MEDICAL UNIVERSITY - PLOVDIV
DEPARTMENT OF GENERAL AND CLINICAL PATHOLOGY

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THE ROLE OF HYPOXIA AND EPITHELIAL-MESENCHYMAL TRANSITION IN CARCINOGENESIS IN ENDOMETRIOSIS, ENDOMETRIAL AND OVARIAN CARCINOMAS

ABSTRACT

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All materials related to the dissertation defense are available at the Department of General and Clinical Pathology and on the official website of the Medical University of Plovdiv.

CONTENTS

ABBREVIATIONS USED

ARID1A – A gene that encodes a protein involved in chromatin remodeling and is associated with various types of cancer.
CDH1 – gene encoding E-cadherin (cell adhesion molecule)
CTNNB1 – gene encoding Beta-catenin (involved in cell adhesion and signaling)
EGFR – Epidermal Growth Factor Receptor
EMT – epithelial-mesenchymal transition
ER – estrogen receptor
ET1 – endothelin 1
Fascin – protein involved in cell migration and invasion
HIF1α – hypoxia-induced factor 1 alpha
IHC – immunohistochemical study
Ki-67 – nuclear protein, marker of cell proliferation
MEP – mesenchymal-epithelial transition
p53 – oncoprotein controlling the cell cycle and apoptosis
PIK3CA – A gene that encodes a protein involved in cell signaling.
PR – progesterone receptor
PTEN – A gene that encodes a protein that plays a role as a tumor suppressor.
SMA – smooth muscle actin
TGF-β – transforming growth factor beta
VEGF – vascular endothelial growth factor
WNT1 – proto-oncogene, ligand of the Wnt signaling pathway

I. INTRODUCTION

Endometriosis, endometrial and ovarian carcinoma represent three significant medical problems with a serious impact on women's health globally. The complexity of these diseases and the frequency with which they occur predetermine their high social importance, as well as the need for a thorough understanding of the pathogenetic mechanisms underlying them. In modern medicine, attention to the relationship between endometriosis and malignant processes in the uterus and ovaries is increasing, with particular interest being the mechanisms of hypoxia and epithelial-mesenchymal transition (EMT), due to their proven role in the progression and aggressiveness of these diseases (Giannella et al., 2021; Guo et al., 2022).

In recent decades, there has been a substantial increase in the incidence of endometriosis, with the disease affecting between 6% and 10% of women of reproductive age worldwide (Zondervan et al., 2020). The characteristic symptomatology of endometriosis, including chronic pelvic pain, dysmenorrhea and infertility, leads to serious social and economic consequences. The disease affects not only the physical health, but also the psycho-emotional state of the patients, which in turn further aggravates their quality of life and requires a complex and more precise approach to diagnosis and treatment (Johnson et al., 2021).

On the other hand, endometrial carcinoma ranks among the most common malignancies of the female reproductive system worldwide, with the trend in recent years pointing to a steady increase in cases, especially in developed countries. This carcinoma accounts for about 7% of all newly diagnosed cancers in women, and its incidence increases after menopause (Sung et al., 2021). The increase in the age of patients at initial diagnosis represents an additional difficulty for the health care system. Endometrial carcinoma is characterized by great histological and molecular diversity, which calls for the need to refine the diagnostic and prognostic criteria, as well as to personalize the therapeutic approach. Ovarian carcinoma is known as one of the deadliest gynecological diseases due to its frequent detection at an advanced stage. Despite advances in diagnostic methods and therapeutic strategies, five-year survival remains unsatisfactory and does not exceed 40% worldwide (Ferlay et al., 2020). Most cases are diagnosed in stage III or IV, which significantly limits the possibilities of successful therapy and improving the prognosis. This determines the need to study additional markers and mechanisms related to the aggressiveness of the disease and its metastasis.

In the context of the pathogenesis of these diseases, hypoxia plays a significant role by activating specific signaling pathways, key of which is HIF (Hypoxia-Inducible Factor). This factor is directly related to the regulation of gene expression, angiogenesis, cell migration and invasion, processes that determine the aggressive behavior and metastatic ability of malignant tumors (Keith et al., 2019). Epithelial-mesenchymal transition (EMT) is a process where epithelial cells acquire mesenchymal properties, increasing their motility and invasive potential. The combination of hypoxia and EMF is considered a key mechanism contributing to the transition from a benign condition such as endometriosis to malignant tumors.

Based on the current knowledge about epidemiological aspects, new classification approaches, molecular mechanisms and the clinical significance of hypoxia and epithelial-mesenchymal transition in these common gynecological diseases, the modern approaches to diagnosis and therapy, as well as the prospects for future research in the field of personalized medicine based on the integration of molecular and immunohistochemical markers in order to improve diagnostic algorithms, therapeutic strategies and general prognosis in these patients, are reviewed.

II. AIM AND TASKS

1. AIM

The aim of the present study was to investigate the immunohistochemical expression of HIF1alpha, Ki 67, E-cadherin, β-cathenin, SMA and Fascin in endometriosis foci, in endometrial and ovarian carcinomas in relation to hypoxia, carcinogenesis and progression and to seek correlation with histological and clinicopathological parameters.

2. TASKS

1. To study the distribution of cases with endometriosis, endometrial and ovarian carcinomas in the biopsy array of the Clinical Pathology Department of UMBAL Pulmed-Plovdiv for a period of five years 2019-2023.

2. To assess the expression of HIF1alpha, Ki 67, E-cadherin and Fascin in patients with ectopic endometriosis and adenomyosis, stratified by age and location.

3. To investigate the expression of HIF1alpha, Ki 67, E-cadherin, β-cathenin, SMA and Fascin in the three groups in relation to hypoxia and epithelial-mesenchymal transition and to make a comparative analysis.

4. To comparatively study the expression of HIF1alpha, Ki 67, E-cadherin, β-cathenin and Fascin at the invasive tumor front and around necrotic foci in ovarian and endometrial carcinomas and to compare with the expression in eutopic endometrium and endometriosis in relation to carcinogenesis.

5. To compare the expression distribution of HIF1alpha, Ki 67 and Fascin in the invasive front and in the tumor in patients with carcinoma and endometriosis.

6. To study the expression of the studied antibodies in relation to the distribution of tumors according to FIGO stage and to derive a prognostic IHC-panel to assess the risk of progression in the three groups.

III. MATERIALS AND METHODS

1. MATERIALS

The study includes a retrospective study of 343 cases of endometriosis and tumors of the uterus and ovaries from the biopsy array of the Department of Clinical Pathology of the UMBAL "Palmed" - the city of Plovdiv for a period of 5 years (2019 - 2023). All biopsy diagnoses were made on surgical material from the Surgery Clinic and the Obs Gyn Clinic of Pulmed University Hospital. Of these, 100 cases were immunohistochemically studied.

The histological and immunohistochemical studies of the biopsy materials were carried out in the laboratory of the Morphology Center of the Medical University of Plovdiv and in the laboratory of the Department of Clinical Pathology of the "Pulmed" UMBAL - Plovdiv.

The data from the studied clinical-morphological parameters and the results of the performed immunohistochemical studies of all studied patients were coded and entered into an electronic database for subsequent statistical processing.

The materials are divided into 3 main groups:

Group I - Patients with endometriosis

Group II - Patients with endometrial carcinoma

Group III - Patients with ovarian carcinoma

In the first group, there were 32 female patients, with an average age of 48.65 ± 14.87 years. The youngest patient was 24 years old, and the oldest - 69 years old.

In the second group, there were 34 female patients, with an average age of 66.45 ± 10.67 years. The youngest patient was 43 years old, and the oldest was 82 years old.

In the third group, there were 34 female patients, with an average age of 67.21 ± 8.94 years. The youngest patient was 56 years old, and the oldest - 86 years old.

For the main groups of working material, we used inclusion and exclusion criteria for case selection.

Inclusion criteria:

1. Biopsy cases of hospitalized and operated patients with a histologically confirmed clinical diagnosis of endometrial carcinoma.
2. Cases with a histologically confirmed clinical diagnosis of endometriosis with different localizations.
3. Biopsy confirmed cases with clinical and histological diagnosis of ovarian carcinoma.

Exclusion criteria:

1. Patients with histological findings other than endometriosis, endometrial or ovarian carcinoma.

2. Cases of metastases in the ovaries with an unclear primary focus.
3. Non-presentative histological results.
4. Biopsy materials with clinical data of endometriosis, but not histologically proven.

Immunohistochemical studies with the markers HIF1alpha, Ki 67, E-cadherin, β-catenin, SMA and Fascin were performed on surgical biopsy material from cases with histologically diagnosed endometriosis, endometrial and ovarian carcinoma. All immunohistochemical studies were performed in the Morphological Center of the Medical University of Plovdiv and in the Department of Clinical Pathology of the UMBAL - Pulmed.

2. METHODS

2.1. Classic histological technique

Routine staining with hematoxylin-eosin /H-E/

The histological examination of the biopsy materials of all cases was performed by making preparations according to the classic histological technique. The surgically removed tissue material is fixed in 10% buffered formalin. A tissue block is then formed and embedded in paraffin. Paraffin sections 5 µm thick were deparaffinized and rehydrated through alcohols of decreasing concentration. Hematoxylin-eosin staining was performed according to standard methodology. Sections were embedded in Canada balsam and covered with coverslips. A microscopic examination of the obtained preparations follows.

In patients with **endometriosis**, age, location of the process, presence of concomitant pathology, such as endometrial hyperplasia, cervicitis, leiomyomas, endometrial carcinoma, endometrial polyps, cervical carcinoma, endometrial atrophy, ovarian carcinoma, ovarian cysts, and vascular invasion were determined.

In all cases with histologically proven **endometrial carcinoma**, we determined the following clinico-morphological parameters: age of the patient, type of endometrial cancer, Figo stage, grading for type I, histological appearance of type I, tumor emboli, lympho-vascular and perineural invasion, tumor necrosis, stromal reaction and desmoplasia, depth of invasion and metastases.

In all cases with histologically proven **ovarian carcinoma**, we determined the following clinico-morphological parameters: patient age, Figo stage, histological type, histological degree of tumor differentiation, nodal status, lympho-vascular invasion and peritoneal washing for cytological examination.

2.2. Immunohistochemistry technique

The immunohistochemical examination was performed at the Morphological Center of the MU - Plovdiv and at the Department of Clinical Pathology of the UMBAL - Pulmed in accordance with validated standard protocols provided by the manufacturer - DAKO (Denmark) and Merck/Cell Marque (Germany).

Serial 5-µm-thick sections were prepared from selected paraffin blocks with representative tissue changes and mounted on adhesive slides. Sections were deparaffinized and rehydrated in alcohols of decreasing concentration. Washing was performed according to the instructions for use. Before performing the immunohistochemical reaction, antigen retrieval was performed by incubation in EnV FLEX Target retrieval solution, pH9 for E-Cadherin, beta-Catenin and SMA for 20 min., pH6 for Ki-67 for 20 min., pH9 for HIF-1α for 30 min., pH6 for Fascin for 30 min.

Immunohistochemistry was performed according to the manufacturer's standard protocols using the EnVision FLEX Visualization System. The reaction was considered positive when brownish staining occurred. It is observed in different areas of the cell (membrane, cytoplasm, nucleus) depending on the localization of the antigen sought. The antibodies used against E-Cadherin, beta-Catenin, SMA and Ki-67 were from DAKO (FLEX E-Cadherin, clone NCH-38, FLEX beta-Catenin, clone b-Catenin-1, FLEX Ki-67, clone MIB-1 and FLEX SMA, clone 1A4), as well as a rabbit monoclonal antibody against HIF-1α, clone EP118, and a mouse monoclonal antibody against Fascin. Branch 55-k2 of Merck/Cell Marque, Germany.

The IHC reaction was performed on a Dako Autostainer Link 48 apparatus with automated staining.

Serial sections from each of the cases studied were tested for the antibodies used. A positive and negative control was prepared for each test run with the individual antibodies.

The positive control was chosen in accordance with the manufacturer's instructions: prostate - for E-cadherin and beta-Catenin; small intestine - for SMA; lymph node - Fascin, tonsil - for Ki-67 and kidney - for HIF-1α. The negative control for each antibody was prepared by standard immunohistochemical procedure without instilling the test antibody.

Reporting of IHC-study results

The evaluation of biopsy materials with X-E staining and the reporting of IHC results were performed at 2 levels: level I - by the doctoral student and level II consultative - by the scientific supervisor. The final score was taken as the average value of the grades of the two levels.

An Olympus Model № CX21LEDFS2, SN 0E86964 light microscope was used. At least 10 fields were selected in each case - a total of 1000 cells observed at 400 magnification (eyepiece x10, objective x40).

Immunohistochemical expression reporting methods

In the present study, the evaluation of the immunohistochemical expression of the studied markers was performed semiquantitatively, observing the specific localization and staining intensity, with a view to maximum reproducibility and objectivity. Positive immunohistochemical expression for each marker was defined by clear, specific staining in the respective cell localizations - nuclear, cytoplasmic and/or membrane, according to approaches established in the world literature.

To assess E-cadherin expression, we adopted the approach proposed by Takai et al. (2014). Positivity is defined by the presence of clear membrane and/or cytoplasmic staining in more than 10% of tumor cells. Immunostaining intensity was scored semiquantitatively into four grades: absent (0), weak (1+), moderate (2+), or strong (3+). We additionally used the staining model proposed by Giurgea et al. (2012), classifying it as focal, heterogeneous or diffuse. Only cases with a score of 3+ and a diffuse pattern were considered negative for epithelial-mesenchymal transition (EMP) status, while cases with scores of 0, 1+, 2+ or the presence of areas of missing expression were defined as positive EMP-status corresponding to reduced or lost E-cadherin expression (Giurgea et al., 2012). Evaluation of β-catenin immunoexpression was performed analogously to that of E-cadherin, according to the approaches described by Takai et al. (2014). Clear positive membranous and cytoplasmic staining was observed in more than 10% of the tumor cells. Besides intensity (absent, weak, moderate, strong expression) and staining pattern (focal, heterogeneous, diffuse), special attention was paid to the cellular localization of expression - membrane, cytoplasmic or nuclear, since nuclear localization is associated with higher malignant potential and activated EMF (Takai et al., 2014; Giurgea et al., 2012).

Immunohistochemical expression of hypoxia-inducible factor HIF-1α was assessed according to the clear presence of nuclear and/or cytoplasmic staining in more than 10% of tumor cells, using the widely accepted approach of classifying staining intensity into four grades: negative, weak (1+), moderate (2+), and strong (3+). In addition, the percentage of positive cells was divided into categories: no expression, less than 10%, 10–25%, 25–50%, 50–75%, and more than 75% positive cells. The staining pattern was scored as perinecrotic, diffuse, mixed, or heterogeneous, according to the criteria outlined in the publication by Seeber et al. (2011), which considered HIF-1α expression as a key marker of tumor hypoxia and aggressiveness in gynecological malignant tumors. For the proliferative marker Ki-67, the method of semiquantitative counting of positive tumor nuclei relative to the total number of examined cells was applied. A positive expression was defined by clear and specific nuclear staining. Due to manual counting and the need for a pragmatic approach, positive cases were divided into the following categories: missing positive cells (0), 1–10% positive cells (1), 10–50% positive cells (2), and more than 50% positive cells (3). This method, widely adopted in diagnostic pathology and endometrial and ovarian carcinoma studies, is in accordance with international recommendations for Ki-67 reporting in tumors with high heterogeneity

(Dowsett et al., 2011).

Smooth muscle actin (SMA) expression was scored in the presence of clear positive membranous and/or cytoplasmic staining, and classified by the intensity of absent, weak, moderate, or strong expression. This reporting method is standard for assessing fibrosis and desmoplastic response in the stroma of endometrial and ovarian carcinomas (Yeung et al., 2013).

Fascin assessment was performed with positive cytoplasmic staining in more than 10% of tumor cells. Expression intensity was graded as absent, weak, moderate, and strong, with additional assessment of the pattern of staining in the microvessels (diffuse or heterogeneous) and the localization of expression (stromal cells and microvessels, epithelial cells, or mixed localization). A similar approach has been successfully used in studies on the role of Fascin in tumor invasion and metastasis in a variety of solid tumors, including endometrial and ovarian carcinoma (Hashimoto et al., 2005).

2.3. Statistical methods of investigation

In the present study, statistical hypotheses were formulated and verified with a precise calculation of the p value (p-value), which allowed an objective assessment of the differences between the compared groups. To accept the null hypothesis (H_0), according to which the values of a given parameter in individual groups do not differ statistically, the criterion $p>0.05$ was adopted. The alternative hypothesis (H_1), according to which there is a statistically significant difference between the compared groups, was accepted at $p<0.05$, which corresponds to a confidence limit above 95% and an acceptable standard error below 5%.

Non-parametric methods were applied to estimate quantitative parameters in the absence of a normal distribution, including the Kruskal-Wallis test when comparing more than two independent groups. When a statistically significant difference was found between them, a subsequent Dunn's post hoc analysis was used to specify pairwise differences. In cases where only two independent groups were compared, the Mann–Whitney U test was used.

Analysis of discrete (categorical) variables was performed using the z-test for comparison of relative proportions. If necessary, depending on the number of observations and the distribution of expected values, the χ^2 test (chi-square) and Fisher's Exact Test were also applied, especially in small groups or in the presence of cells with expected values below 5.

Parameters such as median, interquartile range (25th and 75th percentile), mean and standard deviation, and minimum and maximum values were used to present the descriptive statistics. Absolute and relative frequencies were used to describe categorical features such as degree of expression, immunohistochemical staining pattern, and epithelial-mesenchymal transition (EMT).

Graphical visualization of the data was realized by means of boxplot diagrams on continuous values, which present the median, percentiles and dispersion of the values. For discrete metrics, such as different expression categories or frequency ratios between groups, bar and pie charts normalized to 100 percent were used.

Data processing and statistical analysis were performed using specialized software IBM SPSS Statistics, version 26, as well as Microsoft Excel 2019, used mainly for tabular organization and visualization of results. All analyzes are in accordance with the methodological principles of medical statistics and ensure the necessary reliability and reproducibility of the research results.

IV. RESULTS OF OWN INVESTIGATIONS

Task 1 results

The distribution of cases with endometriosis, endometrial and ovarian carcinomas by age in the biopsy array of the Clinical Pathology Department of UMBAL Pulmed - Plovdiv for a period of five years 2019-2023 was studied.

A total of 343 biopsy cases were reviewed, of which 233 cases of endometriosis of different localization and 110 cases of endometrial and ovarian carcinomas were found. A selective selection was made according to inclusion and exclusion criteria for tissue samples from the three groups studied. 100 suitable cases - 32 patients with endometriosis, 34 with endometrial and 34 with ovarian carcinomas - were selected for immunohistochemical examination with the respective antibodies.

We retrospectively examined 100 cases of women, with a median age of 64.5 years (54; 71 years), divided into three groups: with endometriosis; with endometrial carcinoma and with ovarian carcinoma in the period 2019-2023. The distribution of the number and percentage of patients in the three groups for the indicated period are presented in Table 1.

Table 1. Descriptive statistics of the patient sample - frequency distribution by group and age distribution (means, measures of dispersion)

	Patients with endometriosis	Patients with endometrial cancer	Patients with ovarian cancer
Patients n (%)	32 (32)	34 (34)	34 (34)
Age (years); arithmetic mean ± SD years	48.65 ± 14.87	66.45 ± 10.67	67.21 ± 8.94
Age (years); min - max	24 - 69	43 - 82	56 - 86
Age (years); median	51	68	71
Age (years); 25th percentile	33.75	59.5	57.5
Age (years); 75th percentile	59.25	74	72.5

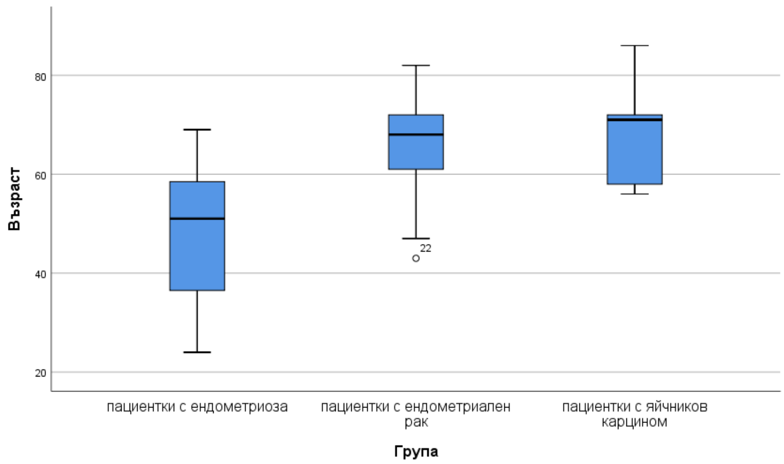


Figure 1. Box-plot diagram with age distribution by group

A statistically significant difference was found in the age of the examined patients by group (H=16.44, p=0.000). After a post hoc test, patients with endometriosis (51 years) were statistically significantly younger than those with endometrial cancer (68 years) (p=0.002) and those with ovarian carcinoma (71 years) (p=0.001). No difference was observed between the median ages of patients with endometrial and ovarian carcinomas.

The distribution of patients with endometrial carcinoma by median age and by FIGO stage is as follows: 5.9% - IA; 47.1% - IB; 11.8% - II; 35.2% - IIIB. No significant difference was observed between median age according to FIGO stage (H=3.52, p=0.318).

