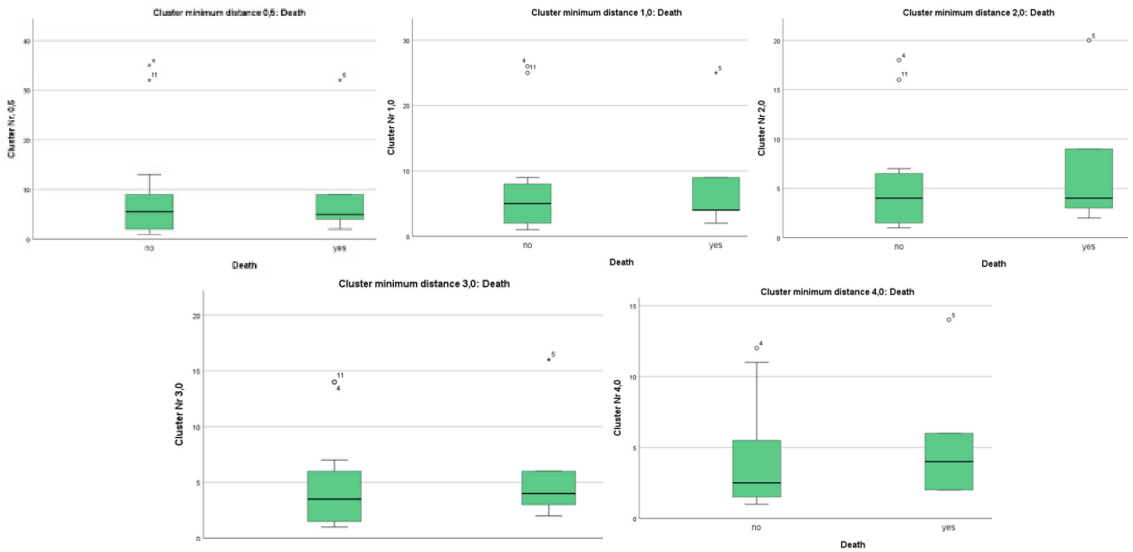

Figure 9: Number of LVSI-Clusters (minimum distance) and death with variously defined diameters (0,5mm, 1mm, 2mm, 3mm, 4mm)

3.4 Clusters vs. absolute number of LVSI

For each tested cluster diameter, the total number of clusters in each case has been counted and the minimum and maximum number of LVSI within these clusters has been stated, as well as the average cluster number has been calculated. As already mentioned above the total number of LVSI, independent of cluster diameters, has been counted and stated in a table as well, corresponding to the cases. Exemplary the 2mm Cluster diameter measured from the minimum distance with the corresponding clinical date and the absolute number of LVSI per case is stated in table 18 below.

Table 18: LVSI cluster 2mm (minimum distance) and corresponding clinical data and LVSI number (RD= Recurrence and/or death; D= Death; R= cancer recurrence; y=yes; n=no; d=diffuse)

Case	Cluster Nr. 2,0	LVSI ∅ 2,0	LVSI max.	LVSI min.	RD	LVSI >20	LVSI >5	LVSI Nr.
1	20	3,7	14	1	RD	y	d	>20
2	2	1	1	1	D	n	n	2
3	5	1,4	3	1	n	n	y	7
4	16	4,0625	10	1	n	y	d	>20
5	7	2,14	7	1	n	n	y	15
6	1	1	1	1	n	n	n	1
7	9	1,11	2	1	D	n	y	10
8	4	1,25	2	1	D	n	y	5
9	2	3,5	6	1	R	n	y	7
10	6	1,83	5	1	n	n	y	11
11	5	1	1	1	n	n	y	5
12	6	1,5	3	1	R	n	y	9
13	7	1,714	5	1	n	n	y	12
14	2	1	1	1	n	n	n	2
15	1	5	5	5	n	n	y	5
16	7	1	1	1	R	n	y	7
17	1	1	1	1	n	n	n	1
18	5	5,2	11	1	n	y	d	>20
19	3	1,66	3	1	n	n	y	5
20	3	3	5	1	n	n	y	9
21	3	2	3	1	D	n	y	6
22	1	2	2	2	n	n	n	2
23	1	1	1	1	n	n	n	1
24	18	2,5	6	1	R	y	d	>20
25	2	1	1	1	n	n	n	2

As seen in the table above, there has been no case with unifocality (= one cluster with <5 LVSI) and either recurrence or death. On the other hand, all cases with noted disease recurrence or death showed multifocality.

