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Original Research

Expression Analysis of *COPB2* and *Bcl-2* in Early Stages of Endometrial Carcinoma

Branko Andrić[1,*], Danijela Cvetković[2], Stefan Blagojević[3], Marko Stanković[4], Nenad Kokošar[4], Dragutin Sretenović[4], Dragiša Šljivančanin[5], Branislav Milošević[5], Danijela Milošev[6], Petar Arsenijević[7]

[1]Department for Women's Health Care, Health Center Raska, 36350 Raska, Serbia
[2]Institute of Genetics Faculty of Medical Sciences, University of Kragujevac, 34000 Kragujevac, Serbia
[3]Department for Biology and Ecology, Faculty of Science, University of Kragujevac, 34000 Kragujevac, Serbia
[4]The Obstetrics and Gynaecology Clinic Narodni Front, 11000 Belgrade, Serbia
[5]Clinic for Gynecology and Obstetrics, University Clinical Center of Serbia, 11000 Belgrade, Serbia
[6]Department of Pathological-anatomical Diagnostics, University Clinical Center Kragujevac, 34000 Kragujevac, Serbia
[7]Department of Obstetrics and Gynecology, Faculty of Medicine, University of Kragujevac, 34000 Kragujevac, Serbia
*Correspondence: drbranko83@gmail.com (Branko Andrić)
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Abstract

Background: Coatomer protein complex subunit β2 (COPB2) is a subunit of the intracellular transport system between cell organelles that participates in the regulation of cell division and differentiation. Bcl-2 is a protein that participates in regulating the process of apoptosis. We aimed to examine and establish expression of these two genes in endometrial cancer at an early stage. **Methods**: In order to examine the relative expression of the gene for the COPB2 subunit and Bcl-2, we sampled endometrial tissue from 40 patients with endometrial cancer (experimental group) and from 20 patients without cancer (control group). All patients in the experimental group had early-stage cancer without metastases at the time of sample collection. Gene expression was performed using the polymerase chain reaction (PCR) method at the Faculty of Science, University of Kragujevac. Relative quantification of *COPB2* and *Bcl-2* gene expression was obtained in relation to the expression of *GAPDH* ("housekeeping gene"). Based on the results of the analysis of the normality of the data distribution (Shapiro-Wilk test), the Mann-Whitney U test was used for the analysis of these variables. **Results**: Using Mann-Whitney U test, we determined that there is a statistically significant difference ($p < 0.05$) in the expression values of the *COPB2* and *Bcl-2* gene in women with endometrial carcinoma (EC) compared to women without cancer. Expression value for the *COPB2* gene in the experimental group (0.18) was lower compared to the value of the control group (0.65). Also, the relative expression value of *Bcl-2* was lower in the examined group (0.15) than in the control group (0.54). Receiver operating characteristic (ROC) curve showed statistically significant diagnostic potential of gene expression for *COPB2* (area under the curve (AUC) 0.878; $p < 0.001$) and *Bcl-2* (AUC 0.666; $p = 0.038$). **Conclusions**: In the initial stages of endometrial cancer, there is a significant reduced expression of the *Bcl-2* and *COPB2* gene compared to cells of normal endometrial tissue. This study showed that the expression value of these two genes in the early stages of endometrial cancer is low. Diagnostic potential in segregation of cancer from non- cancer patients is achieved through expression of these two genes, with *COPB2* being more specific biomarker. **Clinical Trial Registration**: The study has been registered with registration number NCT05951426 on https://classic.clinicaltrials.gov/ct2/home.

Keywords: endometrial carcinoma; apoptosis; *Bcl-2*; *COPB2* (Coatomer protein complex subunit β2); quantitative PCR (quantitative polymerase chain reaction/qPCR)

1. Introduction

Endometrial carcinoma (EC) is the most common malignant tumor of the female genital organs [1]. In the last year, Europe alone recorded over 130,000 cases of women afflicted by this condition. Roughly 5% of these cases were aged 44 or younger, underscoring the tumor's prevalent impact on older women [2]. Gynecological examinations are less frequent among women in less developed countries. For this reason, women report only when irregular bleeding or pain in the lower abdomen and back occurs [2]. The diagnosis of EC is made on the basis of pathohistological analysis of tissue obtained after exploratory curettage (Fig. 1A,B).