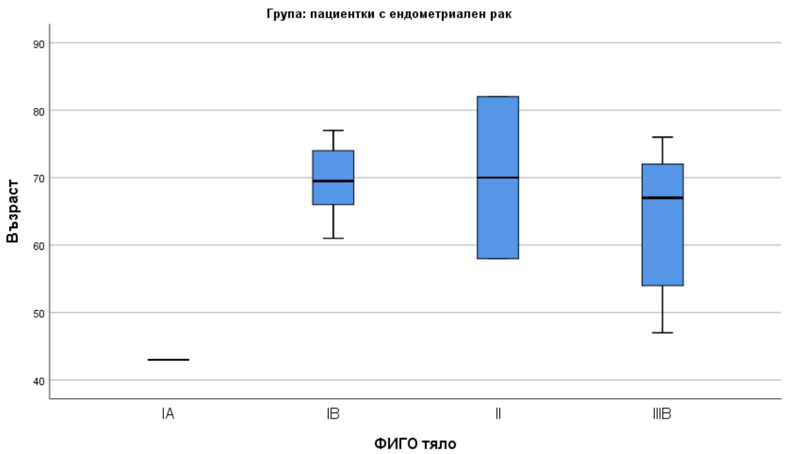


Figure 2. Distribution of patients with endometrial carcinoma (n=34) by age and FIGO stage

The distribution of patients with ovarian carcinoma by median age and by FIGO stage is as follows: 29.4% - IA; 35.3% - IC; 5.9% - II; 23.5% - IIIA; 5.9% - IIIB. No significant difference was observed between median age according to FIGO stage (H=5.90, p=0.207).

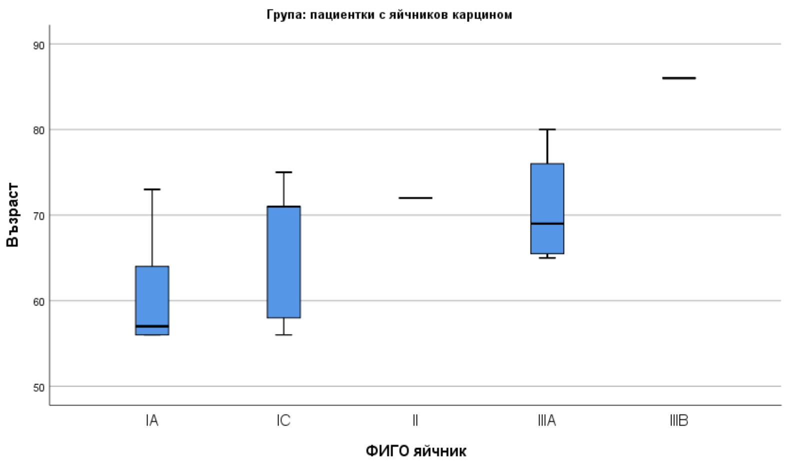


Figure 3. Distribution of patients with ovarian carcinoma (n=34) by age and FIGO stage

The distribution of patients with endometrial carcinoma by median age and depth of invasion of the myometrium is as follows: 23.5% - up to ½ of the thickness of the myometrium; 76.5% - over ½ of the thickness of the myometrium. After conducting the Mann-Whitney test, patients with invasion up to ½ of the myometrium were statistically significantly younger than those with invasion over ½ of the myometrium (p=0.015).

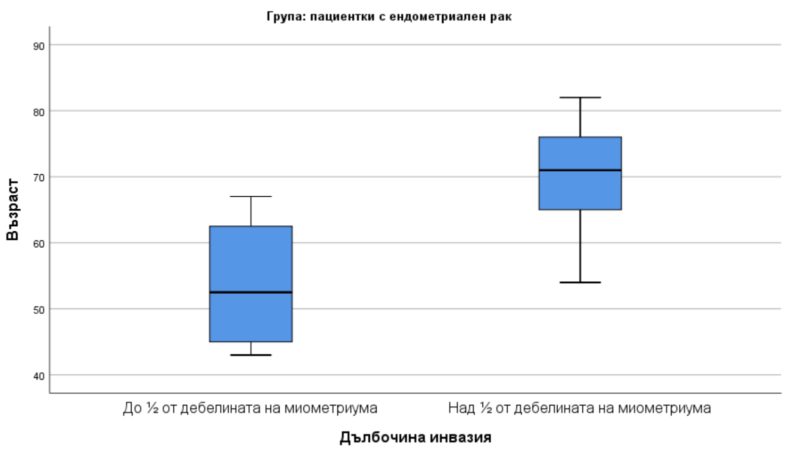


Figure 4. Distribution of patients with endometrial carcinoma (n=34) by age and depth of invasion

Combination of endometriosis with other uterine pathology presented in percentage shares. The leading combinations are endometriosis with atrophy of the endometrium and chronic cervicitis. Percentages are above 100 because the variable includes more than one possible answer.

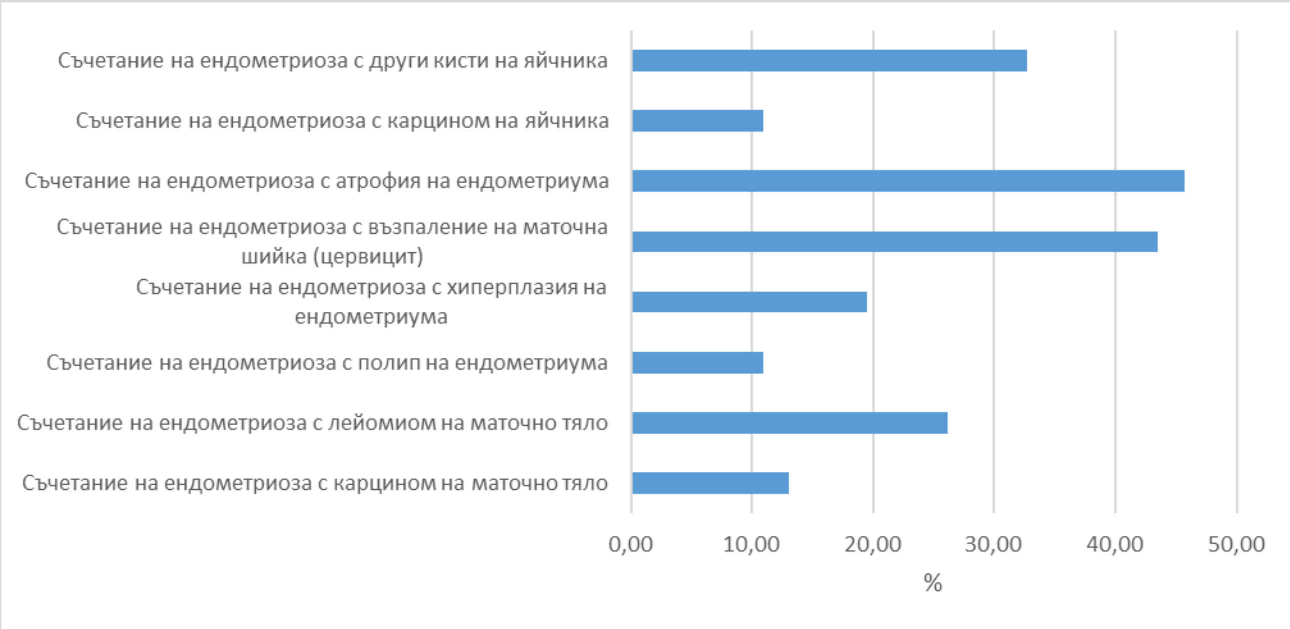


Figure 5. Percentage share of endometriosis accompanying uterine pathology.

The distribution of patients with endometrial carcinoma according to the median age and histological type of endometrial carcinoma is as follows: 82.4% - type 1 endometrioid carcinoma; 17.6% - type 2 non-endometrioid carcinoma. There was no significant difference between the median ages by histological type of endometrial carcinoma (H=1.150, p=0.284).

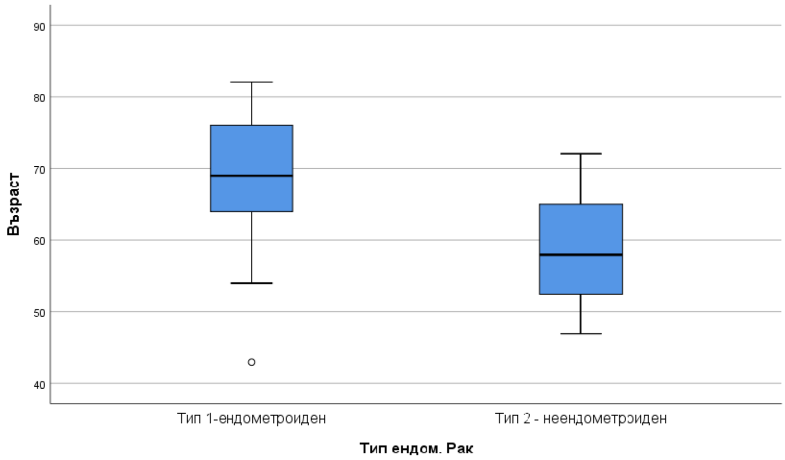


Figure 6. Distribution of patients with endometrial carcinoma (n=34) by age and by histological type of carcinoma

The pie chart shows the distribution of the relative shares of patients with ovarian carcinoma by histological type of carcinoma, the highest being the relative share of high grade serous (41.18%), followed by clear cell (23.53%) and low grade serous (17.65%). No statistically significant difference was demonstrated between the pairwise relative proportions (z-test, p≥0.05).

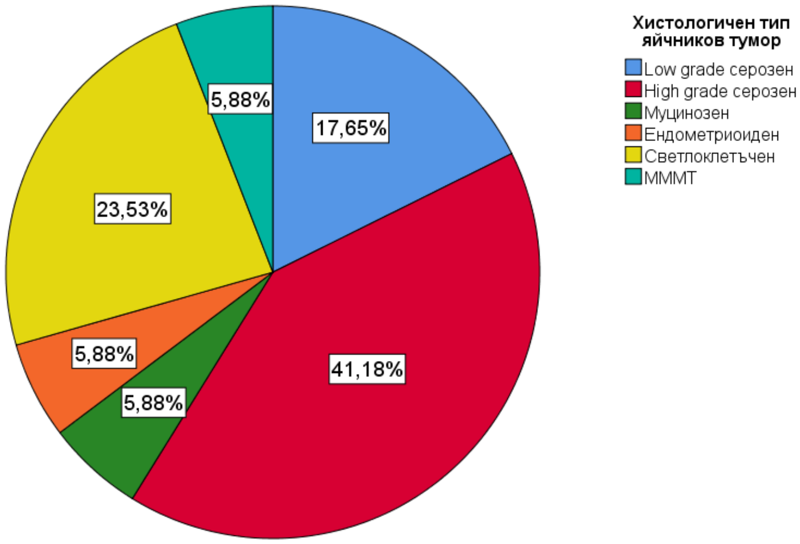
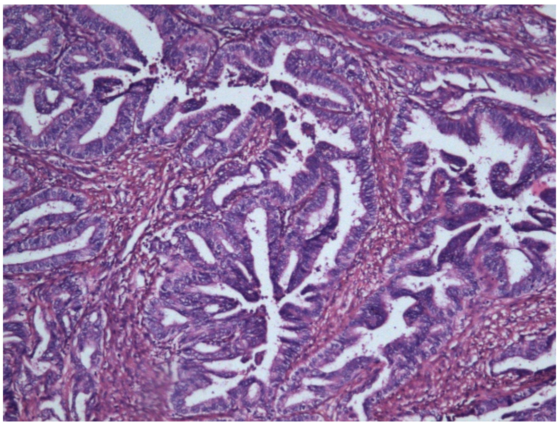
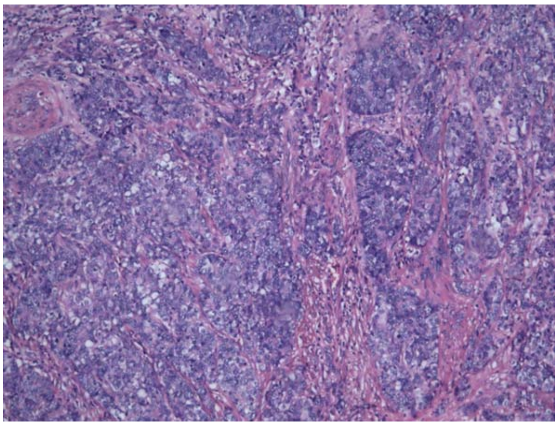


Figure 7. Relative proportions of patients with ovarian carcinoma (n=34) by histological type of carcinoma

Picture 1. Moderately differentiated endometrioid adenocarcinoma of the endometrium, H-E x100

Picture 2. High-grade serous ovarian carcinoma, H-E x100

Task 2 results

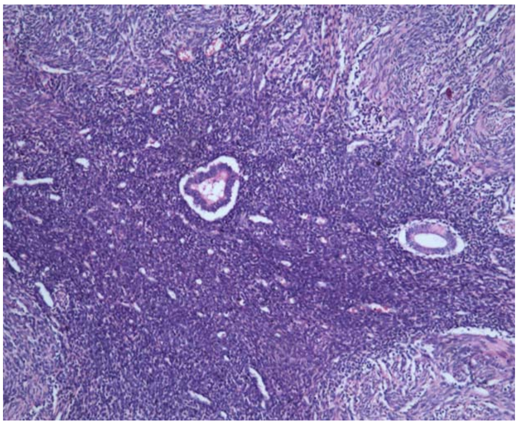
The expression of anti HIF1alpha, Ki 67, E-cadherin and β-cathenin in endometriosis patients by age and location was studied.

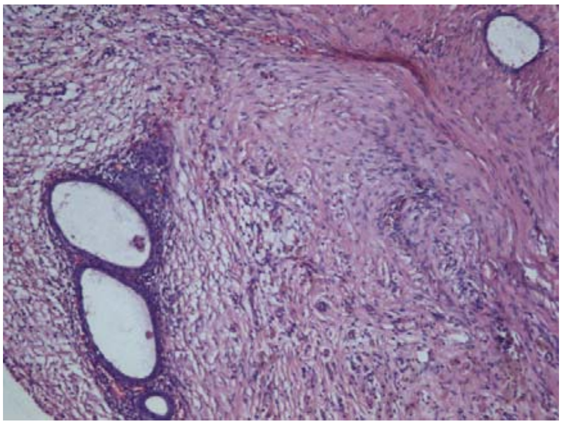
2.1. Distribution by age

Due to the fact that endometriosis develops more often in young women, in reproductive age, we divided the studied patients into two age groups, under and over 45 years, respectively, in order to compare the immunohistochemical expression of the studied markers.

The first group included 12 women of reproductive age under 45 years. The second group consisted of 20 women over 45 years. in menopause.

The age variable was grouped into two groups - up to and including 45 years (n=12; 37.5%) and over 45 years (n=20; 62.5%). No statistically significant difference was demonstrated between the pairwise relative proportions (z-test, p≥0.05).

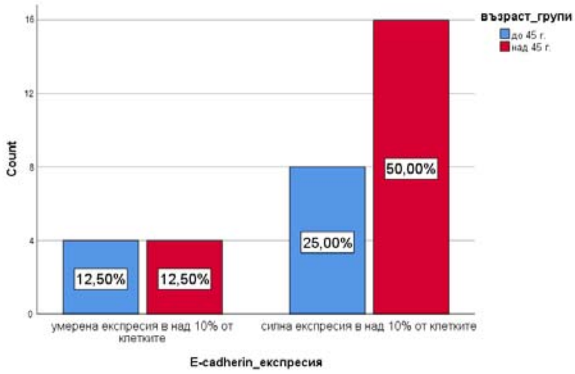




Picture 3. Adenomyosis, H-E x100

Picture 4. Foci with endometriosis in a postoperative cicatrix, H-E x100

Strong expression of E-cadherin was found in female patients over 45 years of age (50%) compared to women under 45 years of age (25%). A more common diffuse pattern of staining was found - in patients over 45 years old (56.25%), and in women under 45 years old (37.5%). In both age groups, the same percentage of positive EMT status was found (6.25%).



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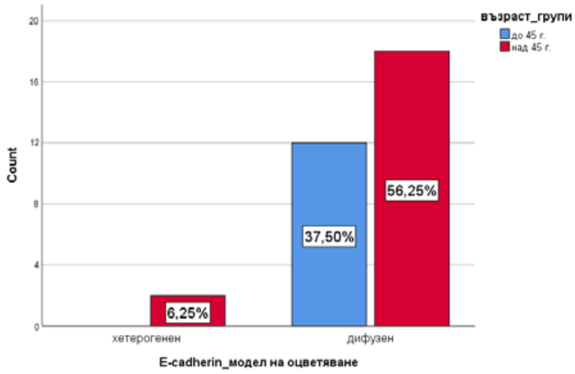

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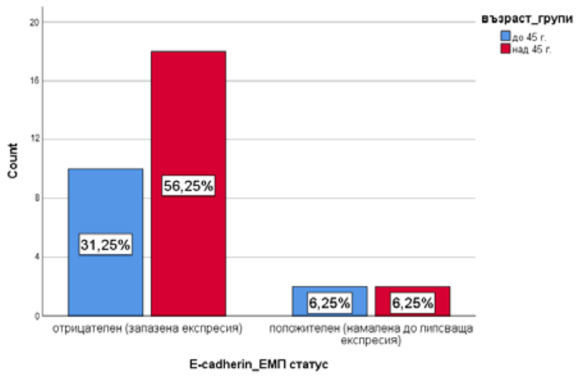
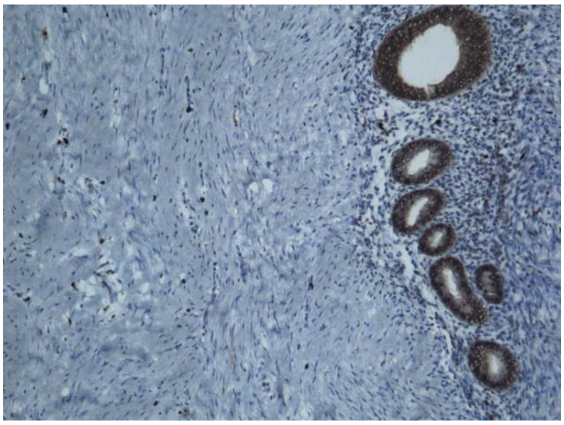
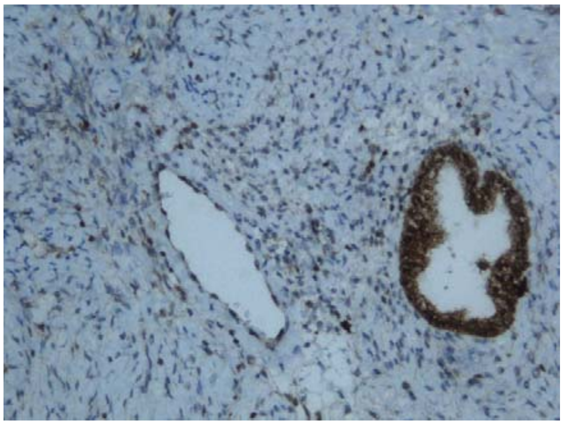


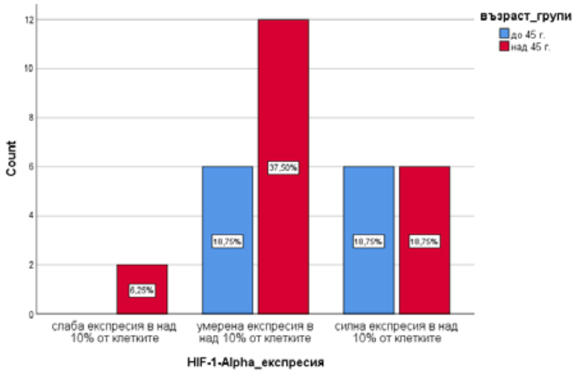
C

Figure 8. (A, B, C) E-Cadherin expression, staining pattern and EMT status in endometriosis patients by age group

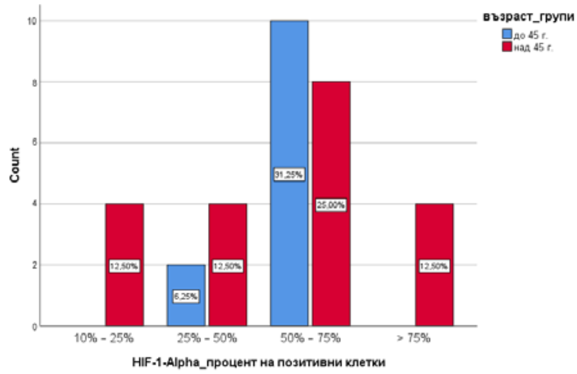
In patients over 45 years of age, moderate (37.5%) and strong (18.75%) anti HIF-1-alpha expression prevailed in more than 10 percent of cells, as well as in those under 45 years of age with moderate (18.75%) and strong (18.75%) anti HIF-1-alpha expression. A high percentage of positive cells was found in both age groups, as follows in patients under 45 between 50 and 75 percent (31.25%), and in patients over 45 between 50 and 75 percent (25%). More than 75 percent positive cells were reported only in patients over 45 years of age (12.5%). In the staining pattern, diffuse was most often reported in women under 45 (18.75%), as well as in patients over 45 (43.75%).