3.5 Grading and LVSI distribution

Out of all 25 cases 18 cases presented with low grade histology (G1 or G2) and 7 cases with high grade histology (G3).

When taking a closer look at different LVSI distribution 6 cases fell into the substantial category and one case into the focal category. This is portrayed in the table below.

Table 19: number of LVSI (per case) in the group of high-grade EC

		Nr. of cases	Percent
LVSI number	<5	1	14,3%
	≥5 <20	6	85,7%
	≥20	0	0,0%

On the other hand, in the low-grade group 6 cases displayed focal LVSI and 12 cases showed substantial or diffuse LVSI. The distribution and corresponding percentage are shown in table 20.

Table 20: number of LVSI (per case) in the group of low-grade EC

		Nr. of cases	Percent
LVSI number	<5	6	33,3%
	≥5 <20	8	44,4%
	≥20	4	22,2%

Regarding the number of LVSI out of the cases with high-grade LVSI 85,7% presented substantial LVSI and in the low-grade LVSI group 66,67% showed substantial/diffuse LVSI.

3.6 Depth of LVSI in the myometrium

In a further analysis the depth of LVSI in the myometrium and the correlation between disease recurrence and overall survival was made. In total 9 cases showed LVSI in the outer myometrium, 13 cases LVSI in the inner half and 3 cases could not be precisely assigned.

The results are displayed in the three tables below.

Table 21: cases with LVSI in the outer myometrium in correlation to recurrence or death

		Nr. of cases	Percent
Recurrrence or death	no	3	33,37%
	yes	6	66,67%

Table 22: cases with LVSI in the inner myometrium in correlation to recurrence or death

		Nr. of cases	Percent
Recurrence or death	no	11	84,6%
	yes	2	15,38%

Table 23: cases with unsure myometrial invasion of LVSI and recurrence and death

		Nr. of cases	Percent
Recurrrence or death	no	2	66,66%
	yes	1	33,33%

It can be seen that in the group with LVSI in the outer myometrium 66,6% of the cases had either cancer recurrence or death, while on the other hand in the group with LVSI in the inner myometrium just 15,38% had documented disease recurrence or death and 84,6% showed a disease-free survival.

4 Discussion

In low grade endometrioid adenocarcinoma of the uterine corpus, FIGO-Stage I, the presence of lymph vascular space invasion is a negative prognostic factor (51), and can determine in some cases, whether adjuvant therapy like brachytherapy or conventional radiotherapy are indicated. In line with the important role in therapy planning, reference criteria for the evaluation of LVSI are provided by both the World Health Organisation and the conjunct guidelines of the European institutions ESGO/ESTRO/ESP. Bosse et al. already emphasized the importance of implementing a scoring system for LVSI. In their study they used a 3-tiered scoring system and found out, that only substantial LVSI has an impact regarding distant metastasis, recurrence and overall survival in 129 patients, indicating the importance of implementing a scoring system, as not only the presence of LVSI is important, but rather their number and distribution (45). Accordingly, lymph vascular space invasion is required in both WHO and ESGO/ESTRO/ESP guidelines to be extensive/substantial to be considered prognostically significant and a cut-off of at least 5 LVSI is given to distinguish between “focal” (no risk) and “substantial” LVSI (increased risk of recurrency and metastatic spread) (2). When the cited sources (45) are thoroughly read, the choice of this particular cut-off appears more as an arbitrarily established rule than a statistically determined value. What is more, only the ESGO/ESTRO/ESP guidelines require a distinction between unifocal and multifocal LVSI. The latter should be automatically considered as “substantial”, hence a discrepancy to the WHO-criteria exists. In routine assessment differentiation between unifocal and multifocal is also problematic and extremely scarce examples of “borderline” multifocality, lik two single LVSI in a single slide and with a distance of 2-4 mm, or only two single LVSI in 2 different slides, could be biologically quite hard to be accepted as risk associated. On the other hand, neglecting multifocality at all could lead to an oversimplification of the biological behaviour of lymph vascular space invasion, when it is not supported by statistical evidence. Apart from that, multifocality implies the need of a definition of “focus”, both in terms of extension (diameter, maximal number of admitted LVSI) and distance between the foci. In absence of a clear definition, it could be in some cases impossible to determine, if two or more (< 5) LVSI which are found in close proximity are unifocal or multifocal. Depth of LVSI in myometrium is a further parameter which has been completely neglected by both guidelines, even if described in diverse works as potentially associated with worse prognosis.