Treatment success is correlated with stage of the disease, histological grade and type of tumor; accordingly, timely detection of this disease is critical [3]. Toward this end, a transvaginal ultrasound examination and doppler flow are performed [3–5] (Fig. 2). Other than this method, there currently exists no sufficient screening technique or specific laboratory analysis to establish a diagnosis of endometrial cancer prior to the onset of symptoms.

Numerous cell mechanisms prevent the formation of malignant cells in the body. In addition to DNA repair

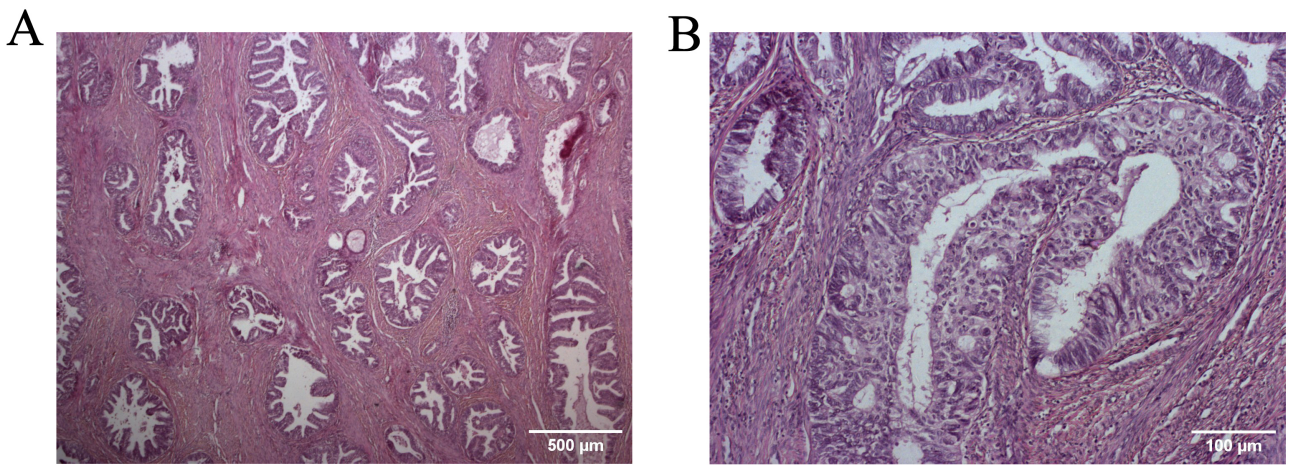

Fig. 1. Histological picture of endometrial carcinoma (EC) on microscopic magnification 25× (A) and 100× (B) (Dr Danijela Milošev, Department of pathological-anatomical diagnostics, University Clinical Center Kragujevac, Kragujevac).