Picture 5. HIF-1-alpha, strong nuclear expression in adenomyosis, IHC, x100
Picture 6. HIF-1-alpha, diffuse nuclear expression in ectopic endometriosis, IHC, x100

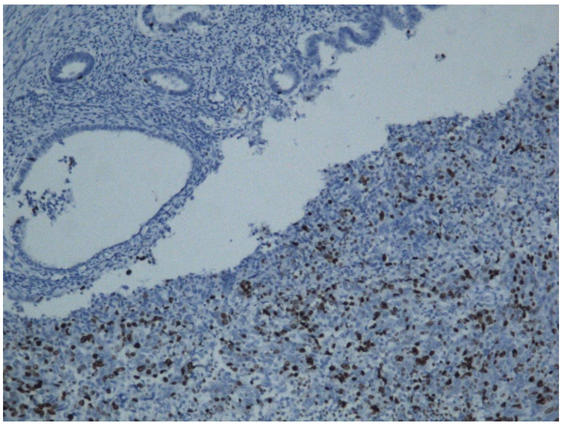
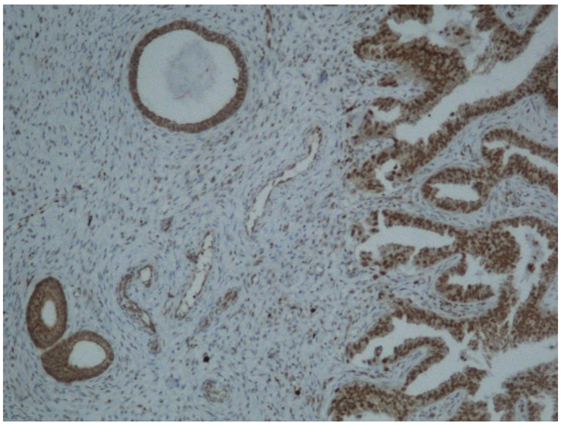


A



B

Figure 9. (A, B) Anti HIF-1-alpha expression, percentage of positive cells and staining pattern in endometriosis patients by age group

Picture 7. Ki 67, comparative expression in endometriosis and in poorly differentiated endometrial carcinoma, IHC, x100

Picture 8. HIF-1-alpha, comparative expression in endometriosis and moderately differentiated endometrial carcinoma, IHC, x100

Ki-67 expression in 1 to 10 percent was found in patients older than 45 years (56.25%) compared with women younger than 45 years (31.25%). In both age groups, expression was detected in 10 to 50 percent of proliferating cells (6.25%). A more frequent focal staining pattern was found - in patients over 45 years old (56.25%), and in women under 45 years old (31.25%).

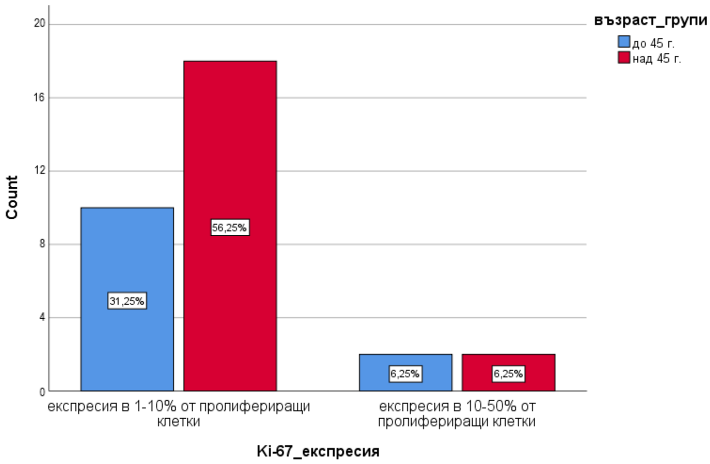
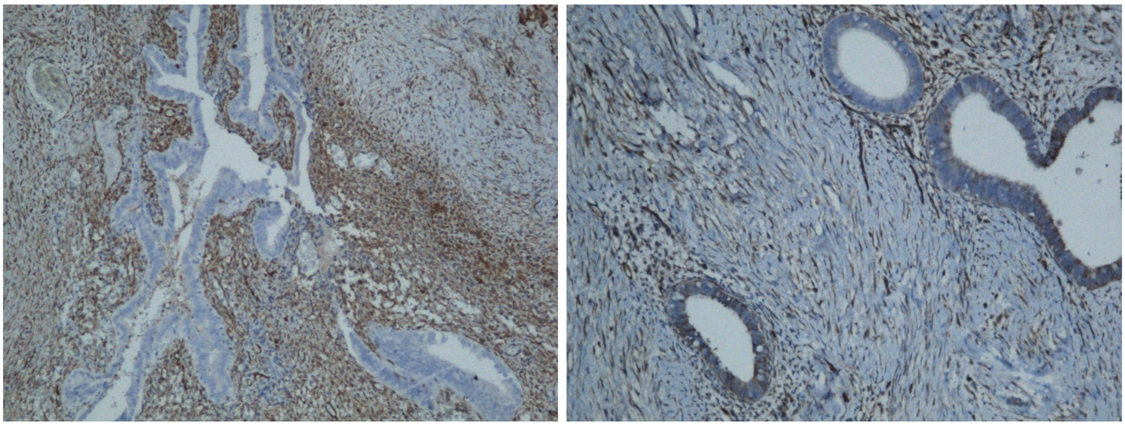


Figure 10. (A) Ki-67 expression and staining pattern in endometriosis patients by age group



Picture 9. Fascin, strong cytoplasmic expression in the stroma of adenomyosis, IHC, x100
Picture 10. Fascin, moderate cytoplasmic expression in ectopic endometriosis in stromal and epithelial cells, IHC, x100

Moderate expression of Fascin was found in female patients older than 45 years (50%) compared with women younger than 45 years (25%). A more frequent heterogeneous pattern of microvessel staining was found - in patients over 45 years of age (50%) and in women under 45 years of age (25%). In both age groups, a different localization was found in patients under 45 years old - in stromal cells and microvessels (25%) and mixed (stromal and epithelial cells, microvessels) (12.5%), and in women over 45 years old - in stromal cells and microvessels (31.25%) and mixed (stromal and epithelial cells, microvessels) (31.25%).

2.2. Distribution by localization

The expression of anti HIF1alpha, Ki 67, E-cadherin, β-cathenin and Fascin was studied in cases with adenomyosis and in ectopic endometriosis of different locations.

We reported the expression of IHC markers in the endometrium of the uterus and in the endometriotic focus. All studied patients had total hysterectomy.

After the comparative analysis, the following differences between the groups were found:

Statistically significantly higher is the relative share of percentage negative for EMT status in adenomyosis (44.4%) compared to ectopic endometriosis (18.5%) (z-test, $p<0.05$). Also, the relative share of percentage positive for EMT status was statistically significantly higher in ectopic endometriosis (29.6%) compared to adenomyosis (7.4%) (z-test, $p<0.05$).

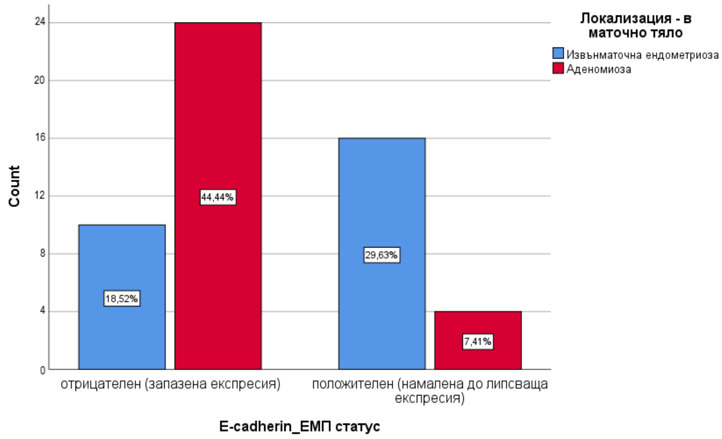
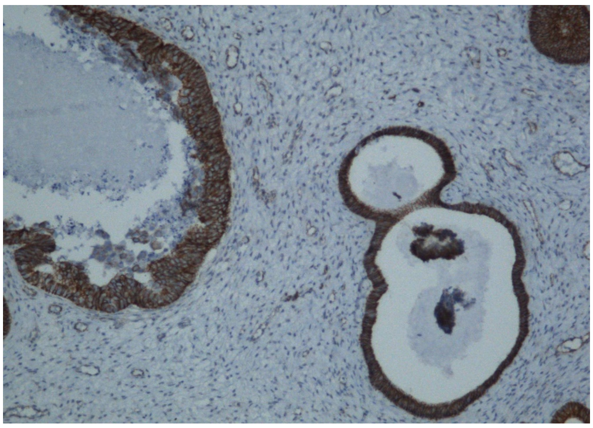
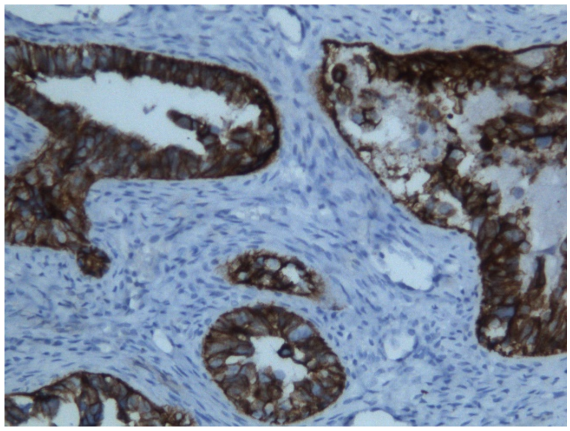


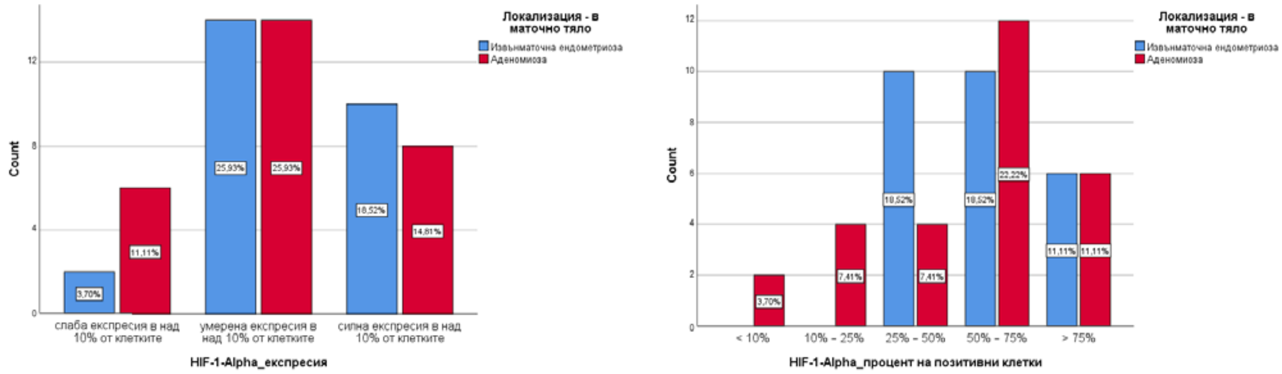
Figure 11. Relative shares of EMT status categories in patients with endometriosis according to its localization

Picture 11. E-cadherin, diffuse membrane expression in adenomyosis, IHC, x100
Picture 12. E-cadherin, heterogeneous expression in ectopic endometriosis in stromal and epithelial cells, IHC, x100

In patients with ectopic endometriosis, moderate (25.9%) and strong (18.5%) anti HIF-1-alpha expression prevailed in more than 10 percent of cells, and in those with adenomyosis, moderate (25.9%) and strong (14.8%) anti HIF-1-alpha expression also prevailed. A high percentage of positive cells was found in both locations, as follows in patients with ectopic endometriosis between 50 and 75 percent (18.5%) and in patients with adenomyosis between 50 and 75 percent (22.2%).

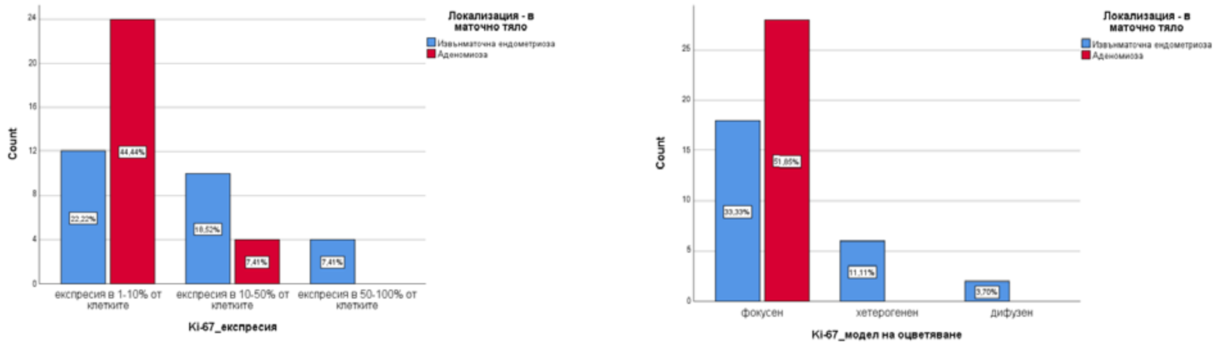
The relative proportion of the diffuse staining pattern was statistically significantly higher in adenomyosis (37%) compared to ectopic endometriosis (11.1%) (z-test, $p<0.05$). The relative share of the mixed staining pattern was higher in ectopic endometriosis (14.8%), while no cases were observed in adenomyosis (0%).



A B

Figure 12. (A, B) Relative shares of anti HIF-1-alpha expression, percentage of positive cells and staining pattern in patients with endometriosis according to its localization

A statistically significant difference in the relative proportions of Ki-67 expression in the 1 to 10 percent range was found in women with adenomyosis (44.4%) compared to women with ectopic endometriosis (22.2%) (z-test, $p<0.05$). A more frequent focal pattern of staining was found - in patients with adenomyosis (51.9%) compared to patients with ectopic endometriosis (33.3%) (z-test, $p<0.05$).



A B

Figure 13. (A, B) Ki-67 expression and staining pattern in patients with endometriosis according to its localization

A more frequent heterogeneous pattern of microvessel staining was found in both localizations of endometriosis.

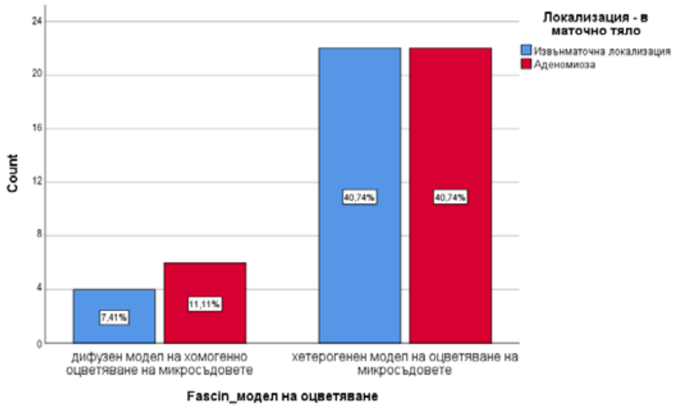


Figure 14. Relative proportions of Fascin expression categories by staining pattern according to the localization of endometriosis

Table 2 presents a distribution of the relative shares of women with ectopic endometriosis by location. Patients with ovarian localization predominated (64.7%).

Table 2. Distribution of the relative shares of women (n=26) with extrauterine endometriosis by location

		N*	**%**
Extrauterine location	In fallopian tube	8	23.5%
	In ovaries	22	64.7%
	In the skin (abdominal wall)	8	23.5%
	In large intestine	2	5.9%
	Total	**40**	**117.6%**

* the number of cases is 40, as a woman can have more than one ectopic location
No statistically significant differences were found for the rest of the investigated markers (z-test, p≥0.05).

Task 3 results

The expression of the studied markers in cases with endometriosis, in endometrial and ovarian carcinomas was studied and a comparative analysis was made of the expression of these markers in the three studied groups.

An analysis of six immunohistochemical markers - anti HIF1alpha, Ki 67, E-cadherin, β-cathenin, SMA and Fascin was performed in the three groups - endometriosis focus, endometrial carcinoma and ovarian carcinoma with Chi-square and z-test to prove statistically significant differences between relative proportions.

No statistically significant difference was observed between the relative shares of the E-cadherin expression categories in the three groups (z-test, p≥0.05).

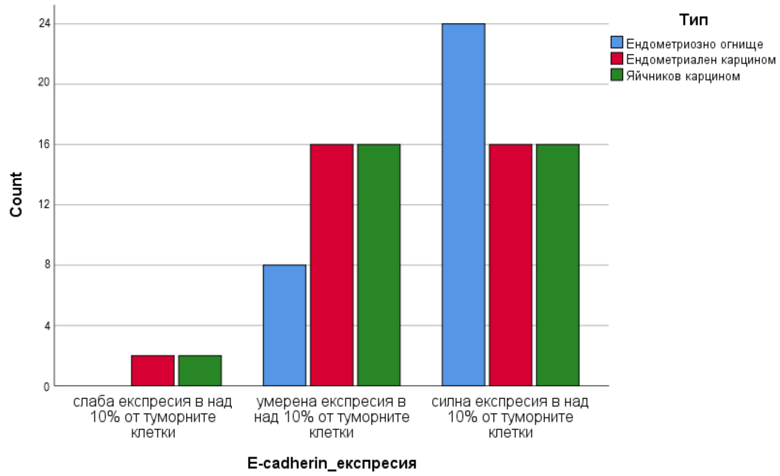
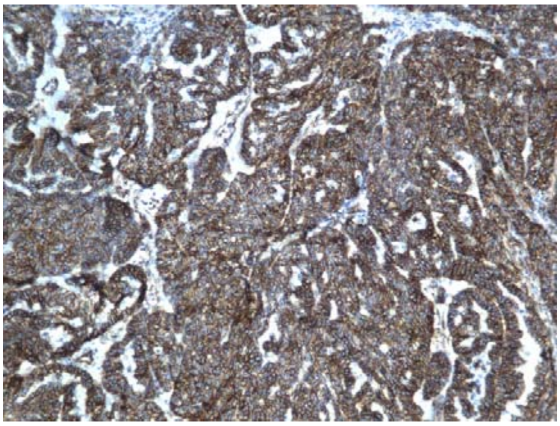
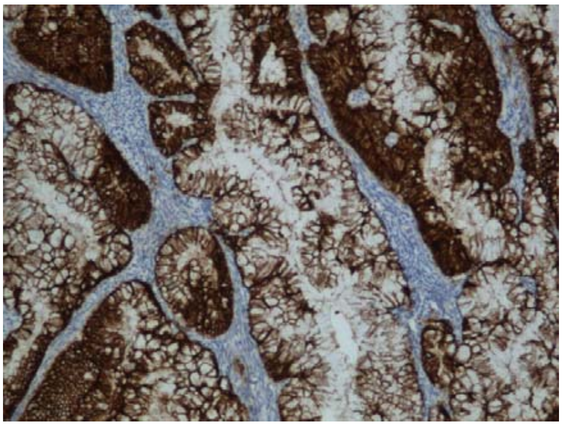


Figure 15. Relative shares of E-cadherin expression categories by groups

Picture 13. E-cadherin, diffuse expression pattern in endometrial carcinoma, IHC, x100
Picture 14. E-cadherin, heterogeneous expression pattern in ovarian carcinoma, positive EMT, IHC, x100

Statistically significantly higher is the relative share of the heterogeneous E-cadherin staining pattern in ovarian carcinoma (52.9%) compared to the other two groups - endometriosis focus (6.3%) and endometrial carcinoma (11.8%) (z-test, $p<0.05$). Also, the relative proportion of diffuse E-cadherin staining pattern was statistically significantly higher in endometriosis (93.8%) and endometrial carcinoma (88.2%) compared to ovarian carcinoma (47.1%) (z-test, $p<0.05$).

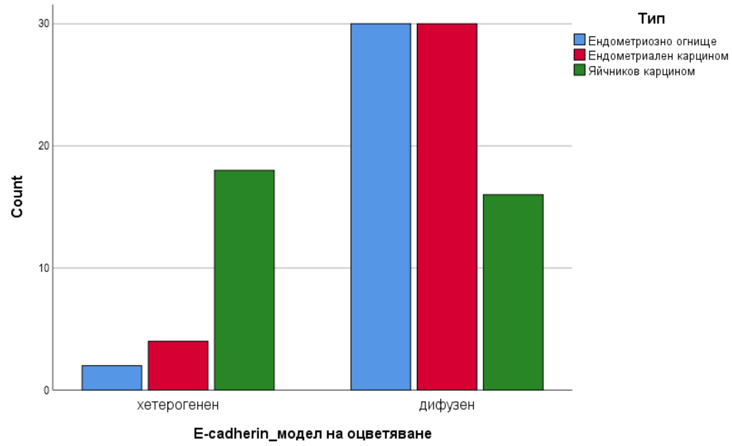


Figure 16. Relative proportions of E-cadherin expression categories by staining pattern by group

Statistically significantly higher is the relative share of negative epithelial-mesenchymal transition (EMT) in endometriosis (87.5%) compared to ovarian carcinoma (23.5%) (z-test, p<0.05). Also, the relative share of positive epithelial-mesenchymal transition (EMT) in ovarian carcinoma (76.5%) compared to endometriosis focus (12.5%) was statistically significantly higher (z-test, p<0.05).

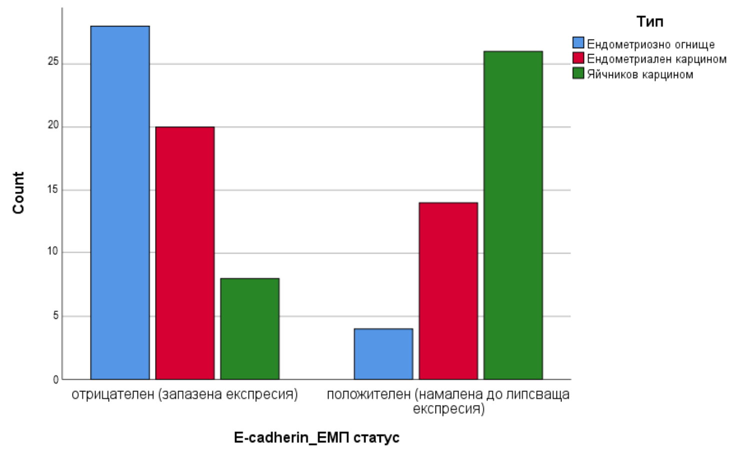


Figure 17. Relative proportions of E-cadherin expression categories by EMT status by group

The relative share of weak expression in more than 10 percent of the cells was highest in ovarian carcinoma (41.2%), no cases were observed in the group of patients with endometriosis focus (0%). Moderate expression of β-catenin was found in all three studied groups with a statistically significantly higher relative proportion of weak and strong expression in endometriosis (87.5%), endometrial carcinoma (58.8%) and ovarian carcinoma (52.9%).