Extent of LVSI and outcome

In our study it could be confirmed that substantial or diffuse LVSI tends to lead to a worse outcome compared to only focal LVSI. The cut-off of 5 LVSI was also verified in our cohort as adequate to separate cases with no additional risk (focal LVSI) from cases with enhanced risk of recurrencies or death (substantial LVSI). In fact, when comparing the two groups of patients with unfavourable vs. uneventful outcome in terms of recurrencies or death, EEC with 5 or more LVSI showed a remarkably higher association with recurrence or death (44,4%) compared to the group with only focal LVSI (14,3%) in our cohort. The latter value is allegedly similar to the rate of recurrencies or death in cases without LVSI, even if a direct comparison here is not possible (there are no cases with 0 LVSI in our cohort) and it was besides not the aim of the study.

Taking a closer look at just the cases with cancer recurrence, similar results can be observed, as 0 cases have been found with focal LVSI and disease recurrence, pointing out the prognostic value of substantial LVSI. Observing just our small group with documented deaths, a tendency can also be seen of substantial or diffuse LVSI to have a slightly higher risk compared to the group without documented deaths. Within the group of documented death 80% of all cases had substantial or diffuse LVSI and just 20% of cases presented focal LVSI. Therefore, it could be proven that the mortality increases with the amount of LVSI.

The results from Tortorella et al. are in line with our study, as the results show that there is a difference between focal and substantial or diffuse LVSI regarding cancer recurrence and overall survival, as they showed that in females with substantial LVSI the overall survival was lower compared to focal LVSI or no LVSI, as well as a higher risk for cancer recurrence in the substantial LVSI group (44). In addition, in their work Restaino et al. proved similar results, that substantial/diffuse LVSI is associated with a higher risk of cancer recurrence and death. The 5-year survival rate in substantial LVSI was 68,3% compared to 88,3% in focal LVSI and 88,9% in no LVSI cases, and on the other hand the risk of cancer recurrence was 24,9% in substantial LVSI and just 6,6% in LVSI negative cases. This study is in concordance with our work, as it could be shown that the risk for recurrence and death is higher in substantial LVSI compared to focal LVSI (50).

Multifocality of LVSI and outcome

Spatial distribution of LVSI, clustering and focality were analysed with the help of technology and digital pathology. A precise mapping and measuring system of all single neoplastic emboli in each slide allowed to analyse if LVSI were grouped in clusters. By setting a maximal diameter of 2 mm as cut-off for the definition of a single cluster (focus) we observed 5 cases with unifocal distribution of the LVSI, including only a single case with at least 5 LVSI in total. None of the 5 cases were associated with unfavourable outcome. Incidentally none of the 5 case was high grade and only one showed LVSI located deeper in the myometrium. 16 cases showed at least 3 clusters of LVSI, all of which had at least 5 LVSI, while recurrencies or death were observed in 7 of these cases (43,7%). Cases with a large amount of clusters are obviously also associated with numerous lymphatic emboli and must be considered examples of "substantial" LVSI, as a result the definition of focality becomes irrelevant in these cases in terms of prognostic prediction. For that reason, we were particularly interested in cases with borderline or very low number of LVSI and bifocal distribution. Our cohort includes 4 cases with only two clusters or foci. 3 cases showed only focal (< 5) and the fourth a substantial LVSI (with 7 LVSI). A worse outcome was seen in 2 out of 4 cases (50%), namely the one with 7 LVSI and a second one with only 2 emboli ("focal LVSI"), however deeply located in the outer half of the myometrium.