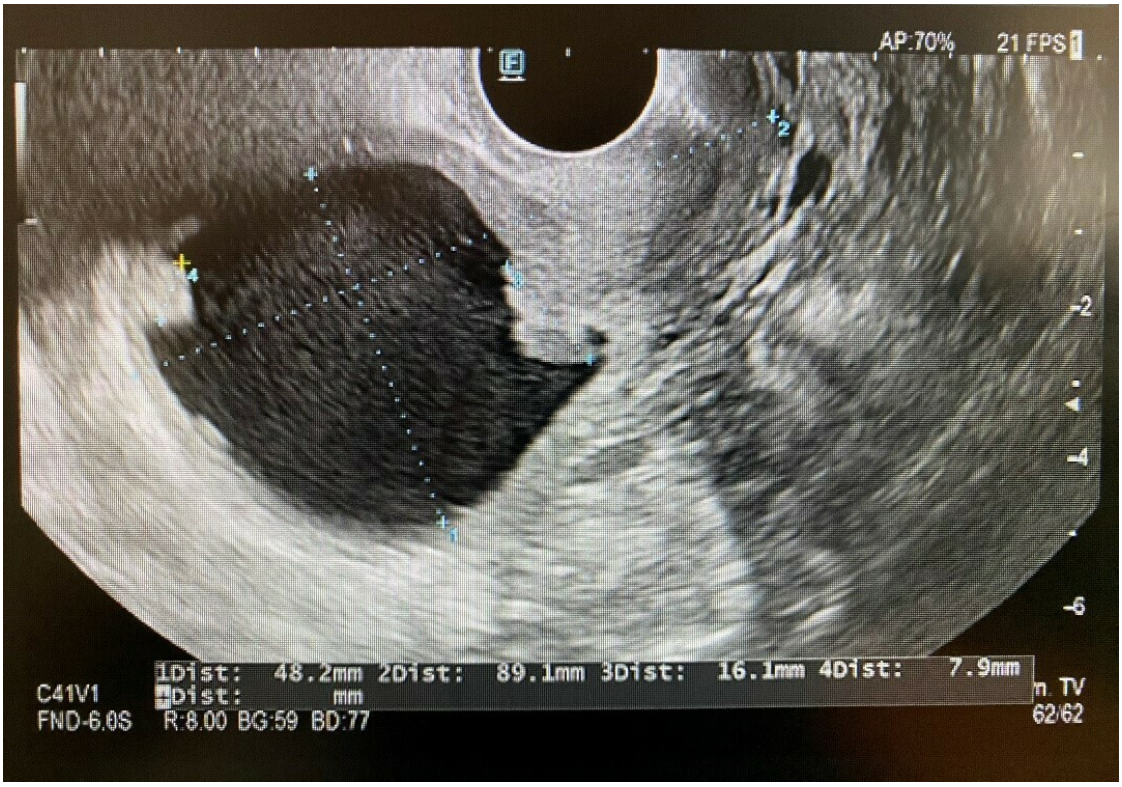

Fig. 2. Transvaginal ultrasound endometrial carcinoma the International Federation of Gynecology and Obstetrics (FIGO) IA (Dr Branko Andrić, Department for Women's Health Care, Health Center Raska, Raska). 1Dist: 48.2 mm (distended uterine cavity), 2Dist: 89.1 mm (uterus length), 3Dist: 16.1 mm (carcinoma endometrii), 4Dist: 7.9 mm (carcinoma endometrii).

mechanisms, apoptosis processes can lead to the destruction of potentially malignantly altered cells, which are mediated by the *Bcl-2* gene family [6–8]. Several studies have examined the expression of the *Bcl-2* gene family, showing low expression of the *Bcl-2* gene, especially in the initial stages of endometrial cancer [9,10]. Numerous intracellular modulators also play an important role. Among them, COPI (Coatomer protein complex I) participates in the transport of proteins between the Endoplasmic reticulum and the Golgi apparatus. The main subunit of this complex is COPB2 (Coatomer protein complex subunit β2) [11]. Decreased activity of this subunit in cells stops cell cycle in the G0/G1 or S phase of cell division and reduces cell growth and differentiation [12]. Numerous previous studies on other tissues (colon, lungs, prostate) have identified heightened expression of the gene associated with this subunit in the malignantly altered tissue of these organs [12–14]. Elevated levels of *COPB2* expression were also

found in other tissues such as cholangiocellular carcinoma and gastric tissues [15,16]. High levels of gene expression were similarly detected in malignant breast cancer cells, which represent estrogen-dependent tissue [17]. Given the absence of prior research on EC patients, who also have an estrogen-dependent tumor, we undertook a study on the early stages of EC. By examining initial molecular and cellular alterations, our aim was to determine whether alterations in gene expression could have potential clinical utility.