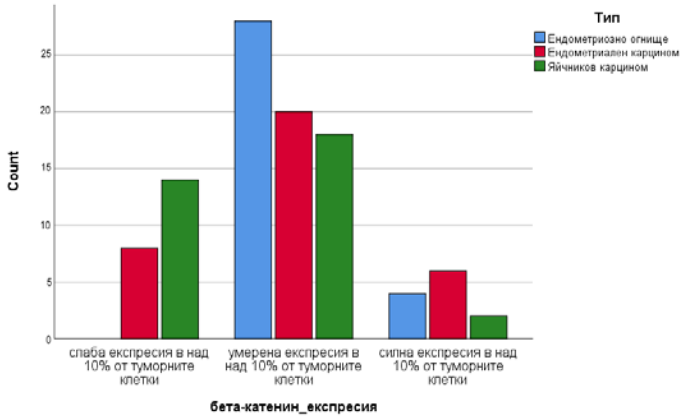


Figure 18. Relative proportions of β-catenin expression categories by group

No statistically significant difference was observed between the relative proportions of β-catenin expression categories by staining pattern in the three groups (z-test, p≥0.05).

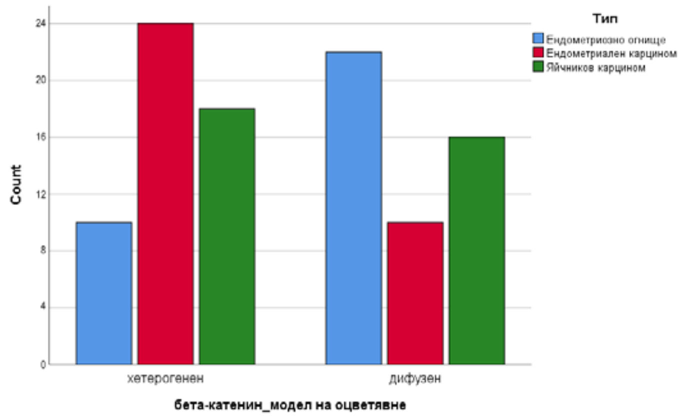
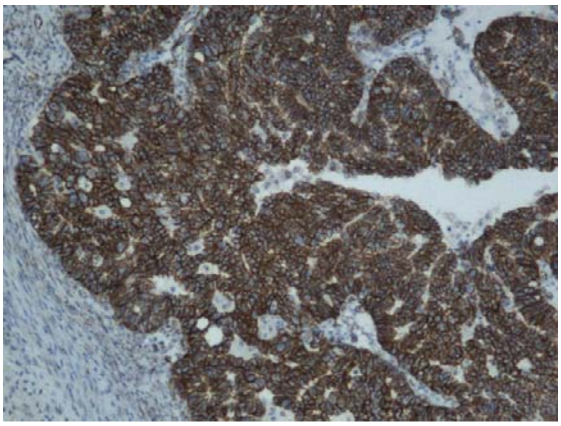
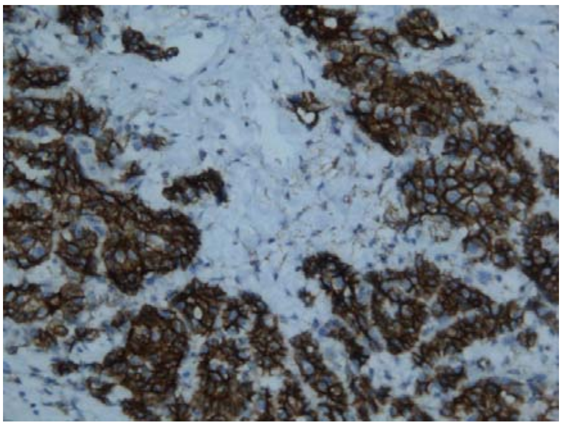


Figure 19. Relative proportions of β-catenin expression categories by staining pattern by group

No statistically significant difference was observed between the relative shares of β-cathenin expression categories by localization in cell membrane, cytoplasm and nucleus in the three groups (z-test, p≥0.05).

Picture 15. β-catenin, strong nuclear expression in ovarian carcinoma, IHC, x100
Picture 16. β-catenin, moderate nuclear expression in ovarian carcinoma, IHC, x100

No statistically significant difference was observed between the relative proportions of the anti HIF-1-alpha expression categories in the three groups (z-test, p≥0.05).

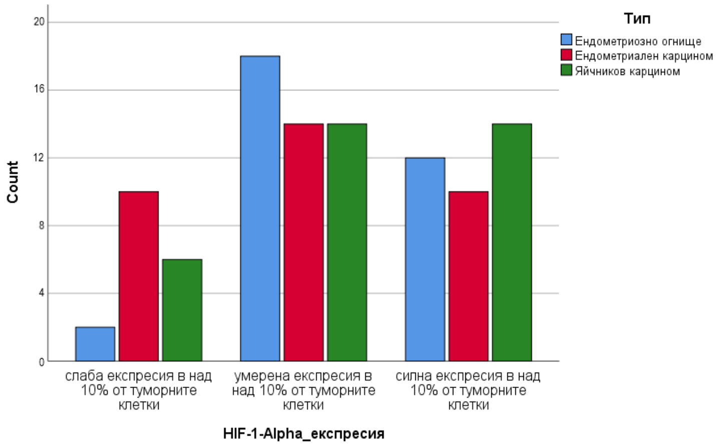


Figure 20. Relative proportions of anti HIF-1-alpha expression categories by group

A statistically significantly higher percentage of HIF-1-alpha positive cells between 25 and 50 percent was found in endometrial carcinoma (58.8%) compared to ovarian carcinoma (11.8%) (z-test, p<0.05). Also, the percentage of HIF-1-alpha positive cells greater than 75 percent was statistically significantly higher in ovarian carcinoma (41.2%) compared to endometrial carcinoma (5.9%) (z-test, p<0.05).

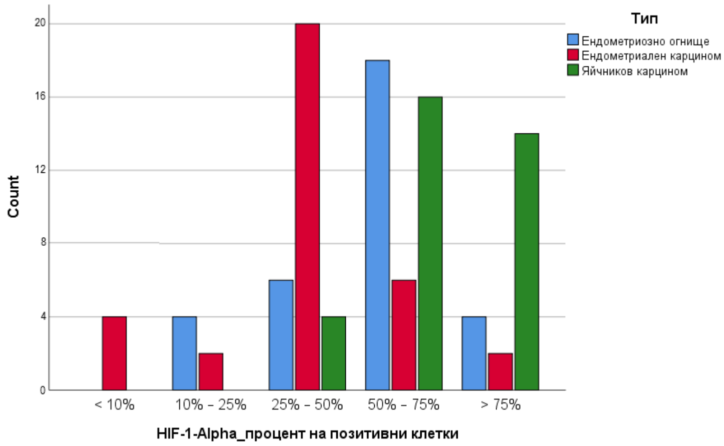


Figure 21. Relative proportions of categories of anti HIF-1-alpha expression by percentage of positive cells by group

The relative proportion of diffuse staining pattern for HIF-1-alpha was statistically significantly higher in endometriotic focus (62.5%) compared to endometrial carcinoma (11.8%) (z-test, p<0.05).

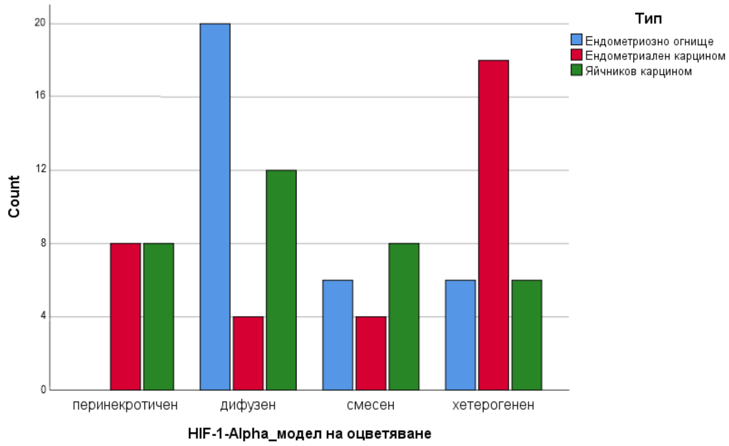


Figure 22. Relative proportions of anti HIF-1-alpha expression categories by staining pattern by group

The percentage of Ki 67 positive cells between 1 and 10 percent was statistically significantly higher in endometriosis (87.5%) compared to the other two groups - endometrial carcinoma (29.4%) and ovarian carcinoma (11.8%) (z-test, $p<0.05$). Also, the percentage of Ki 67 positive cells between 10 and 50 percent was statistically significantly higher in endometrial carcinoma (58.8%) compared to endometriosis (12.5%) (z-test, $p<0.05$). The highest percentage of Ki 67 positive cells is more than 50 percent in ovarian carcinoma (41.2%), and in the remaining two groups of endometrial carcinoma and endometriotic focus, the following relative shares are observed - 11.8% and 0%, respectively.

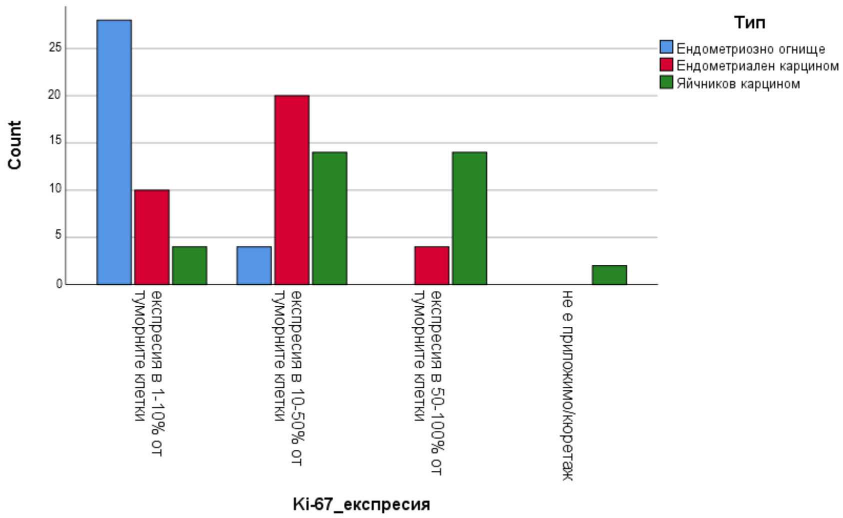
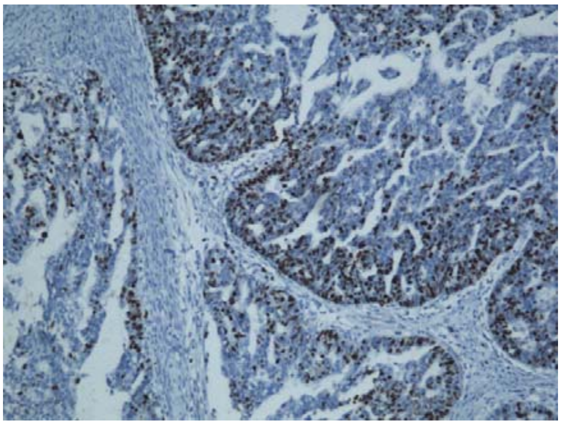
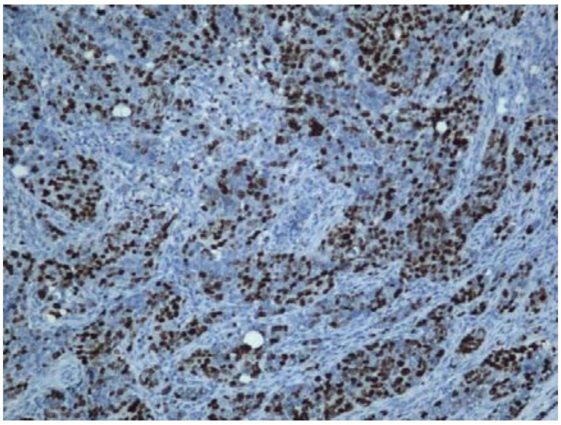


Figure 23. Relative proportions of Ki 67 expression categories by group

No statistically significant difference was observed between the relative proportions of Ki 67 expression categories in the three groups (z-test, $p\geq0.05$).

Picture 17. Ki 67, strong nuclear expression in endometrial carcinoma, IHC, x100
Picture 18. Ki 67, moderate nuclear expression in ovarian carcinoma, IHC, x100

No statistically significant difference was observed between the relative proportions of the SMA expression categories in the three groups (z-test, p≥0.05).

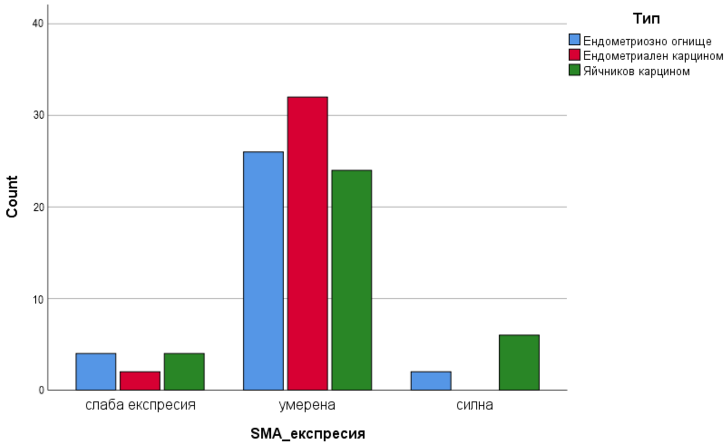


Figure 24. Relative proportions of SMA expression categories by group

No statistically significant difference was observed between the relative proportions of the SMA expression categories in the three groups (z-test, p≥0.05).

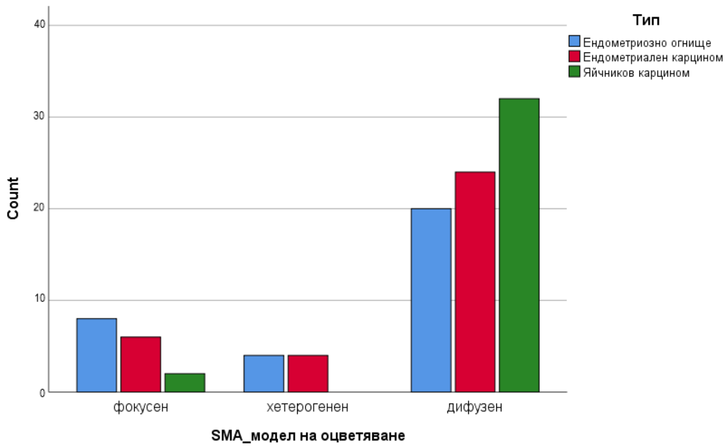
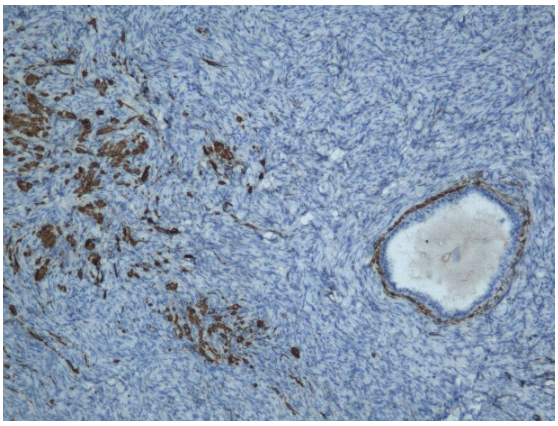
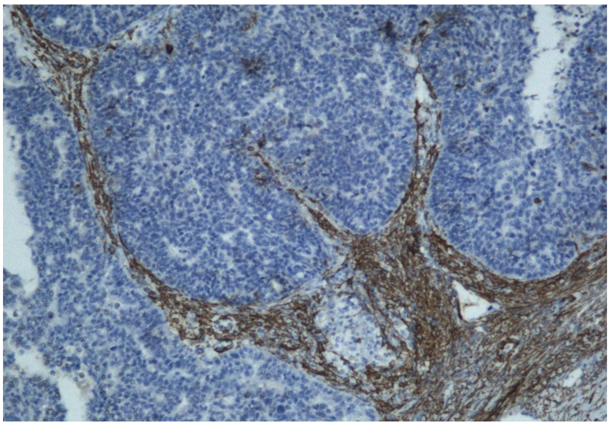


Figure 25. Relative proportions of SMA expression categories by staining pattern by group

Picture 19. SMA, strong cytoplasmic expression in endometriosis, IHC, x100
Picture 20. SMA, moderate cytoplasmic expression in ovarian carcinoma, IHC, x100

No statistically significant difference was observed between the relative proportions of Fascin expression categories in the three groups (z-test, p≥0.05).

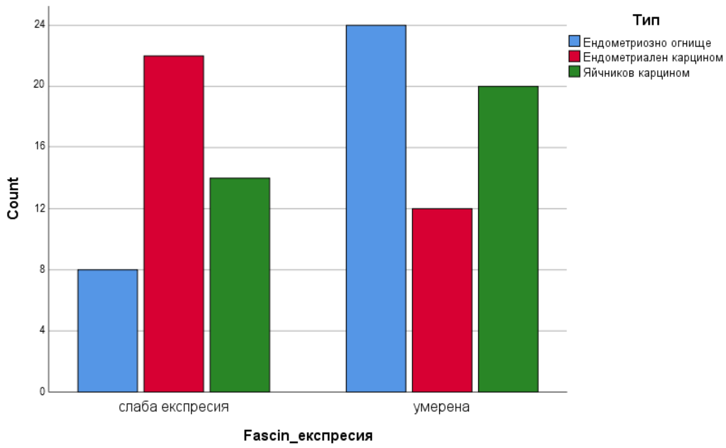


Figure 26. Relative proportions of Fascin expression categories by group

No statistically significant difference was observed between the relative proportions of Fascin expression categories in the three groups (z-test, p≥0.05).

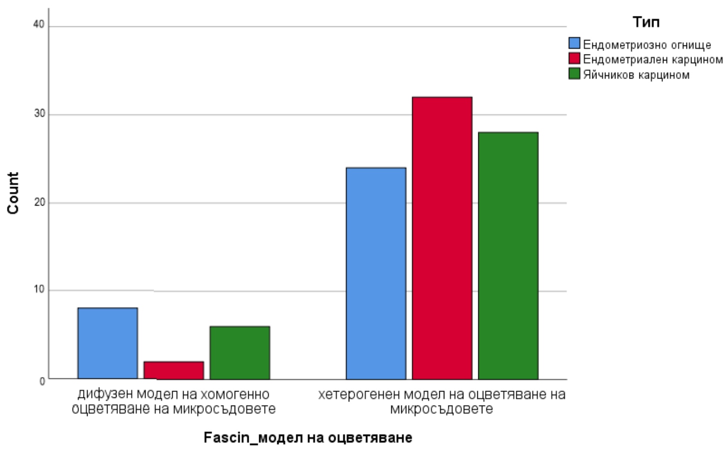


Figure 27. Relative proportions of Fascin expression categories by staining pattern by group

No statistically significant difference was observed between the relative proportions of Fascin expression categories in the three groups (z-test, p≥0.05).