Given the small number of patients of our cohort, a multivariate analysis was not possible, especially in borderline cases regarding focality und absolute number of LVSI, hence we could not find a strong prognostic association of multifocal LVSI, compared to the association that can be simply observed through the absolute number of LVSI per case. As a result, we have not found a strong argument for the implementation of a complex diagnostic work-up to define clustering of LVSI in routine diagnostic.

As there are obviously limitations and as already stated, a definitive conclusion regarding multifocal LVSI can only be drawn through the study of a bigger cohort by means of a multivariate analysis, which should also include other biological features such as depth of LVSI in the myometrium and grading.

Our proposed method, with the employment of the technology of digital pathology, has on the other hand proven to be accurate in terms of standardisation of data collection and prognostic prediction. A sufficiently vast cohort would expectedly allow to verify if

multifocality should be reported and interpreted in all cases as risk-associated, even in borderline cases.

Extent of LVSI and Grading

We found in our cohort a direct association between high grade EC and extent of LVSI. Within the group of high-grade (G3) EEC 85,7% of the cases had 5 or more LVSI. By contrast, out of all low grade (G1 and G2) EEC only 66,6% presented substantial or diffuse LVSI. These results correspond to the study by Wakayama et al. which also found that LVSI positivity appears more often in G3 EC (52).

Even if this observation does not lead to particular practical applications, we found the association between extent of LVSI und high grade intriguing. We can speculate that endometrioid adenocarcinomas with high grade morphology (predominance of solid growth pattern) might be more aggressive because of an increased propensity to lymph vascular space invasion.

Deep location of LVSI in myometrium and outcome

An association between deep location of LVSI and worse outcome has strongly emerged in our study. 6 out of the 9 cases (66,7%) in which LVSI were detectable in the outer half of the myometrium showed either recurrence or death. An unfavourable outcome was seen in contrast only in 2 out of 13 cases (15,4%) with LVSI in the inner part of the uterine wall only. Our study included also 3 cases with no clear cut definable assignment (inner or outer half of the myometrium) in which 2 of them had an uneventful follow up, while the third patient (33,3%) experienced a local recurrence.

Biologically the association is logically, as deep LVSI is located in the lymphatic streams closer to the loco-regional lymph nodes and further away from the primary tumour mass. The depth of LVSI in the uterine wall was also investigated in other studies like Kim et al. who collected 201 LVSI positive patients and observed that LVSI correlates to deeper myometrial invasion (53), but does not appear in any of the official guidelines. The reason might be that a thorough evaluation of LVSI has only recently emerged as indispensable to correctly assign a risk-group and a FIGO-Stage (2023) and as a result has not been mandatory reported with accuracy until now. A correct assessment of the depth of LVSI can also be problematic in not perfect oriented tumour samples or in the ones in which the full thickness of the myometrium cannot be readily identified or reconstructed.

Considering the evidence of a probable impact in terms of prognostic prediction, we expect that the involvement of the deeper parts if the myometrium by LVSI will soon be taken into consideration and integrated in future updates of the guidelines.

In conclusion, the importance of LVSI could be proven in this work, along with other studies, which show similar results. The problem regarding the prognostic value of LVSI is that there is no uniform scoring system. Studies like Bosse et al. already proved the importance of implementing a scoring system like a three-tiered scoring system, where LVSI is distributed in no-LVSI, focal LVSI and substantial LVSI. The importance of implementing a scoring system becomes more clear, when it can be shown that different LVSI pattern need different therapies. As Bosse et al. proved in their study that in females with substantial LVSI the 5-year risk for locoregional recurrence was only 4,3% after external beam radiotherapy and 27,1% after receiving vaginal brachytherapy (45).