2. Materials and Methods

The research was conducted as a retrospective clinical experimental study from 2019 to 2022 on female patients treated at the Gynecology and Obstetrics Clinic in Clinical Center Kragujevac, Serbia. Tissue sections obtained from exploratory curettage and surgical procedures were collected with patients' informed consent, following the principles of the Helsinki Declaration and World Health Organization recommendations for human material experiments. The Ethics Committee also granted approval for the study.

Female patients were divided into two groups:

Group I: 40 patients in whom EC was diagnosed as part of the experimental group, and Group II: 20 patients in whom cancer or atypical hyperplasia of the endometrium was excluded histopathologically. The majority of patients with EC were stage I (29 patients), while only 11 were IIA (the International Federation of Gynecology and Obstetrics (FIGO)). Pathohistological examination of lymph nodes did not reveal the presence of metastases in any patient. Inclusion criteria for participation in the study were: signed informed consent of the patient, pathohistological confirmation of EC for the experimental group or normal endometrial tissue for the control group. Exclusion criteria were the existence of other malignant disease in the patient whose treatment was still ongoing, as well as the pathohistological determination of atypical hyperplasia of the endometrial tissue.

We stored the sample (endometrial tissue) in liquid nitrogen under adequate conditions at the Kragujevac Clinical Center, Department for Gynecology and Obstetrics. We examined the expression of the *COPB2* and *Bcl-2* gene in endometrial tissue cells of these two groups of patients. Genetic processing of the material was carried out at the Faculty of Science in Kragujevac. After thawing the tissue using reverse transcription quantitative PCR (quantitative polymerase chain reaction, qPCR), we determined the expression of the *COPB2* and *Bcl-2* gene from endometrial tissue cells. For Ribonucleic acid (RNA) isolation, we used RNA Extracol (EURx, Gdansk, Poland) according to the manufacturer's instructions. The concentration of each sample was measured on a Eppendorf BioPhotometer Plus (Eppendorf, Hamburg, Germany). An absorbance ratio ranging from 260 to 280 nm, falling between 1.8 and 2.0, indicated the presence of pure RNA. Pure RNA samples were stored at –80 °C until the analysis was started [18]. Reverse transcription (RT-PCR) was then performed [19]. The following equipment and materials were used: First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions and 1 µL of isolated RNA with a concentration of 1 µg/µL. The Eppendorf Mastercycler gradient PCR apparatus was employed for the experiments. The collected DNA samples were stored at –80 °C. For gene expression analysis, we used the AMPLIFYME SG Universal Mix kit (Blirt, Gdansk, Poland), which we used according to the manufacturer's instructions to make the reaction mixture. To prepare the reaction mixture, we incorporated a pair of primers and dyes (Rox Low). We used complementary DNA as the starting molecule; 1 µL of complementary DNA was added to the PCR reaction [20]. PCR plates with complementary DNA and reaction mixture were placed in the Applied Biosystems 7500 Fast Real-Time PCR Systems apparatus according to the manufacturer's instructions. The obtained results were analyzed with the Applied Biosystems 7500 software 2.3 (Thermo Fisher Scientific, Waltham, MA, USA). Relative quantification of *COPB2* and *Bcl-2* gene expression was obtained in relation to the expression of *GAPDH* ("housekeeping gene") in the same sample as the control [21].

Primer sequence used in qPCR was as follows: *GAPDH* forward 5′-AAGCAGGAGTATGACGAGTCCG-3′ and reverse 5′-GCCTTCATACATCTCAAGTTGG-3′; *COPB2* forward 5′-CTTCCTGTTCGAGCTGCAAAG-3′ and reverse 5′-CACTCTAATCTGCATGTCATCC-3′; *Bcl-2* forward 5′-ATCGCCCTGTGGATGACTGAG-3′ and Reverse 5′-CAGCCAGGAGAAATCAAACAGAGG-3′.

3. Results

Analysis of the normality of data distribution was performed using the Shapiro-Wilk test, given that there were 40 patients in the group with EC and 20 in the control group, or fewer than 50 in both cases. As part of the descriptive statistical analysis, we determined the minimum and maximum value as well as the average value and standard deviation for the variables related to protein and gene expression for all measurements.