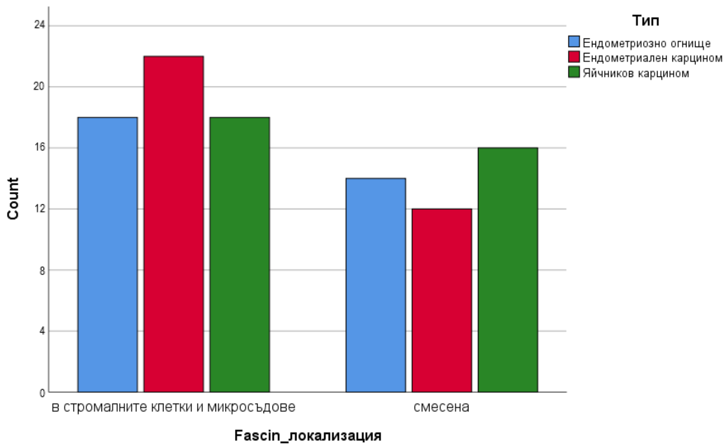
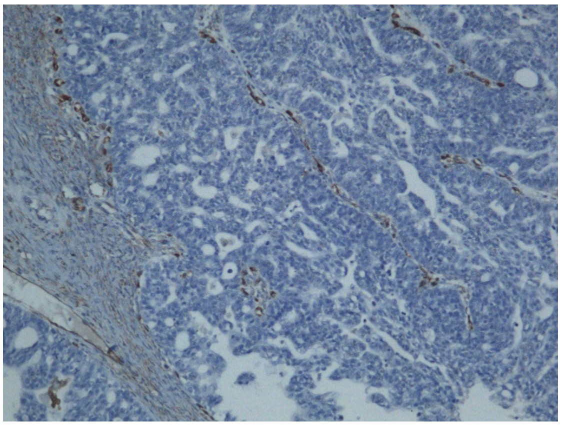
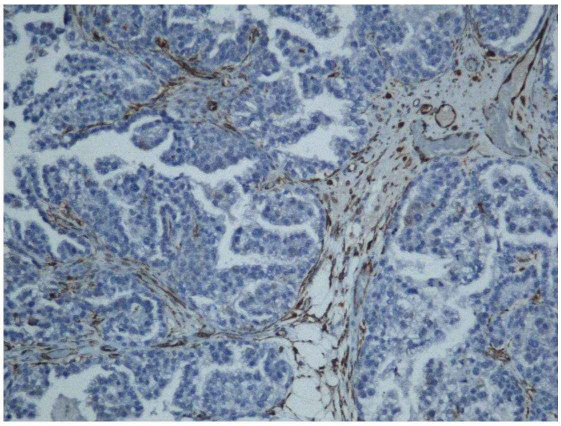


Figure 28. Relative shares of Fascin expression categories by localization by groups

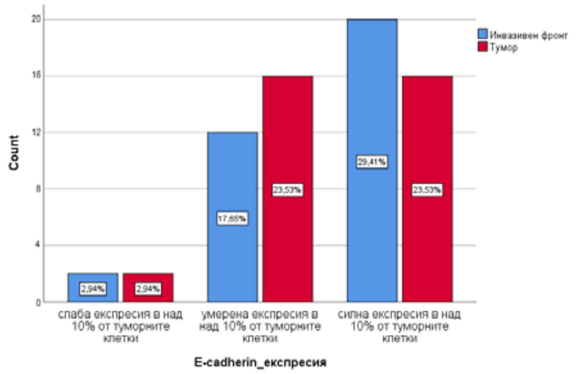



Picture 21. Fascin, strong cytoplasmic expression in endometrioid carcinoma, IHC, x100
Picture 22. Fascin, moderate cytoplasmic expression in ovarian carcinoma, IHC, x100

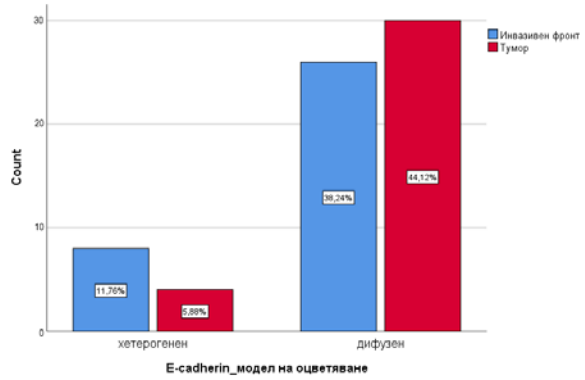
Task 4 results

The expression of anti HIF1alpha, Ki 67, E-cadherin, β-cathenin at the invasive front and around necrotic foci in ovarian and endometrial carcinomas was comparatively studied. The statistical analysis performed demonstrated the following differences:

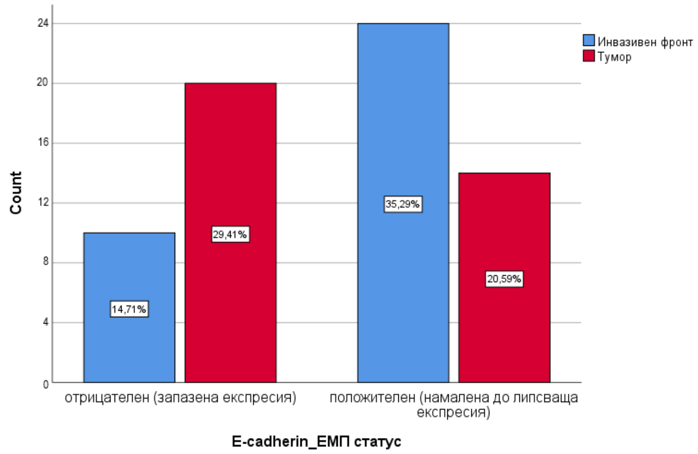
Strong expression of E-cadherin was found in endometrial carcinomas (23.5%) and in their invasive front (29.4%). A more frequent diffuse pattern of staining was found - for endometrial carcinomas (44.1%) and in their invasive front (38.2%). The distribution regarding positive EMT status is as follows - for endometrial carcinomas (20.6%) and for their invasive front (35.3%).



A

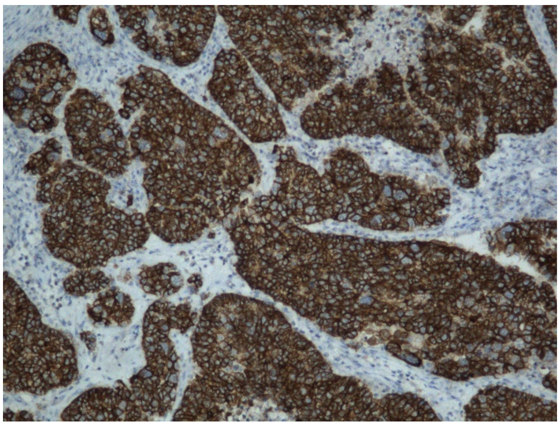


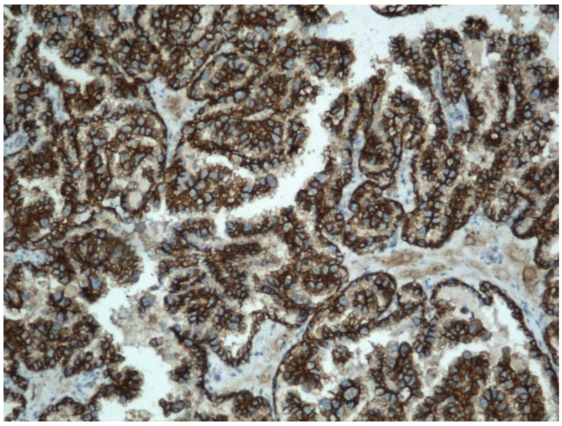
B



C

Figure 29. (A, B, C) E-Cadherin expression, staining pattern and EMF status comparison in endometrial carcinomas and their invasive front

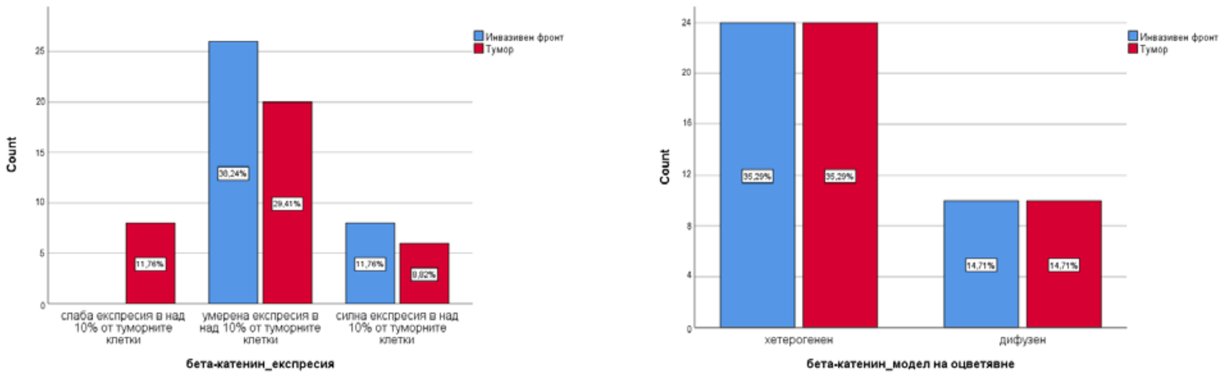




Picture 23. E-Cadherin, strong membranous expression in endometrioid carcinoma, IHC, x100

Picture 24. E-Cadherin, moderate membranous expression in ovarian carcinoma, IHC, x100

More frequent moderate expression was found in more than 10 percent of tumor cells - for endometrial carcinomas (29.4%) and in their invasive front (38.2%). The distribution in the two groups showed a more frequent heterogeneous pattern of staining and was as follows - for endometrial carcinomas (35.3%) and for their invasive front (35.3%).

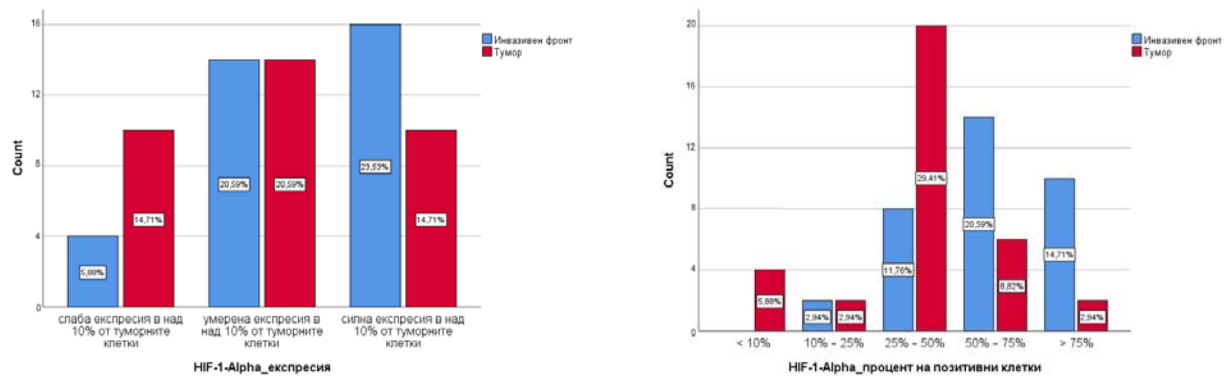


A B

Figure 30. (A, B) Relative proportions of β-catenin expression categories by intensity and staining pattern in endometrial carcinomas and their invasive front

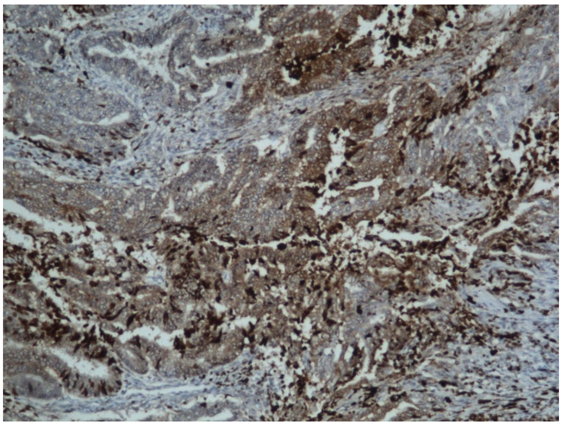
In endometrial carcinomas, moderate (20.6%) and strong (14.7%) anti HIF-1-alpha expression prevailed in more than 10 percent of cells, and in their invasive front, moderate (20.6%) and strong (23.5%) anti HIF-1-alpha expression also prevailed. In endometrial carcinomas, the largest share is with positive cells between 25 and 50 percent (29.4%), and in their invasive fronts, the largest share is occupied by those between 50 and 75 percent positive cells (20.6%).

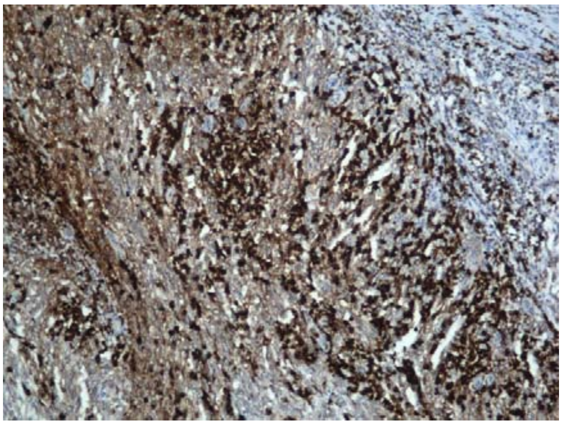
In both groups, the heterogeneous pattern of staining prevails - in endometrial carcinomas (26.5%) and in the invasive front (26.5%)



A B

Figure 31. (A, B) Relative shares of anti HIF-1-alpha expression, percentage of positive cells and staining pattern in endometrial carcinomas and their invasive front





Picture 25. HIF-1-alpha, strong nuclear expression in G2 endometrioid carcinoma, IXX, x100

Picture 26. HIF-1-alpha, moderate nuclear expression in G3 endometrioid carcinoma, IXX, x100

Heterogeneous expression pattern of HIF-1α in endometrial carcinoma type 1 in the nucleus and cytoplasm of tumor cells. We observed increased expression in the invasive front of the tumor.

A statistically significant difference in the relative proportions of Ki-67 expression in 50 to 100 percent was found in the invasive front of the tumor (23.5%) compared to the endometrial carcinoma (5.9%) (z-test, $p<0.05$).

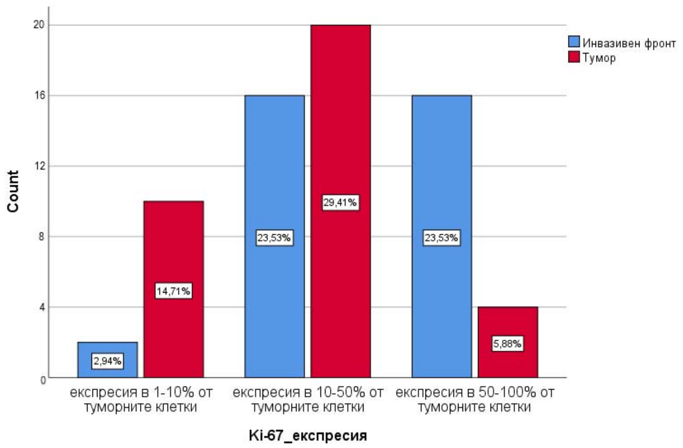


Figure 32. Ki-67 expression in patients with endometrial carcinomas and their invasive front

A statistically significant difference in the relative proportions of Fascin expression strength was found in weak expression, respectively, at the invasive front of the tumor (11.8%) compared to endometrial carcinoma (32.4%) (z-test, $p<0.05$). Also, a statistically significant difference in the relative proportions of Fascin expression strength was also found in moderate expression intensity at the invasive front of the tumor (38.2%) compared to endometrial carcinoma (17.6%) (z-test, $p<0.05$).

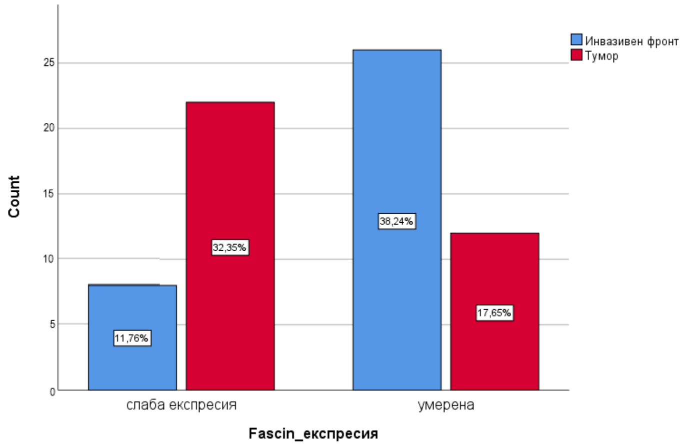


Figure 33. Relative proportions of Fascin expression categories by staining intensity in endometrial carcinomas and in its invasive front

No statistically significant differences were found for the rest of the investigated markers (z-test, p≥0.05).

Strong E-cadherin expression was found in ovarian carcinomas (23.5%) and in their invasive front (11.8%). The moderate expression was as follows - in ovarian carcinomas (23.5%) and in their invasive front (26.5%).

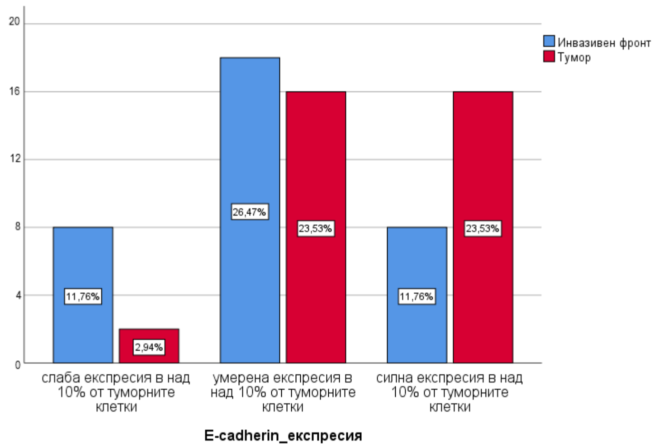


Figure 34. E-Cadherin expression in ovarian carcinomas and their invasive front

A statistically significant difference in the relative proportions of β-cathenin expression strength was found in the weak expression, respectively, at the invasive front of the tumor (2.9%) compared to the ovarian carcinoma (20.6%) (z-test, p<0.05). Also, a statistically significant difference in the relative proportions of β-catenin expression strength was also found in moderate expression intensity at the invasive front of the tumor (44.1%) compared to ovarian carcinoma (26.5%) (z-test, p<0.05).

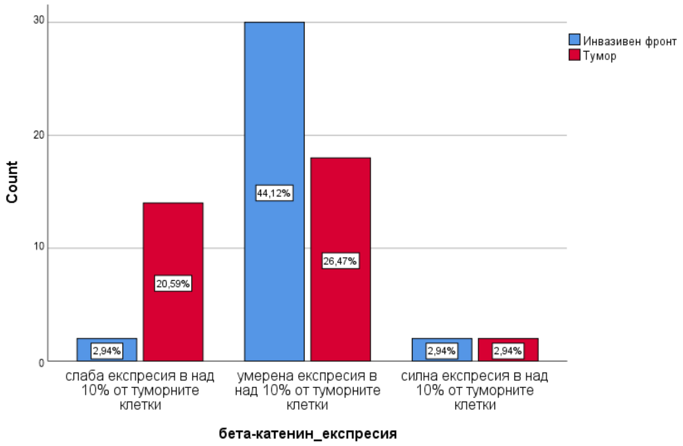
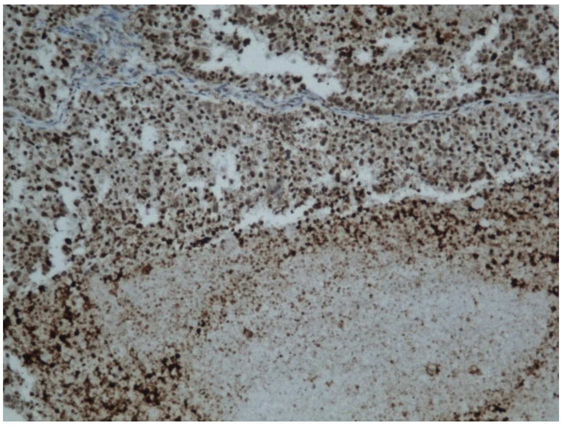
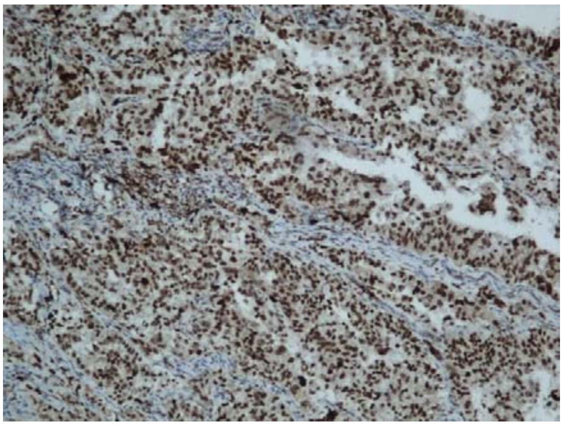


Figure 35. Relative proportions of β-catenin expression categories in ovarian carcinomas and their invasive front

The expression of HIF-1α in type 1 endometrial carcinoma with different grading (expression strength increases with increasing tumor grading).

Picture 27. HIF-1-alpha, presence of perinecrotic pattern of expression, moderate nuclear expression in endometrioid carcinoma G3, IHC, x100

Picture 28. HIF-1-alpha, strong diffuse nuclear expression in endometrioid carcinoma G2, IHC, x100

In ovarian carcinomas, moderate (20.6%) and strong (20.6%) anti HIF-1-alpha expression prevailed in more than 10 percent of cells, and in their invasive front, moderate (17.6%) and strong (29.4%) anti HIF-1-alpha expression also prevailed. In ovarian carcinomas, the largest share is with positive cells between 50 and 75 percent (23.5%), and in their invasive fronts, the largest share is occupied by those between 50 and 75 percent positive cells (26.5%).

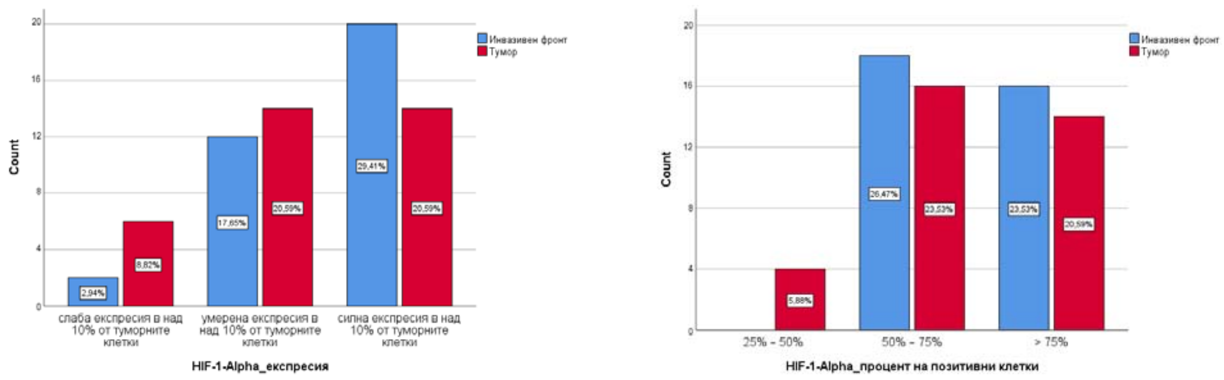


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Figure 36. (A, B) Relative proportions of anti HIF-1-alpha expression in ovarian carcinomas and their invasive front

Table 3 presents a distribution of the relative proportions of patients with presence or absence of tumor necrosis and positive epithelial-mesenchymal transition (EMT status). Patients with positive EMT status and absence of tumor necrosis predominate (47.1%).

Table 3. Distribution of the relative shares of patients with positive EMF status and tumor necrosis

			Tumor necrosis		**Total**
			no	**yes**	
E-cadherin EMT status	negative (preserved expression)	% in tumor necrosis	0.0%	44.4%	23.5%
	positive (reduced to absent expression)	% in tumor necrosis	100%	55.6%	76.5%
Total		% in tumor necrosis	100%	100%	100%
		% Total	47.1%	52.9%	100%

The relative share of percentage negative for EMT status in the presence of tumor necrosis was studied (44.4%), no cases were observed in the group of patients with absent tumor necrosis (0%).