The ESGO/ESTRO/ESP guidelines already included LVSI in their prognostic risk groups. They also used a three-tiered scoring system and defined the cut-off between focal and substantial LVSI at 5 or more LVSI. Females with substantial LVSI and Stage I EEC are being classified as high-intermediate risk, independently of grade or depth of myometrial invasion (2). In the 5th Edition of the WHO the three-tiered scoring system has also been mentioned and they are indicating that substantial LVSI may have an impact on the prognosis of EC (23).

In their newest update (2023) FIGO incorporated LVSI as parameter for stage assignment pointing out the importance of differentiating between focal and substantial LVSI. In their 2023 classification FIGO uses the same definition for substantial LVSI as the 2020 WHO classification (5 LVSI or more), while on the other hand they mention the problem of different cut-offs in other systems and the need for further evidence to confirm or eventually change the cut-off (29). In their critical review McCluggage et al. discus the following points. Due to the lack of a uniform scoring system difficulties in including LVSI as prognostic parameter still exist. Further, none of these recommendations (including FIGO 2023, WHO and ESGO/ESTRO/ESP) state if the distribution of LVSI is relevant or just the total number of LVSI, as well as if the extent of LVSI is measured of the maximum LVSI count in just a single tissue probe or if it is a cumulative number of all tissue sections. Because of this, comparability between different centres and institutions regarding LVSI remains difficult, on account of different approaches, which leads to

different correlation with outcome (54). Moreover, the ESGO/ESTRO/ESP guidelines are the only ones in which a focal, prognostic irrelevant lymph vascular space invasion requires to be also unifocal, thus adding a further element (uni- or multifocality) of potential poor agreement (2). As there has been no uniform approach implemented, the reproducibility of LVSI quantification still remains a subject for further studies.

In this work an approach of trying to implement a scoring system for LVSI has been made. It could be proven that a three-tiered scoring system equally to ESGO/ESTRO/ESP guidelines, defining substantial LVSI as 5 or more vessel, is of good practical use. Furthermore, a different approach has been made in trying to test if LVSI distribution also plays an important factor regarding prognosis in EEC. A significant correlation between different LVSI cluster diameters could be proven, but due to the low case number, these results need to be further observed in a bigger cohort. There has been no significant correlation between LVSI clusters and death, but in the absolute number a correlation could be proven in the substantial LVSI group, so these results should be further observed in a bigger study.

The weakness of this study is the low case number. As already mentioned, we found a lot more cases in the data base but unfortunately not all cases were available as histological slides and therefore we could not perform an analysis of the LVSI. Another reason why the case number is low is due to the very specific parameters of the EC. We only used endometrioid EC, Stage I, with lymph vascular space invasion but without lymph node metastasis. This is a very rare case due to the biology of the EC, as LVSI and lymph node metastasis are often diagnosed together and cases without lymph node involvement are rare.

Nonetheless, despite the low case number, significant results could be observed. This shows that the used method in analysing LVSI has proven itself to be reliable and could be used for further studies, like in a multicentre study with a bigger case size. Furthermore, this study shows the importance of digital pathology, as it allows a new and more in-depth approach to analyse different histopathological parameters such as LVSI in this study. What is more, that now LVSI has been more prominent in different guidelines the importance of implementing a universal scoring system for LVSI has risen, as many studies, including this work, prove the importance of LVSI dependent on different distribution and number. LVSI therefore plays an important role in the outcome of patients,

even in low-risk EC and without lymph node involvement and therefore these patients would benefit from a different therapy approach compared to patients without LVSI, or with focal LVSI.

