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**The influence of the expression of steroid receptors on angiogenesis,  
proliferation and apoptosis in myomas of pre- and postmenopausal women**

Утицај експресије стероидних рецептора на ангиогенезу,  
пролиферацију и апоптозу у миомима пре- и постменопаузних жена

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## The influence of the expression of steroid receptors on angiogenesis, proliferation and apoptosis in myomas of pre- and postmenopausal women

Утицај експресије стероидних рецептора на ангиогенезу, пролиферацију и апоптозу у миомима пре- и постменопаузних жена

### SUMMARY

**Introduction/Objective** The aim of this study was to determine the effects of the estrogen and progesterone receptor status on angiogenesis, proliferation and apoptosis of myoma cells in premenopausal and postmenopausal women.

**Methods** This was a cross section; clinical-experimental, retrospective, non-interventional study in the field of the study of fundamental pathogenesis mechanisms of disease using pathohistological materials from the existing archive. The research included 76 patients diagnosed with uterine leiomyomas, operatively treated in the Clinic for Gynecology and Obstetrics, Clinical Centre Kragujevac, Serbia. According to the menstrual status, two experimental subgroups were formed. The first group was premenopausal women (PreM) ( $n=35$ ;  $46.2 \pm 5.02$  year old), and the second group was postmenopausal women (PostM) ( $n=41$ ;  $60.25 \pm 5.41$  year old). H&E staining for myoma and myometrium was conducted as well as immunohistochemistry for ER $\alpha$ , ER $\beta$ , PR $\alpha$ , VEGF, CD105, Ki678 and Cas-3.

**Results** Progesterone receptor was over expressed in myoma and myometrium of premenopausal compared to myoma and myometrium of postmenopausal women. Expression of Caspase 3 was statistically significant increased in PostM women compared to PreM group. ER $\alpha$  and ER $\beta$  were not changed among groups neither in myoma nor in myometrium samples.

**Conclusion** According to our data, PR $\alpha$  had higher influence on apoptosis and cell growth than estrogen receptors. Since PR $\alpha$  was increased in PreM in both myoma and myometrium, probably this expression led further to lower expression of apoptotic marker in PreM women.

**Keywords:** steroid receptors; apoptosis; angiogenesis; premenopausal; postmenopausal

### САЖЕТАК

**Увод/Циљ** Циљ ове студије је био да се утицај експресије стероидних рецептора на маркере ангиогенезе, пролиферације и апоптозе ћелија миома код жена у пременопаузи и постменопаузи.

**Метод** Ово је била студија пресека, клиничко-експериментална, ретроспективна, неинтервенцијска студија у пољу истраживања фундаменталних механизма патогенезе болести, коришћењем патохистолошких материјала из постојеће архиве. Истраживањем је обухваћено 76 пацијената са дијагностикованим лејомиома утеруса, оперативно леченим на Клиници за гинекологију и акушерство, Клиничког центра Крагујевац, Србија. Према менструалном статусу, формиране су две експерименталне подгрупе. Прва група биле су жене у пременопаузи (ПреМ) ( $n = 35$ ;  $46,2 \pm 5,02$  година), а друга група биле су жене у постменопаузи (ПостМ) ( $n = 41$ ;  $60,25 \pm 5,41$  година). Коришћено је *H & E* бојење за миом и миометријум, као и имунохистохемија за *ER $\alpha$* , *ER $\beta$* , *PR $\alpha$* , *VEGF*, *CD105*, *Ki678* и *Cas-3*.

**Резултати** Прогестеронски рецептор је био више изражен у миому и миометријуму у пременопаузи у поређењу са миомом и миометријумом жена у постменопаузи. Експресија *Caspase 3* је статистички значајно повећана у групи ПостМ жена у поређењу са ПреМ групом. *ER $\alpha$*  и *ER $\beta$*  нису били различити између група ни у узорцима миома ни миометријума.

**Закључак** Према нашим подацима, *PR $\alpha$*  је имао већи утицај на апоптозу и раст ћелија него на естрогенске рецепторе. Пошто је *PR $\alpha$*  повећан код жена у ПреМ у миому и миометријуму, вероватно је ова експресија довела до смањења експресије апоптотског маркера код ПреМ жена.