Statistically significantly higher is the relative share of percentage positive for EMT status in the absence of tumor necrosis (47.1%) compared to patients with the presence of tumor necrosis (29.4%) (z-test, $p<0.05$).

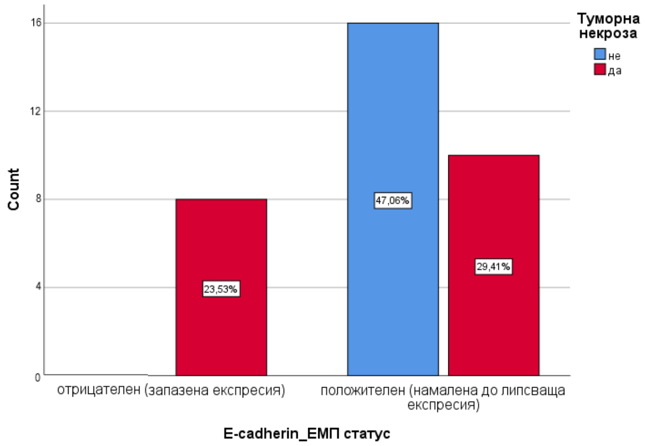


Figure 37. E-Cadherin EMP status (transition) - comparison according to the presence of tumor necrosis

No statistically significant differences were found for the rest of the investigated markers (z-test, p≥0.05).

Task 5 results

The groups with endometrial and ovarian carcinoma combined or not with endometriosis were compared and a relationship was sought between endometriosis and the development of carcinomas based on the expression of the studied IHC-markers.

In 1925, Sampson proposed 3 criteria for the diagnosis of malignancy occurring in endometriosis:

- demonstration of both benign and neoplastic endometrial tissues in the tumor,
- histology compatible with endometrial origin
- no other primary tumors were detected in the uterine body and/or the ovary.

Later, Scott introduced a fourth criterion: metaplasia between endometriosis and carcinoma.

Due to the small number of women with combined carcinoma and endometriosis in our study and the inability to collect data on the expression of relevant markers at diagnosis with prior endometriosis, it was not possible to make comparisons between groups. Descriptive statistics of the expression distribution at the invasive front and in the tumor in carcinoma patients in whom endometriosis foci were also found are presented. We followed up 12 patients with endometrial carcinoma and endometriosis and 10 patients with ovarian carcinoma and endometriosis, comparing the results of immunohistochemical examination.

Table 4. HIF-1-Alpha Expression - Invasive Front

		Number	%
Endometrial cancer patients	weak expression in more than 10% of the tumor cells	2	16.7
	moderate expression in more than 10% of the tumor cells	4	33.3
	strong expression in more than 10% of the tumor cells	6	50.0
	Total	**12**	**100.0**
Ovarian cancer patients	moderate expression in more than 10% of the tumor cells	4	40.0
	strong expression in more than 10% of the tumor cells	6	60.0
	Total	**10**	**100.0**

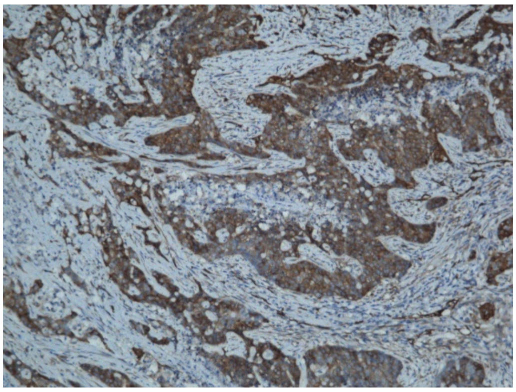
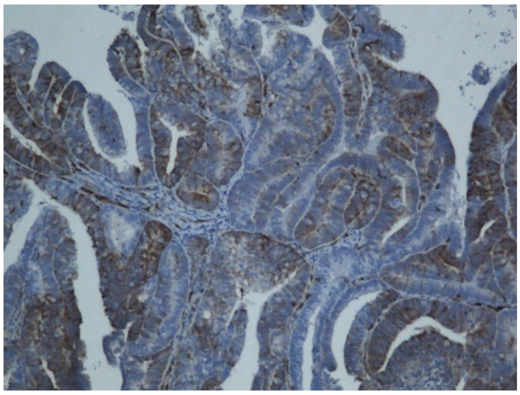
Table 5. HIF-1-Alpha Expression - Tumor

		Number	%
Endometrial cancer patients	weak expression in more than 10% of the tumor cells	4	33.3
	moderate expression in more than 10% of the tumor cells	4	33.3
	strong expression in more than 10% of the tumor cells	4	33.3
	Total	**12**	**100.0**
Ovarian cancer patients	weak expression in more than 10% of the tumor cells	2	20.0
	moderate expression in more than 10% of the tumor cells	6	60.0
	strong expression in more than 10% of the tumor cells	2	20.0
	Total	**10**	**100.0**

Moderate and strong HIF-1-Alpha expression prevailed in both groups of carcinomas (endometrial and ovarian).

Table 6. Fascin_expression - invasive front

		Number	%
Endometrial cancer patients	weak expression	2	16.7
	moderate expression	10	83.3
	Total	**12**	**100.0**
Ovarian cancer patients	weak expression	4	40.0
	moderate expression	6	60.0
	Total	**10**	**100.0**

Picture 29. Fascin, strong diffuse cytoplasmic expression in endometrioid carcinoma G3, IHC, x100
Picture 30. Fascin, moderate cytoplasmic expression, heterogeneous expression pattern in endometrioid carcinoma G1, IHC, x100

Table 7. Fascin_expression - tumor

		Number	%
Endometrial cancer patients	weak expression	6	50.0
	moderate expression	6	50.0
	Total	**12**	**100.0**
Ovarian cancer patients	weak expression	4	40.0
	moderate expression	6	60.0
	Total	**10**	**100.0**

A moderate expression of Fascin prevailed in both groups of carcinomas (endometrial and ovarian).

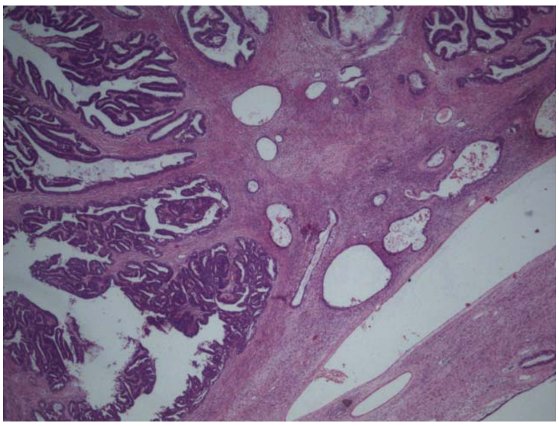
Table 8. Ki-67 Expression - Invasive Front

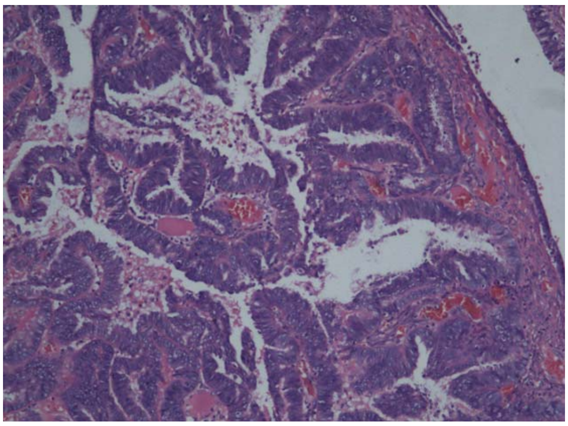
		Number	%
Endometrial cancer patients	expression in 1–10% of the tumor cells	2	16.7
	expression in 10–50% of the tumor cells	8	66.7
	expression in 50–100% of the tumor cells	2	16.7
	Total	**12**	**100.0**
Ovarian cancer patients	expression in 1–10% of the tumor cells	2	20.0
	expression in 10–50% of the tumor cells	4	40.0
	expression in 50–100% of the tumor cells	4	40.0
	Total	**10**	**100.0**

Table 9. Ki-67 Expression - Tumor

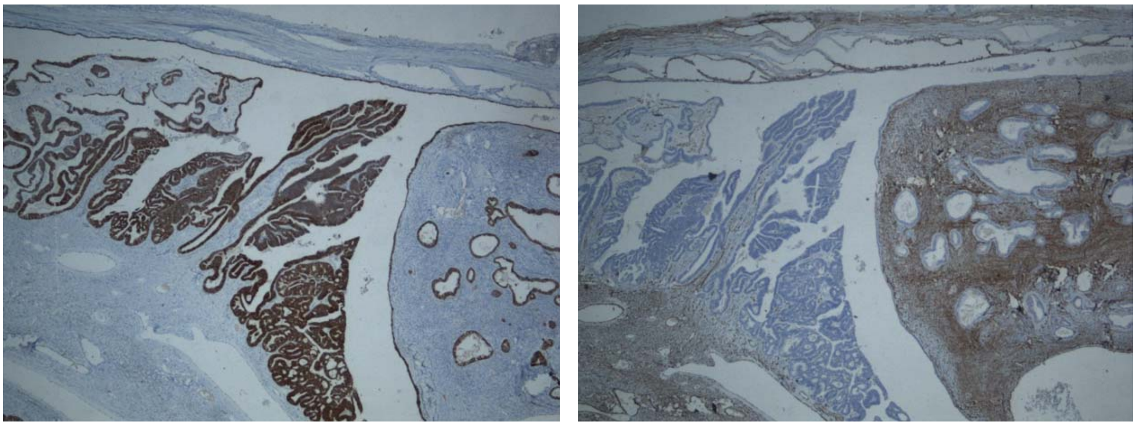
		Number	%
Endometrial cancer patients	expression in 1–10% of the tumor cells	6	50.0
	expression in 10–50% of the tumor cells	6	50.0
	Total	**12**	**100.0**
Ovarian cancer patients	expression in 1–10% of the tumor cells	2	20.0
	expression in 10–50% of the tumor cells	4	40.0
	expression in 50–100% of the tumor cells	4	40.0
	Total	**10**	**100.0**

Predominant expression in 50-100% of tumor cells of Ki-67 in both groups of carcinomas (endometrial and ovarian).



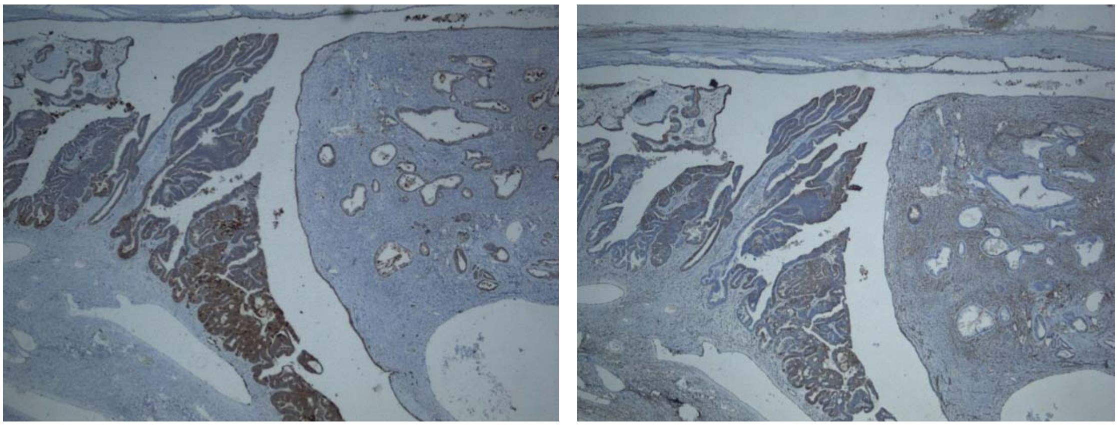


Pictures 31 and 32. Case of endometrial carcinoma arising in a focus of deep pelvic endometriosis.



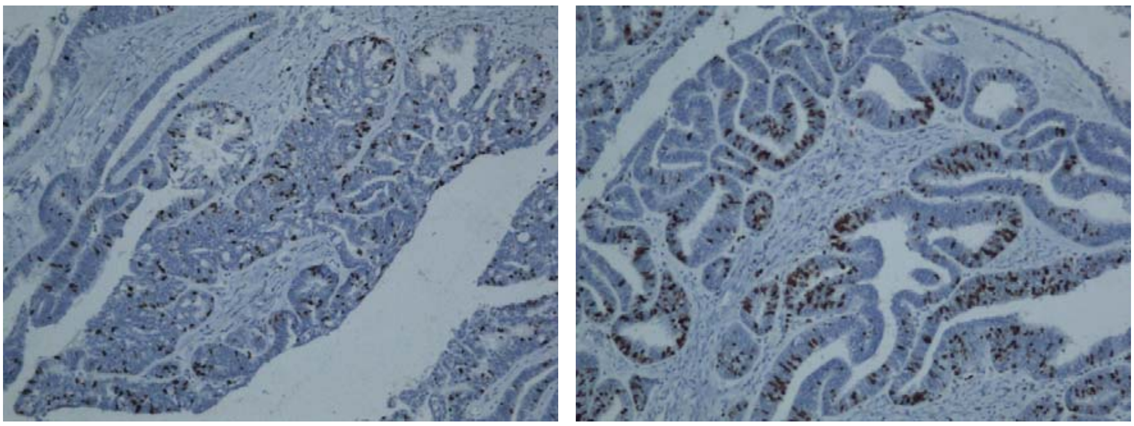
Picture 33. Endometrial adenocarcinoma foci and endometriosis foci are presented in parallel IHC-expression of E-Cadherin, X100

Picture 34. Endometrial adenocarcinoma foci and endometriosis foci are presented in parallel IHC-expression of SMA, X100



Picture 35. Endometrial adenocarcinoma foci and endometriosis foci are presented in parallel. IHC-expression of HIF-1 Alpha, X100.

Picture 36. Endometrial adenocarcinoma foci and endometriosis foci are presented in parallel. IHC-expression of Fascin, X100.



Picture 37. IHC expression of Ki 67, strong nuclear expression in endometrioid carcinoma of the ovary, X100

Picture 38. IHC expression of Ki 67, strong nuclear expression in endometrioid carcinoma of the endometrium, X100

Task 6 results

The immunohistochemical expression of the indicated markers was compared in relation to the distribution of tumors by FIGO stage, depth of invasion and histological subtype and an IHC panel was derived to assess the risk of progression in the three groups.

Statistically significantly higher was the relative proportion of weak expression of anti HIF-1-alpha in more than 10 percent of cells with invasion up to ½ of myometrial thickness (17.6%) compared to invasion of more than ½ of myometrial thickness (11.8%) (z-test, $p<0.05$).

In patients with invasion up to ½ of the myometrial thickness, weak (17.6%) and moderate (5.9%) anti HIF-1-alpha expression prevailed in more than 10 percent of cells, as in those with invasion over ½ of the myometrial thickness, moderate 35.3%) and strong (29.4%) anti HIF-1-alpha expression prevailed.

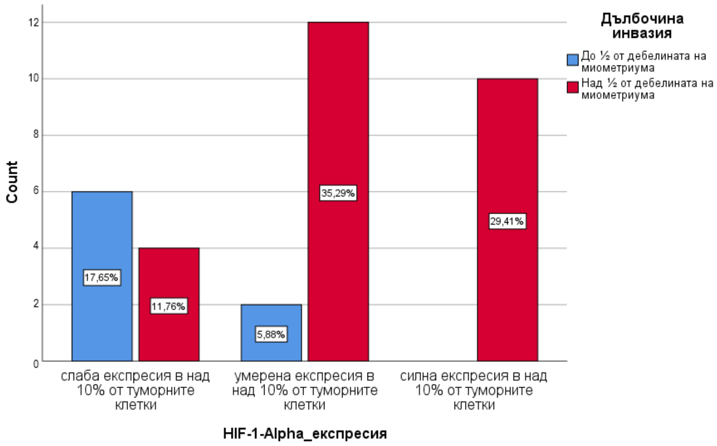


Figure 38. Relative proportions of anti HIF-1-alpha expression in endometrial carcinomas according to the depth of myometrial invasion

No statistically significant differences were found for the rest of the investigated markers (z-test, $p\geq0.05$).

Due to the small sample size and the large number of categories for each variable - grading of the first type of endometrial carcinoma and the expressions of the corresponding markers, it is not possible to make comparisons between the groups.

Statistically significantly higher is the relative share of moderate expression of β-catenin in more than 10 percent of cells in low-grade and high-grade serous tumors and mucinous tumors (47.1%) compared to endometrioid, clear cell and MMMT (5.9%) (z-test, $p<0.05$).

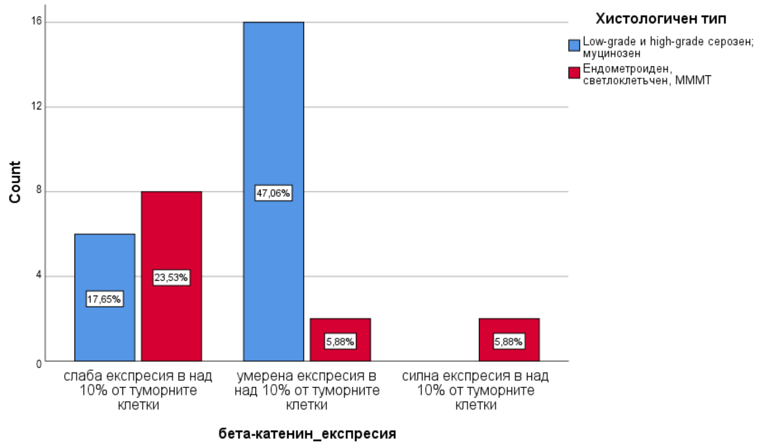


Figure 39. Relative shares of β-catenin expression by histological type of ovarian carcinoma

No statistically significant differences were found for the rest of the investigated markers (z-test, p≥0.05).

Based on the results of our IHC-study, we have attempted to compile **Prognostic Immunohistochemical Panels for the assessment of the risk of malignancy** in endometriosis and the risk of progression in endometrial carcinoma and ovarian carcinoma in the following three tables:

Table 10. Prognostic immunohistochemical panel for assessing the risk of progression in endometriosis

Marker	Low risk of malignancy	Intermediate risk of malignancy	High risk of malignancy
HIF-1α	<10%, weak expression; absence of clear perinecrotic zone	10-50%, moderate expression; small hypoxic foci are possible	>50%, strong and diffuse expression with clearly defined hypoxic areas
Ki-67	<5% positive cells	5-10% positive cells	>10% positive cells with foci of cellular atypia
E-cadherin	Strong membranous expression	Partially reduced membrane expression	Significantly reduced or absent membrane expression (a hallmark of EMT)
β- catenin	Membrane expression, without nuclear positivity	Weak focal nuclear positivity	Aberrant, significant nuclear positivity
α-SMA (stroma)	Absent or weak fibrous reaction	Moderate focal stromal fibrosis	Marked desmoplastic stromal reaction with diffuse expression
Fascin	Lack of expression	Focal weak expression	Diffuse or strong expression with invasive phenotype

Table 11. Prognostic immunohistochemical panel to assess the risk of progression in endometrial carcinoma

Marker	Low risk (FIGO IA, G1)	Average risk (FIGO IB-II, G2)	High Risk (FIGO III-IV, G3, Non-endometrioid)
HIF-1α	<10%, weak expression	10–50%, moderate expression	>50%, strong expression and the presence of a hypoxic zone
Ki-67	<10% positive cells	10-40% positive cells	>40%, strong expression
E-cadherin	Retained membrane expression	Partially reduced expression	Significantly reduced or absent expression
β- catenin	Membrane expression, weak nuclear	Moderate nuclear positivity	Diffuse nuclear expression (active Wnt pathway)
α-SMA (stroma)	Slight or focal fibrosis	Moderate desmoplastic reaction	Severe reactive fibrosis
Fascin	Absent or very weak expression	Focal moderate expression	Diffuse and strong expression with invasive pattern

Table 12. Prognostic immunohistochemical panel for assessing the risk of progression in ovarian carcinoma

Marker	Low Risk (FIGO I, Low Grade)	Medium risk (FIGO II-III, G2)	High risk (FIGO III-IV, high-grade serous/clear cell)
HIF-1α	<25%, low expression	25-50%, moderate expression	>50%, strong expression with hypoxic areas
Ki-67	<15%, low proliferative index	15-40% moderate index	>40%, high proliferative index
E-cadherin	Retained membrane expression	Partially reduced	Markedly reduced or absent expression
β-catenin	Membrane expression	Moderate nuclear positivity	Pronounced nuclear expression (Wnt activation)
α-SMA (stroma)	Weak or absent reaction	Moderate desmoplastic reaction	Marked stromal reaction and invasion
Fascin	Weak or focal expression	Moderate focal expression	Diffuse strong expression - marker for invasiveness

V. DISCUSSION

According to Task 1

The high frequency of endometriosis and its role as a potential precursor of endometrial and ovarian carcinomas (Sampson, 1927; Nezhat et al., 2014) necessitate a detailed study of the age distribution of these diseases. Endometriosis is a chronic inflammatory condition that affects 10–15% of women of reproductive age and has been found to increase the risk of developing carcinomas, particularly of the ovaries (Guo, 2009). According to our data, the average age of patients with endometriosis was 48.65 ± 14.87 years, while that of patients with endometrial carcinoma was 66.45 ± 10.67 years, and for ovarian carcinoma - 67.21 ± 8.94 years. These results are consistent with data in the literature showing that endometriosis predominantly affects women of reproductive age, while endometrial and ovarian carcinomas are diagnosed more frequently in the postmenopausal period (Zaino et al., 2011; Pearce et al., 2012).