5 References

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6 Appendix

Table 24: Cluster LVSI 0,5mm (minimum distance)

Case	Cluster Nr. 0,5	LVSI ∅0,5	LVSI min. 0,5	LVSI max. 0,5
1	32	2,31	1	5
2	2	1	1	1
3	6	1,16	1	2
4	32	2,03	1	4
5	10	1,5	1	3
6	1	1	1	1
7	9	1,11	1	2
8	5	1	1	1
9	4	1,75	1	3
10	8	1,375	1	3
11	5	1	1	1
12	8	1,125	1	2
13	10	1,2	1	3
14	2	1	1	1
15	3	1,666	1	3
16	7	1	1	1
17	1	1	1	1
18	13	2	1	5
19	4	1,25	1	2
20	6	1,5	1	3
21	4	1,5	1	2
22	1	2	2	2
23	1	1	1	1
24	35	1,214	1	3
25	2	1	1	1

Table 25: Cluster LVSI 1mm (minimum distance)

Case	Cluster Nr. 1,0	LVSI ∅ 1,0	LVSI min. 1,0	LVSI max. 1,0
1	25	2,96	1	10
2	2	1	1	1
3	6	1,16	1	2
4	25	2,6	1	5
5	9	1,6	1	5
6	1	1	1	1
7	9	1,11	1	2
8	4	1,25	1	2
9	2	3,5	1	6
10	8	1,375	1	3
11	5	1	1	1
12	6	1,5	1	3
13	8	1,5	1	4
14	2	1	1	1
15	3	1,66	1	3
16	7	1	1	1
17	1	1	1	1
18	9	2,88	1	7
19	4	1,25	1	2
20	5	1,8	1	3
21	4	1,5	1	2
22	1	2	2	2
23	1	1	1	1
24	26	1,769	1	5
25	2	1	1	1

Table 26: Cluster LVSI 3mm (minimum distance)

Case	Cluster Nr. 3,0	LVSI∅ 3,0	LVSI min. 3,0	LVSI max. 3,0
1	16	4,625	1	20
2	2	1	1	1
3	4	1,75	1	3
4	14	4,462	1	10
5	6	2,5	1	8
6	1	1	1	1
7	6	1,6	1	2
8	4	1,25	1	2
9	2	3,5	1	6
10	6	1,83	1	6
11	5	1	1	1
12	6	1,5	1	3
13	7	1,714	1	5
14	2	1	1	1
15	1	5	5	5
16	7	1	1	1
17	1	1	1	1
18	4	6,5	1	16
19	2	2,5	1	4
20	3	3	1	5
21	3	2	1	3
22	1	2	2	2
23	1	1	1	1
24	14	3,28	1	13
25	2	1	1	1

Table 27: Cluster LVSI 4mm (minimum distance)

Case	Cluster Nr. 4,0	LVSI ∅ 4,0	LVSI min. 4,0	LVSI max. 4,0
1	14	5,285	1	23
2	2	1	1	1
3	3	2,3	1	4
4	11	5,909	1	14
5	6	2,5	1	8
6	1	1	1	1
7	6	1,6	1	2
8	4	1,25	1	2
9	2	3,5	1	6
10	5	2,2	1	7
11	4	1,25	1	2
12	5	1,8	1	4
13	6	2	1	5
14	2	1	1	1
15	1	5	5	5
16	6	1,16	1	2
17	1	1	1	1
18	2	13	9	17
19	2	2,5	1	4
20	3	3	1	5
21	2	3	2	4
22	1	2	2	2
23	1	1	1	1
24	12	3,83	1	13
25	2	1	1	1

Table 28: Cluster LVSI 0,5mm (centre distance)