Based on the results of the analysis of the normality of the data distribution, the Mann-Whitney U test was used for the analysis of these variables. Using this test, we determined that there is a statistically significant difference ($p < 0.05$) in the expression values of the *COPB2* and *Bcl-2* gene in women with EC compared to women without cancer. It is observed that significantly lower *COPB2* and *Bcl-2* gene expression values were found in patients with EC compared to those without cancer (control group) (Table 1, Fig. 3).

By analyzing the expression of the *COPB2* gene in endometrial tissue (measurement performed in two repli-

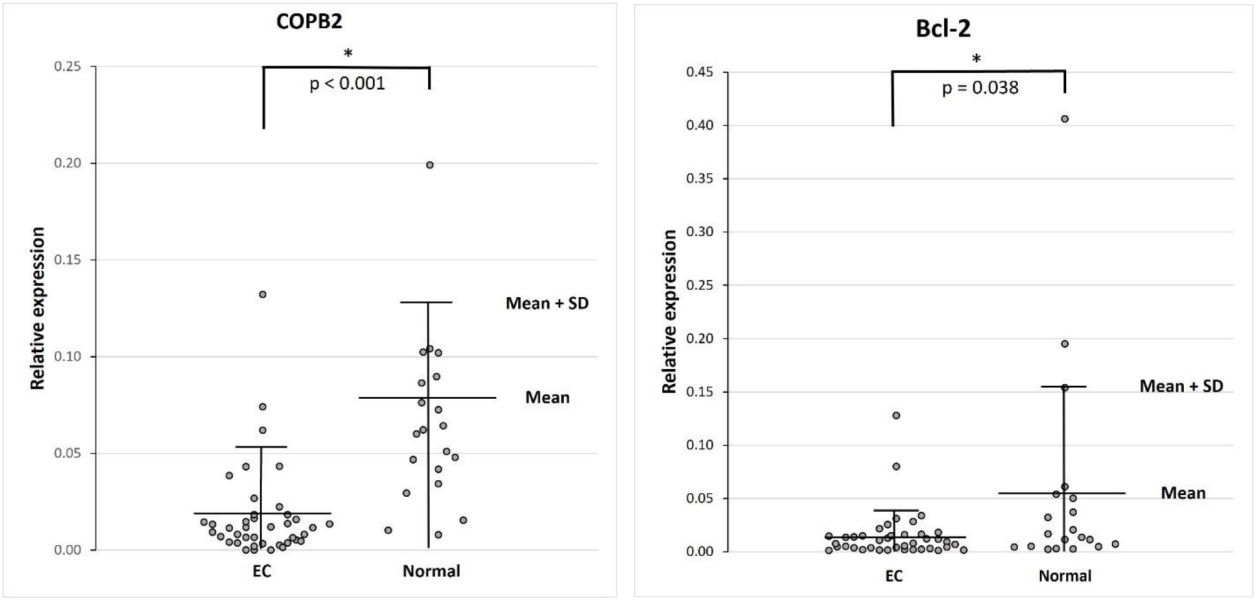

Fig. 3. Scatter plot figures: Relative expression of *COPB2* and *Bcl-2* (EC-endometrial cancer, Normal patients without carcinoma). *COPB2*, Coatomer protein complex subunit β2; SD, standard deviation. *, statistically significant difference ($p < 0.05$).

Table 1. Descriptive statistics in the expression values of the *COPB2* and *Bcl-2* gene in women with EC compared to women without cancer (Normal).

Sample		N	Mean	Std. Deviation	Std. Error Mean
Bcl2	Carcinoma	40	0.015	0.023	0.004
	Normal	20	0.054	0.097	0.022
COPB2	Carcinoma	40	0.018	0.025	0.004
	Normal	20	0.065	0.043	0.010

COPB2, Coatomer protein complex subunit β2; EC, endometrial carcinoma; N, number; Std., standard.

cates), we obtained values indicating decreased relative expression of this gene in malignantly altered endometrial cells: *COPB2* (0.18). The values obtained in both repetitions in the control group with normal tissue indicated a higher relative expression of *COPB2* (0.65). The values we obtained for the relative expression of *Bcl-2* were similar: relative expression of *Bcl-2* (0.15) in patients with EC and *Bcl-2* (0.54) in the control group.