Looking at the age distribution in our study, we found that patients with endometrial carcinoma most often fell into the age range of 61–70 years, which is consistent with the observations of Morice et al. (2019). This result reflects the typical profile of patients with this type of neoplasia, which is associated with hormonal and genetic factors, such as mutations in PTEN, PIK3CA and β-catenin (β-catenin) pathway (McCluggage, 2020). It is important to emphasize that patients with ovarian carcinoma also fall into a similar age range, with the most common subtype being high-grade serous carcinoma (41.18%). These data are consistent with the studies of Kurman & Shih (2016), which found that this aggressive subtype is the leading one among ovarian carcinomas and is usually diagnosed at advanced stages.

Furthermore, FIGO stage analysis in our study showed that in endometrial carcinoma, the most common stage was IB (47.1%), while in ovarian carcinomas, IA (29.4%) and IC (35.3%) predominated. This is in line with clinical observations that endometrial carcinoma is often diagnosed at an earlier stage due to symptoms such as abnormal uterine bleeding, while ovarian carcinoma remains asymptomatic until more advanced stages (McCluggage, 2020).

Additionally, analyzing the histological subtype of the carcinomas, we found that the endometrioid variant predominated in the endometrial carcinoma (82.4%), while the high-grade serous subtype was the most common in the ovarian carcinomas (41.18%). This is consistent with literature data where serous carcinomas account for over 60% of all ovarian cancer cases (Bodnar et al., 2014). It is important to note that in part of the patients with ovarian carcinoma concomitant endometriosis was also found, which supports the theory of possible malignant transformation of endometriotic lesions in some cases (Pearce et al., 2012). The analysis of our results confirms that the age distribution of patients with endometriosis, endometrial and ovarian carcinoma corresponds to the literature data and emphasizes the need for earlier follow-up of patients with a long history of endometriosis. Improving diagnostic strategies and recognizing risk factors may help early detection of malignant transformations, especially in patients with atypical endometriosis (Pavone et al., 2018).

According to Task 2

Data from the conducted study show important differences in the expression of HIF-1α, Ki-67, E-cadherin and β-catenin in endometriosis in different age groups, as well as their significance for assessing the risk of malignant progression. Endometriosis is recognized as a chronic, systemic inflammatory disease of women of reproductive age with a prevalence of approximately 10% (Allaire et al., 2023). Although benign in nature, this heterogeneous pathology shares some characteristics with neoplastic processes - infiltrative growth, recurrent course, and even the potential for malignant transformation. Malignant degeneration has been found to occur in approximately 1-2% of endometriosis cases, with the risk increasing with age (Hablase et al., 2024). In our study, no significant difference was found in the frequency of HIF-1α expression between the two age groups, but a trend towards higher expression in postmenopausal patients was striking. They more often had moderate to strong expression of HIF-1α in more than 10% of cells, and in this group alone there were cases with more than 75% positive cells (12.5% of menopausal cases) - something that was not encountered in younger women. Furthermore, the diffuse staining pattern for HIF-1α was more characteristic of postmenopausal endometriotic foci (in 44% of them vs. 19% in reproductive age). These observations point to more pronounced hypoxia or hypoxic adaptation in the lesions of older patients. Endometriotic implants are known to often exist in a hypoxic microenvironment, especially in deeper infiltrating foci and ovarian endometriomas. Periodic engorgement and hemorrhages in ectopic endometrial tissue (eg, in “chocolate” cysts) lead to accumulation of iron ions and local anoxia, which stimulates the expression of the hypoxia-inducible factor HIF-1α. HIF-1α in turn triggers a cascade of genes related to proangiogenesis (eg, vascular endothelial growth factor, VEGF), local estrogen production (via increased aromatase expression), enhanced inflammatory response, and metabolic adaptation of cells. As a result, endometriosis foci manage to survive and progress even outside the normal immune and hormonal environment of the uterus. In the literature, HIF-1α is increasingly established as a key factor in the pathogenesis of endometriosis and a possible target for therapy (Dai et al., 2024), analogous to its role in multiple tumors. It is important to note that hypoxia not only supports the survival of ectopic endometrial cells, but can also drive their phenotypic transition to a more invasive one. One of the key consequences of chronic hypoxic stress is the initiation of epithelial–mesenchymal transition (EMT) in endometriotic cells (Liu et al., 2018). Such a mechanism facilitates the invasion of the endometriotic glands into the surrounding tissues and the progression of the disease (Martire et al., 2024). It is these long-term hypoxic-remodeled endometriotic tissues that are considered to be the foci at the highest risk of malignant transformation, an observation that is consistent with the clinical fact that endometriosis-associated carcinomas are predominantly found in women around and after menopause (Hablase et al., 2024). The cell proliferation marker Ki-67 is a well-established prognostic marker in a number of tumors including endometrial carcinoma (Kitson et al., 2017). Our results showed a low proliferative activity in the majority of examined cases, especially in menopausal patients - in 56.3% of them Ki-67 was positive in only 1-10% of cells, while this proportion in younger women was 31.3%. Although the proportion of cases with more than 10% Ki-67-positive cells was not significantly different between the two groups, it is striking that in the postmenopausal group no case showed high proliferation (>50% Ki-67 positive cells). This corresponds to clinical expectations - after cessation of cyclic hormonal stimulation, endometriosis foci usually atrophy or become inactive (Vallée et al., 2024). In the absence of estrogens, the epithelial gland cells remain quiescent, which explains the minimal Ki-67 expression in our menopausal group. In contrast, in the reproductively active patients in our study, even ectopic endometrial tissue is periodically stimulated by estrogen, which may manifest with focal proliferation. In our study, 6.3% of the younger women had Ki-67 expression in a moderate degree of cell proliferation (10–50%), whereas none of the menopausal cases were above 10%. These differences are consistent with the notion that postmenopausal endometriosis is "less active and more limited" (Benagiano and Guo, 2022). From a clinical point of view, however, it is precisely the rare cases with higher proliferative activity in endometriotic foci that are of interest, as they may signal more aggressive behavior or precancerous changes. In terms of localization, the results of the conducted research reveal significant differences in the expression of the investigated markers between adenomyosis and extrauterine endometriosis with different localizations. Adenomyosis is not simply "endometriosis of the uterus" but is characterized by a distinctive molecular profile (Guo S-W., 2020). The observed differences in the degree of hypoxia, proliferative index, adhesion properties of glandular cells and stromal reorganization support the notion that the individual types of endometriotic lesions (peritoneal, ovarian, deep infiltrating) are heterogeneous in their biology (Fukunaga, 2000) and are distinct from adenomyosis.

Immunohistochemical expression of hypoxia-inducible factor 1α (HIF-1α) was strongly positive in the majority of the studied cases, with no significant differences between individual subgroups. In adenomyotic foci, as in all variants of ectopic endometriosis, diffuse expression of HIF-1α in glandular and stromal cells was observed. This reflects the condition of chronic tissue hypoxia characteristic of ectopically implanted endometrial tissue. Our data are consistent with the literature, according to which endometriotic lesions demonstrate a predominantly diffuse distribution of HIF-1α, in contrast to malignant tumors, where hypoxia and HIF-1α are mainly confined to areas of perinecrotic necrosis. Analysis of the Ki-67 marker showed significantly higher proliferative activity in the glandular epithelium of adenomyosis compared to ectopic endometriosis implants. In adenomyotic lesions, dividing cells (with Ki-67-positive nuclei) are in a higher percentage. We observed a high proliferative index in this group, statistically significantly exceeding that of extrauterine endometriosis (Istrate-Ofiţeru et al., 2022). In contrast, superficial peritoneal and ovarian endometriosis foci showed lower Ki-67, indicating poorer cell proliferation. It is possible that the intrauterine location and better blood supply of adenomyosis foci support higher cell proliferation. Some studies indicate that even eutopic endometrium in patients with endometriosis shows increased mitotic activity compared to controls, which supports the hypothesis of congenital or early acquired properties of the endometrium predisposing to the development of ectopic lesions. In adenomyotic glands, membrane expression of E-cadherin is relatively well preserved (diffusely positive in most glandular cells), whereas in ovarian and especially in peritoneal endometriosis, weaker and often heterogeneous E-cadherin immunoreactivity is observed. Our data indicate that the glandular epithelium in ectopic lesions more often undergoes a loss of E-cadherin, which is a marker of the onset of epithelial-mesenchymal transformation and the acquisition of a more invasive phenotype. The literature supports such an observation - a moderate decrease in E-cadherin (as well as related catenins) was found in the epithelium of patients with endometriosis, with the decrease being most pronounced in the endometriosis foci themselves (Yoo et al., 2020). Stromal reaction and invasiveness (SMA and Fascin). One of the distinguishing findings when comparing adenomyosis to extrauterine endometriosis is the nature of the stromal component. In adenomyosis foci, the endometrial stroma is located among the myometrium and is in direct contact with the smooth muscle cells of the uterus. In classic deeply infiltrating endometriosis (eg, retrocervical) there is also extensive fibrosis and infiltration of smooth muscle fibers into the lesion, which is why these lesions are often referred to as "adenomyomatous" nodules. Our results confirm the marked smooth muscle transformation of the stroma in extrauterine endometriosis: in almost all studied cases of pelvic (peritoneal and deep) and ovarian endometriosis, stromal cells diffusely express α-smooth muscle actin (α-SMA), accompanied by pronounced collagen fibrosis. In contrast, in superficial peritoneal implants without a massive fibrotic

reaction, SMA expression may be focal or weak. Regardless of the origin, the presence of α-SMA positive myofibroblasts is a marker of remodeling of the microenvironment - the stroma thickens and shrinks the lesion, but at the same time can support invasion through its contractile properties. In adenomyosis, the situation is similar in appearance, although different in origin: there, the endometrial stroma enters between the smooth muscle bundles of the myometrium.
In normal endometrium, Fascin-1 is practically not expressed, but in malignant endometrial formations, an increased expression was found, associated with a more aggressive behavior of the tumor (Kabukcuoglu et al., 2006). Data from our study indicate a trend toward stronger fascin expression in more invasive lesions. In adenomyotic foci and superficial peritoneal implants, fascin expression was low and focal. These results are consistent with the biological behavior of the lesions - deeply infiltrating endometriosis has the highest invasiveness, while adenomyosis grows more restricted within the confines of the uterine wall.

According to Task 3

In the series we studied, we confirmed certain parallels between endometriotic lesions and carcinomas, with hypoxia being the connecting link. HIF-1α (Hypoxia-Inducible Factor 1-alpha) was diffusely expressed in the majority of endometriotic foci, while in carcinomas its expression was heterogeneous with areas of strong positivity. This reflects the state of the microenvironment: endometriotic implants survive chronic hypoxia and HIF-1α promotes their survival and invasion (Liu et al., 2017). It is known that in the ectopic endometrium of patients with ovarian endometriosis, HIF-1α is overexpressed and stimulates stromal cell migration by inducing autophagy (Liu et al., 2017). This contributes to the "invasive" behavior of endometriosis, despite its benign nature. At the same time, carcinoma cells often experience acute oxygen deprivation during tumor growth, resulting in mosaic HIF-1α expression that is strongly positive in hypoxic areas (usually perinecrotic) and weaker in better vascularized parts of the tumor (Al-Sharaky et al., 2016). Our results reflect exactly this picture: in endometrial carcinomas, the relative proportion of cases with intermediate HIF-1α positivity (25–50% of tumor cells) was significantly higher, while ovarian carcinomas more often showed extensive HIF-1α expression (>75% positive cells). This pattern correlates with the more aggressive phenotype of ovarian carcinoma and the more marked hypoxia in these tumors. In support of this, meta-analyses have shown that HIF-1α overexpression is associated with advanced stage and worse prognosis in endometrial carcinoma (Zhu et al., 2020), as well as shorter survival in ovarian carcinoma (Rahman et al., 2025). In our study, high HIF-1α levels statistically correlated with deeper myometrial invasion—endometrial carcinomas infiltrating >1/2 of the myometrial thickness were dominated by strong

HIF-1α expression, while limited invasion was dominated by weak expression. This is consistent with the literature where high HIF-1α expression statistically correlates with higher histological grading, deep myometrial invasion, and presence of lymphovascular invasion (Abouhashem et al., 2016). HIF-1α stimulates tumor progression by activating angiogenesis, metabolic adaptation, and even epithelial-mesenchymal transition (EMT) (Rahman et al., 2025). Indeed, in our study, we observed an association between HIF-1α and EMF: all cases with marked loss of E-cadherin (positive EMF status) showed high HIF-1α expression. These data support the hypothesis that tumor hypoxia (via HIF-1α) can induce EMT-changes and thus promote tumor cell invasiveness (Abouhashem et al., 2016). It has been described in the literature that HIF-1α activates EMT-mechanisms through regulators such as Snail, leading to suppression of E-cadherin (Abouhashem et al., 2016). Our results accordingly show a significantly more frequent "positive EMT" (reduced E-cadherin expression) in tumors with high HIF-1α. They even found a tendency for these tumors to less often develop extensive necrosis. We found a statistically significantly lower proportion of EMT-positive tumors among carcinomas with the presence of necrosis compared to those without necrosis. It is possible that tumors that survive without extensive necrosis achieve invasion via EMT-pathways, while in bulky, rapidly growing tumors, necrosis is a sign of hypoxia, but they may still retain some epithelial junctions. Further studies are needed to elucidate this inverse relationship between tumor necrosis and EMT-status.

Cell proliferation expectedly differed between benign and malignant lesions. Ki-67 - a marker of proliferative activity - showed low values in endometriosis (in 87.5% of cases <10% Ki-67-positive nuclei). This corresponds to the clinical behavior of endometriotic foci, which are usually hormone-dependent and with slow cell growth, resembling normal endometrium in the secretory phase. Conversely, in carcinomas, the Ki-67 index was significantly higher: about 60% of endometrial carcinomas demonstrated moderate proliferation (10-50% positive cells), and 41.2% of ovarian carcinomas extremely high proliferation (>50% Ki-67-positive). These differences are statistically significant and highlight the importance of the proliferative index demonstrated by the Ki-67 marker as a hallmark of aggressive tumors. Higher proliferative activity is known to correlate with higher tumor grading and more advanced stage in endometrial carcinoma (Kitson et al., 2017), as well as with a worse prognosis (in some studies, Ki-67 ≥35% was associated with lower survival). Our data confirm this trend, we observed the highest Ki-67 index in serous ovarian carcinomas, which are clinically the most aggressive. Also of interest is the local increase in proliferation in the frontal zone of invasion in the carcinomas in our study. In endometrial carcinoma, the proportion of tumors with very high Ki-67 (over 50% positive) increased significantly in their invasive front (23.5% of cases) compared to the central tumor part (only 5.9%). This supports the concept that the peripheral tumor cells leading the invasion divide more actively, probably under the influence of the stimuli of the tumor environment (hypoxia, growth factors, etc.). Increased peripheral proliferation may facilitate tumor spread to neighboring tissues.

The expression of E-cadherin also showed significant differences between the studied groups, reflecting the extent of loss of cell adhesion during malignant transformation in the tumors. In the endometriotic foci, E-cadherin was stored diffusely on the membranes of glandular cells (diffuse positive pattern in 93.8% of cases). Diffuse membrane expression of E-cadherin prevailed in endometrial carcinomas (88.2% of cases), especially in highly differentiated tumors, although with some heterogeneous attenuation of staining in individual foci. However, ovarian carcinomas demonstrated significant heterogeneity: in as many as 52.9% of them, E-cadherin had a mosaic/focally absent expression pattern. Statistically, the proportion of cases with heterogeneous or absent E-cadherin expression was significantly higher in ovarian carcinoma versus endometriosis and endometrial carcinoma. This clearly correlates with the biology of these tumors - in particular, serous ovarian carcinoma, which is prone to discohesive spread in the abdominal cavity, often loses E-cadherin as a result of EMT (Bozhkova D, Poryazova E, 2019). In our study, a positive EMT-status (ie, reduced or absent E-cadherin expression) was found in 76.5% of ovarian carcinomas versus only 12.5% in endometriotic lesions. This confirms that epithelial-mesenchymal transition is characteristic of malignant tumors and is absent in benign endometrial cells.

Regarding the stromal components and cytoskeletal proteins investigated in our study by the markers α-SMA (smooth muscle actin) and Fascin, SMA was expressed in endometriosis in the majority of cases studied. Stromal smooth muscle metaplasia is often seen in endometriotic foci, and endometrial stromal cells can differentiate into myofibroblasts and even true smooth muscle cells, especially in long-standing and deeply infiltrating lesions (Fukunaga, 2000). In contrast to SMA, our endometriosis cases virtually did not express Fascin (in 0% of cases with significant expression). In endometrial carcinomas, however, we found Fascin positivity in tumor cells in a significant proportion of cases (about 74% overall, if we include all expression grades), although mostly of low to moderate intensity. Similarly, Kabukcuoglu et al. reported that 74% of endometrioid carcinomas were Fascin-positive, versus only 39% of benign endometrial biopsies (Kabukcuoglu et al., 2006). Our results showed a tendency for high-grade endometrial carcinomas to have stronger Fascin expression, which coincides with the significant upregulation of Fascin in G3 tumors described in the literature. In ovarian carcinomas, we observed a similar phenomenon: in their invasive fronts, the proportion of tumors with moderate Fascin expression was higher than in the center of the tumor. Although these differences did not reach statistical significance for Fascin between groups, the dynamics within the tumor highlight a role for Fascin in invasion. The clinical consequences of high Fascin expression have been the subject of increased interest in the literature, being associated with more aggressive behavior and poorer outcome.

According to Task 4

Differences in the tumor microenvironment between the invasive tumor front and the surrounding necrotic areas have important biological significance in ovarian and endometrial carcinomas. In our study, tumor cells adjacent to necrotic foci showed increased expression of HIF-1α (Hypoxia-Inducible Factor 1α). This is consistent with the known pattern of "perinecrotic" HIF-1α activation seen in a number of solid tumors (Proestling et al., 2015). Birner et al. described increased expression of HIF-1α in ovarian carcinomas, concentrated in tumor cells around areas of necrosis. Similarly, in endometrial carcinoma, high HIF-1α levels often coincide with the presence of tumor necrosis and aggressive tumor behavior. A study by Seeber and colleagues showed that perinecrotic expression of HIF-1α in endometrioid carcinomas of the endometrium is a significant adverse prognostic marker—the presence of tumor necrosis and associated HIF-1α activation correlates with shorter survival (Seeber et al., 2010). Stabilization of HIF-1α in hypoxic sites activates the transcription of a number of genes supporting tumor survival. This stimulates angiogenesis, metabolic adaptation and cell invasiveness. Our results of increased HIF-1α expression around necrotic foci confirm the role of the hypoxic microenvironment as a stimulus for tumor progression. These support data from the literature that overexpression of HIF-1α is a common phenomenon in solid tumors and is associated with worse prognosis (Braicu et al., 2014).

The association between hypoxia and epithelial-mesenchymal transition (EMT) in our study is interesting. In the perinecrotic areas, alongside increased HIF-1α, we observed EMT marks with decreased expression of E-cadherin in tumor cells and changes in cytoskeletal proteins. Hypoxic conditions can stimulate EMT in tumor cells. This is supported by literature that demonstrates that HIF-1α is directly involved in the suppression of E-cadherin through the regulation of EMT-transcription factors. For example, Cheng et al. found that in ovarian tumor cells, HIF-1α mediates EGF-induced Snail/Slug activation with subsequent loss of E-cadherin leading to increased invasion and metastasis (Cheng et al., 2013). Our observation of attenuation of E-cadherin expression around necroses is consistent with this mechanism. Additionally, in these areas of low oxygen saturation, tumor cells likely experience stress that leads to the acquisition of a mesenchymal phenotype in order to more easily invade the surrounding tissue. Our results found significant differences in the proliferative activity of tumor cells in different regions of the tumor. Immunoreactivity for Ki-67 was higher in the peripheral invasive front of the tumor compared to the cells surrounding the necrotic foci. Literature data confirm this heterogeneity in endometriosis, for example, peripherally located tumor cells show strong Ki-67 immunostaining, while central parts of the lesion have a significantly lower proliferative index (Yalcin et al., 2017). They also described a similar pattern in endometrial carcinoma with strong Ki-67 expression in the periphery of the tumor glands and reduced in their central parts (Yalcin et al., 2017). In the context of prognosis, a high proliferative index (eg, Ki-67) and the presence of extensive tumor necrosis are independently associated with a worse outcome in a number of carcinomas. Our data confirm the importance of these markers and highlight that their spatial distribution in the tumor (periphery vs. center) reflects different evolutionary strategies of tumor cells.