Case	Cluster Nr. 0,5	LVSI ∅ 0,5	LVSI min. 0,5	LVSI max. 0,5
1	40	1,85	1	5
2	2	1	1	1
3	39	1,6	1	4
4	12	1,25	1	2
5	6	1,16	1	2
6	1	1	1	1
7	9	1,11	1	2
8	5	1	1	1
9	4	1,75	1	2
10	8	1,375	1	3
11	5	1	1	1
12	8	1,125	1	2
13	10	1,2	1	3
14	2	1	1	1
15	4	1,25	1	2
16	7	1	1	1
17	1	1	1	1
18	17	1,529	1	3
19	4	1,25	1	2
20	8	1,125	1	2
21	5	1,2	1	2
22	2	1	1	1
23	1	1	1	1
24	41	1,1219	1	2
25	2	1	1	1

Table 29: Cluster LVSI 1mm (centre distance)

Case	Cluster Nr. 1,0	LVSI ∅1,0	LVSI min. 1,0	LVSI max. 1,0
1	28	2,6428..	1	9
2	2	1	1	1
3	29	2,24	1	5
4	10	1,5	1	3
5	6	1,16	1	2
6	1	1	1	1
7	9	1,11	1	2
8	4	1,25	1	2
9	2	2,333	1	4
10	8	1,375	1	3
11	5	1	1	1
12	7	1,285	1	2
13	9	1,333	1	4
14	2	1	1	1
15	3	1,66	1	3
16	7	1	1	1
17	1	1	1	1
18	10	2,6	1	6
19	4	1,25	1	2
20	5	1,8	1	3
21	4	1,5	1	2
22	1	2	2	2
23	1	1	1	1
24	30	1,533	1	5
25	2	1	1	1

Table 30: Cluster LVSI 2mm (centre distance)

Case	Cluster Nr. 2,0	LVSI ∅2,0	LVSI min. 2,0	LVSI max. 2,0
1	20	3,7	1	12
2	2	1	1	1
3	18	3,6	1	6
4	7	2,14	1	7
5	5	1,4	1	3
6	1	1	1	1
7	9	1,11	1	2
8	4	1,25	1	2
9	2	3,5	1	6
10	7	1,57	1	5
11	5	1	1	1
12	6	1,5	1	3
13	7	1,714	1	5
14	2	1	1	1
15	1	5	5	5
16	7	1	1	1
17	1	1	1	1
18	6	4,333	1	11
19	3	1,666	1	3
20	5	1,8	1	4
21	3	2	1	3
22	1	2	2	2
23	1	1	1	1
24	22	2,09	1	6
25	2	1	1	1

Table 31: Cluster LVSI 3mm (centre distance)

Case	Cluster Nr. 3,0	LVSI ∅3,0	LVSI min. 3,0	LVSI max. 3,0
1	17	4,35	1	20
2	2	1	1	1
3	14	4,64	1	10
4	7	2,14	1	7
5	4	1,75	1	3
6	1	1	1	1
7	7	1,42	1	2
8	4	1,25	1	2
9	2	3,5	1	6
10	6	1,83	1	5
11	5	1	1	1
12	6	1,5	1	3
13	7	1,714	1	5
14	2	1	1	1
15	1	5	5	5
16	7	1	1	1
17	1	1	1	1
18	4	6,5	1	16
19	2	2,5	1	4
20	3	3	1	5
21	3	2	1	3
22	1	2	2	2
23	1	1	1	1
24	15	3,06	1	12
25	2	1	1	1

Table 32: Cluster LVSI 4mm (centre distance)

Case	Cluster Nr. 4,0	LVSI ∅ 4,0	LVSI min. 4,0	LVSI max. 4,0
1	15	4,933	1	20
2	2	1	1	1
3	11	5,909	1	14
4	6	2,5	1	8
5	3	2,3	1	4
6	1	1	1	1
7	6	1,6	1	2
8	4	1,25	1	2
9	2	3,5	1	6
10	5	2,2	1	7
11	4	1,25	1	2
12	6	1,5	1	3
13	6	2	1	5
14	2	1	1	1
15	1	5	5	5
16	6	1,16	1	2
17	1	1	1	1
18	2	13	9	17
19	2	2,5	1	4
20	3	3	1	5
21	2	3	2	4
22	1	2	2	2
23	1	1	1	1
24	12	3,833	1	13
25	2	1	1	1