Receiver operating characteristic (ROC) curve was used to test the diagnostic potential of *COPB2* and *Bcl-2* gene expression for discrimination of cancer from noncancer patients. Expression of *Bcl-2* gene as area under the curve was 0.666, a result that was statistically significant ($p = 0.038$) (Fig. 4). For expression of *COPB2* gene, the area under the curve was 0.878, and this result was statistically significant ($p < 0.001$) (Fig. 5). For both genes, diagnostic potential in discrimination of cancer from noncancer patients was demonstrated, with *COPB2* being more specific.

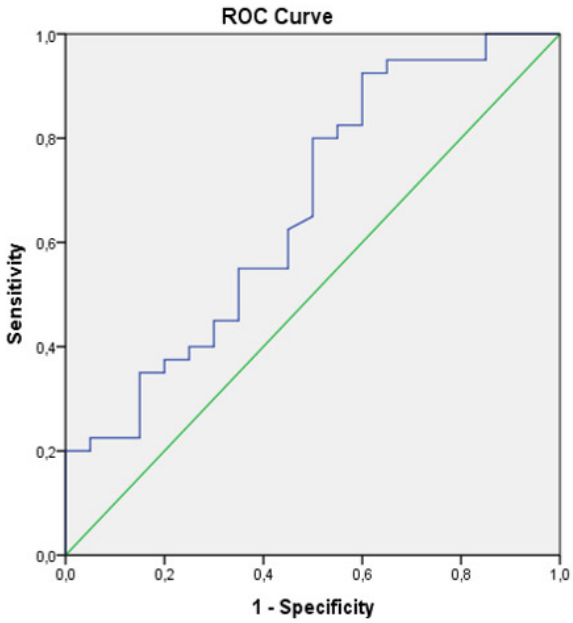

Fig. 4. ROC curves for the *Bcl-2* to discriminate cancer from noncancer patients: AUC = 0.666, $p = 0.038$, sensitivity = 77.5%, specificity = 50.0%. ROC, Receiver operating characteristic; AUC, area under the curve.

4. Discussion

Numerous factors can lead to endometrial cancer. Several studies have tried to determine the influence of certain molecular changes depending on the patient's phenotype (Body mass index (BMI), age), which proved to be an

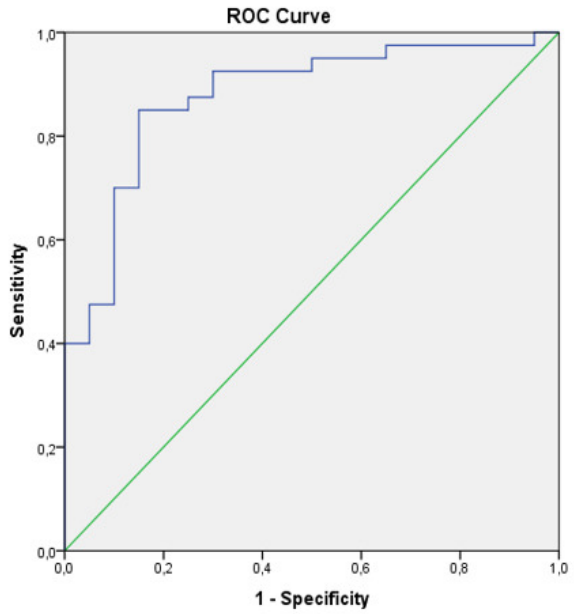

Fig. 5. ROC curves for the *COPB2*, to discriminate cancer from noncancer patients, AUC = 0.878, $p < 0.001$, sensitivity = 85.0%, specificity = 85.0%.