The expression and cellular distribution of the adhesion molecule E-cadherin showed significant changes depending on the tumor area, which correlate with the commented hypoxic and invasive processes. In the well-oxygenated invasive front of the carcinomas, we observed a relatively preserved membrane expression of E-cadherin in the tumor glandular complexes, especially in the highly differentiated tumors. In contrast, around the necrotic foci, E-cadherin was almost completely lost from the tumor cell membranes. This confirms that cell adhesion is disrupted precisely in stressed hypoxic areas, a fact that supports the occurrence of EMT. In the literature, loss of E-cadherin is a universal marker of invasion and metastasis in epithelial tumors. Numerous studies indicate that reduced E-cadherin expression facilitates tumor cell dissociation and invasion into the underlying tissue in carcinomas of the breast, ovary, endometrium, etc. (Mateva et al., 2021). Consistent with this, in our study surrounding necroses, where conditions dictated invasive behavior, tumor cells virtually lost their E-cadherin-mediated connections. In endometrial carcinomas, it has been specifically described that areas of active invasion (eg, MELF-structural changes of invasion) are characterized by a focal loss of membrane E-cadherin, in contrast to conventional glandular areas where E-cadherin remains intact expressed. Mateva et al. found that it was the areas of deep invasion in the myometrium that showed greatly reduced E-cadherin expression, accompanied by the appearance of mesenchymal markers, which is direct evidence of EMT (Mateva et al., 2021). Closely associated with E-cadherin is β-catenin. Upon loss of E-cadherin or upon activation of the Wnt/β-catenin signaling pathway, β-catenin accumulates in the cytoplasm and translocates to the nucleus, where it acts as a transcriptional coactivator of proliferative and EMT-related genes. In our study, we did not perform a detailed cellular analysis of the subcellular localization of β-catenin, but judging by the reduced E-cadherin expression and the occurring EMT in certain areas, we can assume that β-catenin in these cells was dissociated from the cell membrane and potentially active in the nucleus. Myofibroblasts play an active role in tumor progression by remodeling the extracellular matrix, secreting growth factors and cytokines (eg, TGF-β1, HGF, VEGF), and promoting invasion and angiogenesis (Otranto et al., 2012). Lewis et al. demonstrated that tumor cells can directly induce the differentiation of normal fibroblasts to myofibroblasts through secretion of TGF-β1, and the resulting myofibroblasts accelerate tumor cell invasion (Lewis et al., 2004). Therefore, our finding of enhanced SMA-expression in the invasive periphery in ovarian and endometrial carcinomas is fully consistent with the concept of a "reactive stroma" that actively supports the malignant process. In our paper, we did not directly assess the prognostic significance of stromal indices, but the differences obtained suggest that aggressive carcinomas probably induce a stronger stromal reaction at their periphery. Fascin-1 is expressed de novo, particularly in invasive cell subpopulations (Zhang et al., 2022). Our results confirm this: immunohistochemical positivity for Fascin was highlighted more strongly in tumor cells at the invasive front, while in areas surrounding necrosis, tumor cells showed weaker or absent Fascin expression. This contrast between periphery and center reflects the functional specialization of cells - at the invasive edge, tumor cells acquire motility and invasive ability, which is contributed by the enhanced expression of Fascin, facilitating the formation of stable actin bundles and filopodia for migration (Zhang et al., 2022). In the poorly differentiated, actively invasive components of the ovarian and endometrial tumors, Fascin was significantly more expressed. This suggests that Fascin is a marker of invasive potential of tumor cells and its expression may distinguish more aggressive cell clones.

According to Task 5

In the present study, we compared cases with endometriosis, with ovarian carcinoma, and with endometrial carcinoma regarding the expression of the following immunohistochemical markers - HIF-1α, Ki-67, E-cadherin, β-catenin, α-SMA, and fascin-1. We sought to identify distinctive expression patterns that may suggest a relationship between the presence of endometriosis and the development of carcinoma. The obtained results revealed significant differences between the groups, reflecting the biological characteristics of benign and malignant tissue.

First, hypoxia and related molecular pathways appear to play a role in both endometriosis and cancer. HIF-1α is known to be the main transcriptional regulator by which cells adapt to low oxygen saturation. In the tissue samples we examined, we found increased expression of HIF-1α in all three studied groups compared to normal endometrium, with varying intensity and distribution. In endometriosis foci, HIF-1α was moderately expressed diffusely in the glandular and stromal component, whereas in carcinomas, much more intensely positive areas were observed, especially in tumor invasive fronts and around necrotic areas. Strongest and diffuse HIF-1α immunoreactivity (>75% of tumor cells) was observed in a significant proportion of ovarian carcinomas, while endometrial carcinomas more often showed moderate levels of positive cells (25-50%). Quantitatively, the proportion of cases with moderate HIF-1α positivity was statistically significantly greater in endometrial carcinoma (eg, 88.2% of cases with 25–50% positive cells) compared with ovarian carcinoma (11.8%). In contrast, strong HIF-1α expression (over 75% of cells) predominated in the ovarian carcinoma group (about 41.2% of cases, compared to only 5.9% in endometrial carcinoma). These differences also correlate with histological differentiation - the cells of poorly differentiated adenocarcinomas in our series demonstrated a stronger HIF-1α signal, accompanied by active proliferation (high Ki-67) and reduced adhesion (reduced E-cadherin), which is characteristic of the more aggressive phenotype. Our findings are consistent with literature data that advanced and high-grade carcinomas often present with significant tumor hypoxia and HIF-1α overexpression associated with worse prognosis (Park et al., 2018). Increased HIF-1α activity stimulates new vessel formation and promotes cell survival in an adverse environment, which likely also plays a role in the persistence and progression of endometriotic implants. In fact, ectopic endometriosis is accompanied by chronic recurrent bleeding, leading to local tissue hypoxia - considered one of the key factors for its invasive growth and potential malignant evolution. Our study demonstrated increased levels of HIF-1α in endometriotic tissue compared to normal endometrium, supporting the hypothesis of a "hypoxic trigger mechanism" in endometriosis similar to that in solid tumors.

The expression of proliferative activity (with the Ki-67 marker) between groups clearly highlighted the benign nature of endometriosis versus malignant processes. In almost all cases of endometriosis (87.5%), we found extremely low Ki-67 - less than 10% Ki-67-positive cell nuclei. In contrast, carcinomas demonstrated a significantly higher proliferative index. In about 58.8% of endometrial carcinomas Ki-67 was in the intermediate range of 10-50% positive cells, which is statistically significantly more compared to endometriosis (only 12.5% of the latter showed such activity). The highest Ki-67 values (>50% positive cells) were observed in ovarian carcinomas - 41.2%, and no case of endometriosis reached similar levels. These differences are expected as they reflect the intrinsic acceleration of the cell cycle in malignant transformation. It is known that a higher Ki-67 index generally correlates with a more advanced stage and more aggressive behavior in a number of carcinomas (Chidananda Murthy, 2023). In our series, we also more often observed high Ki-67 values in poorly differentiated and invasive carcinomas (with concomitant loss of E-cadherin) than in well-differentiated tumors. It is important to note that endometriosis is not a hyperplastic lesion - its low proliferative index confirms its benign character.

A curious fact reported in the literature is that even eutopic endometrium in women with endometriosis shows slightly higher proliferative activity compared to controls, which may reflect a shared hormonal imbalance or other systemic factors (Keita et al., 2016). Nevertheless, the malignant transformation of endometriosis requires the accumulation of additional genetic damage - only then is cell proliferation "unlocked" and reaches the levels seen in cancer.

Evaluation of the adhesion molecules E-cadherin and β-catenin showed interesting trends. In our study, diffuse membrane expression of E-cadherin prevailed in endometriosis and in endometrial carcinoma - in 93.8% and 88.2% of cases, respectively, i.e. almost all samples from these groups had uniform E-cadherin along the cell membranes. In contrast, in ovarian carcinomas we observed significantly more often heterogeneous or reduced E-cadherin expression. In more than half of the cases (52.9%), E-cadherin was partially or almost completely lost in tumor glandular structures. These differences are statistically significant and indicate that EMT occurs much more frequently in ovarian carcinomas, especially those associated with endometriosis. Loss of E-cadherin leads to a weakening of cell adhesion and allows tumor cells to more easily infiltrate the surrounding tissue and disseminate. In endometrial carcinoma, preserved E-cadherin expression is known to correlate with better differentiation and better patient survival (Zheng et al., 2019). Although E-cadherin is sometimes paradoxically found co-expressed with high Ki-67 levels (ie, a tumor with active proliferation but intact intercellular junctions), its presence has nevertheless been confirmed as an independent favorable prognostic factor in endometrial cancer. Our results are consistent with this finding - endometriosis and associated endometrial carcinoma, which are usually hormonally driven and more indolent, retain E-cadherin and virtually do not exhibit EMT, while endometriosis-associated ovarian carcinomas (OCCC and endometrioid) much earlier acquire an E-cadherin-negative/EMT-positive phenotype with all the resulting implications for tumor invasiveness. Nuclear expression of β-catenin is observed in a proportion of endometrioid carcinomas of the uterus and ovary and is usually indicative of a mutation in the CTNNB1 gene. About 20% of endometrial endometrioid carcinomas and up to 40-50% of ovarian endometrioid carcinomas have been described in the literature to have a mutation in CTNNB1, which leads to stabilization of β-catenin and its accumulation in the nucleus. We have also observed isolated cases in our series (endometrioid ovarian carcinoma) with aberrant nuclear β-catenin immunoreactivity. Regarding the results of the expression of the stromal and cytoskeletal markers - SMA and Fascin, our data showed that the stroma of endometriosis and carcinomas did not differ significantly in terms of SMA-expression. In all three groups of biopsies examined, there were cases with highly diffusely SMA-positive stromal cells. Quantitatively, we found no significant difference in the distribution of SMA-positivity categories between endometriosis, endometrial and ovarian carcinoma. In addition, marked fibrous stroma with diffuse SMA-immunostaining was present in almost all endometriotic foci examined, including cases of pelvic peritoneal endometriosis and especially ovarian endometriomas. This confirms the well-known phenomenon of smooth muscle (myofibroblastic) metaplasia in the endometriotic stroma, also described in classic histological studies.

The present comparative analysis of immunomarkers in endometriosis, endometrial and ovarian carcinoma shows that endometriosis possesses molecular features overlapping with those of malignant tumors, albeit to a lesser extent. The hypoxic environment (HIF-1α), local hyperplasia (Ki-67), partial loss of adhesion (reduced E-cadherin, β-catenin relocalization) and fibrous stroma (SMA) in endometriotic lesions are evidence of a "neoplastic-like" behavior, possibly explaining the epidemiological association with certain tumor diseases. When these changes are further aggravated by the accumulation of genetic mutations (ARID1A, PTEN, PIK3CA, TP53, etc.), endometriosis can turn into carcinoma - most often ovarian clear cell or endometrioid. The comparison between cases with and without endometriosis supports this concept: in the presence of endometriosis, a predisposition to the development of specific subtypes of malignant tumors is observed, which are distinguished by particular molecular profiles (eg, Wnt-activation, ARID1A mutations) and clinical behavior. A deeper understanding of this relationship will help to better predict and individualized monitoring of patients with endometriosis, with a view to prevention or early detection of associated carcinomas.

According to Task 6

Endometrial and ovarian carcinomas demonstrate characteristic immunohistochemical profiles that are associated with traditional prognostic factors such as FIGO stage, depth of invasion, and histologic subtype. The results of the conducted research show that advanced stages and more aggressive tumor variants have a higher expression of the hypoxic marker HIF-1α and the proliferative index Ki-67, accompanied by a reduced expression of the adhesion molecule E-cadherin. These changes reflect a shift to a more invasive phenotype and correlate with propensity for tumor progression and metastasis (Koyuncuoglu et al., 2012). Consistent with the literature, immunohistochemical analysis in our study confirmed that higher FIGO stages and deeper invasion were characterized by biological markers of higher malignant potential.

We found that HIF-1α expression was increased in the more aggressive tumors. In endometrioid carcinomas of the endometrioid type (type I), diffuse expression of HIF-1α occurs more frequently in poorly differentiated and advanced FIGO stage cases, as reported by other authors. In contrast, in non-endometrioid high-grade carcinomas (type II), uniformly strong HIF-1α expression was observed in all cases examined—including perinecrotic positivity, regardless of depth of invasion, vascular invasion, or tumor stage. This suggests that in type II carcinomas the hypoxic response is maximally activated already in the early stages of development. In our series, high HIF-1α expression was more frequent and more intense in ovarian carcinomas compared to endometrial carcinomas (significantly more cases in our series had >75% positive cells in ovarian tumors). These data are consistent with the known fact that ovarian carcinomas, especially high-grade serous carcinomas, often grow in a hypoxic environment due to their rapid growth and insufficient blood supply. In the literature, it has been noted that increased expression of HIF-1α is associated with a worse prognosis in ovarian carcinoma (Qin et al., 2017). Hypoxia in the tumor microenvironment not only promotes angiogenesis but also promotes invasion and resistance to therapy, which explains why HIF-1α-positive tumors are more aggressive.Consistent with our results, a number of studies have shown that a high Ki-67 index correlates with a more aggressive tumor behavior. In endometrial carcinomas, increased expression of Ki-67 was observed in poorly differentiated and advanced tumors, and the index value increased significantly in more advanced FIGO stages. A higher Ki-67 index was associated with a more unfavorable clinical course and shorter survival (Martin-Salamanca et al., 2023). We found that in cases with high proliferative activity, signs of epithelial-mesenchymal transformation (EMT) are also more often present - for example, areas with a high Ki-67 index in the tumor often coincide with foci of reduced E-cadherin expression and vice versa. This observation suggests that cell proliferation and invasive phenotype go hand in hand in rapidly progressing carcinomas. In support of this, Ki-67 has been identified as an independent adverse prognostic factor in endometrial carcinoma—tumors with high Ki-67 have significantly shorter survival at long-term follow-up (Martin-Salamanca et al., 2023). A similar trend was reported in ovarian carcinomas, where cases with high Ki-67 (especially among high-grade serous carcinomas) showed a higher risk of recurrence and a poorer response to chemotherapy (Ismail et al., 2023).

The present comparative analysis of the expression of HIF-1α, Ki-67, E-cadherin, β-catenin, SMA and Fascin in endometrial and ovarian carcinomas shows that these immunohistochemical markers reflect the degree of tumor aggressiveness and may serve for prognosis. Higher FIGO stages and deep invasion are characterized by activation of hypoxic mechanisms (HIF-1α), enhanced cell proliferation (Ki-67), loss of epithelial adhesion (decreased E-cadherin, aberrant β-catenin) and acquisition of a mesenchymal phenotype (SMA and Fascin expression) in the tumor tissue. Different histological subtypes show a different profile—endometrioid carcinomas more often undergo EMT and have Wnt activation, while serous carcinomas retain epithelial features, which correlates with differences in their biology and dissemination pathways. These results are consistent with current scientific evidence and support the idea that immunohistochemical panels can complement established prognostic factors. By quantifying markers such as Ki-67, HIF-1α, E-cadherin and Fascin, the pathologist could better stratify tumors by risk and help optimize the therapeutic approach in endometrial and ovarian carcinomas.

VI. FINDINGS AND CONCLUSION

FINDINGS

1. Endometriosis mainly affects women of reproductive age, while endometrial and ovarian carcinomas are diagnosed more often in the postmenopausal period and in older age. The mean age of patients with endometriosis was 48.65 ± 14.87 years, while that of patients with endometrial carcinoma was 66.45 ± 10.67 years, and in ovarian carcinoma - 67.21 ± 8.94 years.

2. FIGO staging analysis in our study showed that endometrial carcinoma was diagnosed at an earlier stage. In endometrial carcinoma, the most common stage is IB (47.1%), while in ovarian carcinomas IA (29.4%) and IC (35.3%) predominate.

3. Endometriotic lesions demonstrated a predominantly diffuse distribution of HIF-1α in glandular epithelium and in stromal cells, in contrast to malignant tumors where hypoxia and HIF-1α were mainly confined to perinecrotic areas. The strongest and diffuse HIF-1α immunoreactivity (>75% of tumor cells) was observed in a significant proportion of ovarian carcinomas.

4. High levels of HIF-1α statistically correlated with deeper myometrial invasion in endometrial carcinomas infiltrating >1/2 of the myometrial thickness, strong HIF-1α expression predominated, while weak expression predominated in limited invasion.

5. We observed a relationship between HIF-1α and EMF: all cases with marked loss of E-cadherin (positive EMF status) showed high HIF-1α expression.

6. The relative proportion of cases with heterogeneous or absent E-cadherin expression (positive EMT status) was significantly higher in ovarian carcinomas than in endometriosis and endometrial carcinomas.

7. We found a smooth muscle transformation of the stroma in extrauterine endometriosis: in almost all examined cases, the stromal cells diffusely expressed α-smooth muscle actin (α-SMA).

8. Fascin expression was markedly stronger in tumor cells at the invasive front in tissue samples from carcinomas, while in areas surrounding necrosis it was weaker to absent.

9. Higher FIGO stages and deep invasion are characterized by activation of hypoxic mechanisms (strong HIF-1α expression), enhanced cell proliferation (Ki-67), loss of epithelial adhesion (decreased E-cadherin, aberrant β-catenin) and acquisition of a mesenchymal phenotype (SMA and Fascin expression) in the tumor tissue.

10. We found a statistically significantly lower proportion of EMT-positive tumors among carcinomas with the presence of macroscopic necrosis compared to those without necrosis.

CONCLUSION

Endometriosis, endometrial, and ovarian carcinomas share common key molecular mechanisms related to hypoxia, cell proliferation, changes in cell adhesion, and cytoskeletal remodeling. Analysis of the expression of HIF1alpha, Ki-67, E-cadherin, β-catenin, SMA and Fascin revealed significant differences and commonalities between these diseases that may explain their progression and biological behavior.

VII. CONTRIBUTIONS

Theoretical contributions of original character

1. For the first time in Bulgaria, a comprehensive analysis based on IHC-expression was performed on the role of hypoxia in carcinogenesis in endometriosis, endometrial and ovarian carcinomas with HIF1alpha, Ki-67, E-cadherin, β-catenin, SMA and Fascin.
2. Increased expression of HIF-1α in endometrial and ovarian tumors around necrotic foci confirms the role of the hypoxic microenvironment as a stimulus for tumor progression and EMT.
3. Moderate to high expression of HIF1alpha in deep infiltrative forms of endometriosis confirms that the hypoxic microenvironment supports their survival and plays a role in progression.

Scientific-applied contributions

1. Adenomyosis and ectopic endometriosis have clearly distinct molecular profiles. Ectopic endometriosis demonstrated higher levels of HIF1alpha, Ki-67 and β-catenin, while adenomyosis had higher SMA expression and preserved E-cadherin expression, making it more localized.
2. Endometriosis cases showing abnormal expression of certain markers (eg, increased HIF-1α or focal loss of E-cadherin) could be considered at risk for progression and subject to closer monitoring.
3. In already diagnosed carcinomas, the immunohistochemical profile complements traditional prognostic factors (stage, grading) and can point to the risk of progression - e.g. HIF-1α-positive, E-cadherin-negative tumors with high Ki-67 require monitoring for recurrence.

Contributions with affirmative character

1. The most common localization of ectopic endometriosis is the ovaries, the fallopian tubes and abdominal wall are less frequently affected.
2. The endometrioid variant predominates in endometrial carcinoma, while the high-grade serous subtype is most common in ovarian carcinomas.

VIII. PUBLICATIONS AND PRESENTATIONS RELATED TO THE DISSERTATION

List of scientific publications on the topic of the dissertation

1. **Daniel Markov**, Elena Poryazova, Ralitsa Raycheva, Galabin Markov. ***Expression of HIF-1α, Ki67, SMA and E-Cadherin in Endometriosis, Endometrial and Ovarian Carcinoma***. Folia Medica 2024;66(1):97-103. doi: 10.3897/folmed.66.e112757.
2. **Markov D**, Uchikov P, Timonov P, Markov G, Poryazova E. ***Primary pleomorphic breast sarcoma - a case report***. Folia Medica 2024; 66(4): 568-573. doi: 10.3897/folmed.66.e120391.
3. **Markov D**., Serteva D., Bozhkova D., Poryazova E. ***EXPRESSION OF p53 AND Ki67 IN TYPE 1 AND TYPE 2 ENDOMETRIAL CARCINOMAS AND IN LOW AND HIGH GRADE SEROUS OVARIAN CARCINOMAS.*** Znanstvena misel journal 2023; ISSN 3124-1123 №82 p. 11-16.
4. **Markov Daniel**, Poryazova Elena. ***ENDOMETRIOSIS AND RELATED NEOPLASMS OF THE OVARY AND ENDOMETRIUM. REPORTING 11 CASES.*** Eurasian Union of Scientists (Евразийский Союз Ученых) 2023; ISSN: 2413-9335; №8 (109), p. 3-8.

List of participations in national and international scientific events

1. Innovative approaches in pathology: Expression of immunohistochemical markers in endometriosis and endometrial cancer in relation to hypoxia and epithelial-mesenchymal transition for diagnosis and prognosis with the help of artificial intelligence, **Daniel Markov**, Annual Doctoral Scholarship, Karol Knowledge Foundation 2025. 15.01. Sofia.
2. Expression of anti HIF1alpha, Ki 67, E-cadherin, β-cathenin, SMA in foci of endometriosis, in endometrial and ovarian carcinomas, **Daniel Markov**, Days of Medical Science 04/19/2024, MU-Plovdiv, p.160-161.
3. Expression of HIF1Alfa in endometriosis and endometrial carcinomas, **Daniel Markov**, Elena Poryazova, National Conference on Pathology 01-04.06.2023, Golden Sands, p. 31-32.
4. Presentation of two rare cases of combination of endometriosis with endometrial carcinoma and stromal sarcoma, **Daniel Markov**, Denitsa Serteva, Elena Poryazova, National Conference on Pathology 01-04.06.2023, Golden Sands, p. 32-33.
5. A Rare Case of Undifferentiated Pleomorphic Sarcoma of the Mammary Gland, **Daniel Markov**, Galabin Markov, Denitsa Serteva, Elena Poryazova, SCIENCE and YOUTH Conference 2022, Plovdiv, p. 31-32.
6. EMT in gynecological neoplasms and endometriosis, Elena Poryazova, Desislava Bozhkova, Denitsa Serteva, **Daniel Markov**, National Pathology Conference 17-18.06.2022, Lukovit, p. 19-20.
7. Immunohistochemical expression of p53 and Ki67 in endometrial and ovarian carcinomas, Elena Poryazova, Desislava Bozhkova, Denitsa Serteva, **Daniel Markov**, National Pathology Conference 17-18.06.2022, Lukovit, p. 22.
8. A rare case of undifferentiated pleomorphic sarcoma of the mammary gland, **Daniel Markov**, Galabin Markov, Denitsa Serteva, Elena Poryazova, National Conference on Pathology 17-18.06.2022, Lukovit, p.27-28.

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True initiation begins only when one discovers that life is not only what is seen, but also what is felt.