important factor [22]. Changes at the level of intracellular mechanisms of regulation of cell growth and differentiation clearly affect the process of malignancy. COPB2 was first discovered in 1993 by the Stenbeck *et al*. [23] as the main subunit of COPI. In the work that was carried out *in vitro*, on mice, the importance of reduced expression of *COPB2* on stopping cell growth and further development of malignant disease was examined. A connection has been demonstrated between high expression of *COPB2* and the development of gastric cancer [24]. The same study also confirmed that *COPB2* may be considered a valuable gene therapy target for the treatment of gastric cancer [16]. In addition to laboratory research under *in vitro* conditions, several clinical studies were also conducted. In the research by Sudo *et al*. [24], COPA knockdown induced apoptosis and suppressed tumor growth *in vitro* conditions on mice. Wang *et al*. [12] found that *COPB2* staining was markedly stronger in colon cancer tissues than in normal tissues. These results indicate that *COPB2* may be involved in the pathogenesis of human colon cancer [12].

Increased expression of *COPB2* in malignant prostate cells has also been researched. The results show that *COPB2* expression was higher in cancer tissues than in normal tissues. To determine the clinical significance of COPB2 protein, Mi Y *et al*. [14] found that high expression of *COPB2* was associated with a low 5-year survival rate. In a study comparing *COPB2* expression values in normal *vs*. tumor breast tissue, which is a predominately estrogen-dependent malignancy, it was concluded that elevated *COPB2* expression values are also important for the existence of metastatic changes in breast cancer [17]. In light of the previous research, we hypothesized that such elevated expression could be the basis of tumorigenesis.

The importance of the *Bcl-2* gene family in the process of apoptosis has already been proven by numerous works and clinical trials. There are numerous published works that examined the expression of *Bcl-2* in patients with EC [6–8]. Although high expression of the *Bcl-2* gene was found in the advanced stages of EC, that is not so in the early stages, where expression tends to be lower [9,10]. Considering the importance of Bcl-2 for the process of apoptosis and tumorigenesis, the expression values of the *Bcl-2* gene served as a comparative parameter, since the expression of *COPB2* in cells of the endometrium in patients with initial stages of EC has yet to be determined.

Accordingly, we collected endometrial tissue samples from 40 patients with EC (FIGO I and IIA) and 20 patients in whom the presence of this disease was ruled out. Our primary focus was on assessing the expression of *COPB2* and *Bcl-2* in endometrial cells, specifically in patients at the early stages of the disease, without distant metastases or metastases in lymph nodes. From a total of 40 patients (experimental group), most were FIGO I. In earlier studies, patients with increased expression of the *COPB2* gene in other cancer cells had advanced stages of the cancer.

The expression of the gene for *Bcl-2* in this case should have served us for comparison, considering that the importance of Bcl-2 in apoptosis processes has long been proven. ROC curve showed the diagnostic potential of *COPB2* and *Bcl-2* gene expression (Figs. 4,5). As discussed, this regulatory mechanism plays a major role and can be used as a prognostic parameter for the existence of metastases, survival rate or as a target for gene therapy [14,17,25]. Our results suggest that the presence of elevated expression of *COPB-2* in endometrial cells could indicate the presence of metastases in endometrial carcinoma, which has already been proven in other malignancies.

5. Conclusions

Expression values of *COPB-2* and *Bcl-2* genes in the initial stages of endometrial cancer that we obtained showed a low level of expression. Also, diagnostic potential in segregation of cancer from non- cancer patients is achieved through expression of these two genes, with *COPB2* being more specific biomarker. The results obtained from this study have paved the way for further research, offering potential for new insights in the realms of EC diagnosis, clinical staging, and novel treatment approaches.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Author Contributions

BA, PA, DC designed the research study. BA, PA, DM, BM, DSl, MS performed the research. SB and DC

provided help and advice on the gene expression. NK and DSr analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Clinical Center Kragujevac, Serbia (approval number: 01/19/1438).

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Conflict of Interest

The authors declare no conflict of interest.

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