**Кључне речи:** рецептори за стероиде; апоптоза; ангиогенеза; пременопауза; постменопауза

### INTRODUCTION

Uterine fibroids, also known as uterine leiomyomas or fibroids are well-limited, pseudo-encapsulated, benign, monoclonal tumors, composed mainly of smooth muscle cells of the uterus uterine leiomyomas is one of the most frequent gynecological tumors in the reproductive period of women. Independently or in association with hyperplasia and adenomyosis, they reach an incidence of 77%, often cause clinically complicated bleeding,

which is why they are the leading cause for hysterectomy and a major global health problem [1, 2]. It has been known that uterine leiomyomas is a hormone-dependent disease. However, the mechanism of action is still unknown, and there is increasing evidence that steroid hormones, estrogen (ER) and progesterone (PR) are not the only modulators of myoma growth [2, 3, 4]. This can be explained with a presence of similar level of circulating hormone in women with and without myoma, with the occurrence of hormone independent extrauterine leiomyoma and the possible absence of their regression in postmenopausal women [5].

Through last decade, high effort is being invested to clarify the role of gonadal steroids, the expression of local growth factors, and factors associated with apoptosis in myeloma cells. Recently studies showed local tissue-specific factors (for example, growth factors), as well as somatic mutations in pro and antiapoptotic genomes, participate in the pathogenesis and progression of these tumors, with or without cross-linking with mechanisms of action of steroid hormones. Among the environmental factors, particular attention is drawn to the presence and effect of estrogen and progesterone receptors on endometrium and myometrial cells in the uterine wall with myoma [3].

The key pathological processes involved in myoma growth are proliferation and hypertrophy of leiomyocytes, apoptosis, angiogenesis, stromal and secondary changes [6]. The most reliable marker of cell proliferation is Ki-67 or a proliferation-cell nuclear antigen, which denotes not only the cells in the divide, but all those in the synthetic phase of the cell cycle [7]. A high level of Ki-67 antigen, detected during the secretory phase, suggests that progesterone has a synergistic effect in the pathogenesis of myoma [8].

Apoptosis is a process of programmed cell death that eliminates dysfunctional and undesirable cells. It is highly regulated by the complex interaction between the pro and the anti-apoptotic molecules, is performed in one cell independently of the surrounding, and is induced by the activation of caspases, specific endoproteases that destroy the essential structural components including the genetic material of the cell [9]. Caspase-3, due to its specificity and sensitivity, is a reliable marker of cells that pass the process of programmed cell death. Its activity cannot be detected before apoptosis. It is registered in early stages and detection grows with progression while is reduced only in the final phase of the apoptotic process [10].

Furthermore, angiogenesis is mediated by numerous angiogenetic growth factors; the most powerful among them is the vascular endothelial growth factor (VEGF). VEGF affects the degree of microvascular tumor density by stimulating the proliferation of endothelial cells. Variable concentrations of VEGF, depending on the phase of menstrual cycle, are detected in myometrium, stromal and epithelial endometrial elements [11].

During tumor angiogenesis, endothelial cells intensely express endoglin (CD105), while vascular endothelium, stromal and inflammatory cells barely or do not express at all CD105 [12].

Beside the fact that the prevalence of proliferation over apoptosis is a major condition for myoma growth, with this study we tried to indicate that similarities and differences in angiogenesis, proliferative, and apoptotic indexes in myomas and surrounding tissue, are primarily dependent on the expression of steroid hormone receptors. According to previous, the aim of this study was to determine the effects of the estrogen and progesterone receptor status on angiogenesis, proliferation and apoptosis of myoma cells in premenopausal and postmenopausal women.

## METHODS

This was a cross section; clinical-experimental, retrospective, non-interventional study in the field of the study of fundamental pathogenesis mechanisms of disease using pathohistological materials from the existing archive.

The research included 76 patients diagnosed with uterine leiomyomas, operatively treated in the Clinic for Gynecology and Obstetrics, Clinical Centre Kragujevac, Kragujevac, Serbia, in a three-year period from 2007-2010. According to the menstrual status, two experimental subgroups were formed. The first group was premenopausal women ( $n=35$ ;  $46.2\pm 5.02$  year old), and the second group was postmenopausal women ( $n=41$ ;  $60.25\pm 5.41$  year old).

Clinical data were collected by insight into disease history and operational protocols of examined patients. We collected information related to gynecological status (menstrual cycle, menarche, menopause, number of deliveries, etc.) and data obtained by macroscopic analysis

of the operative preparations (number, position and size of the myoma, changes in the ovaries, morphometric characteristics of the uterus).

The experimental part of the study was carried out on the operative tissue material obtained by hysterectomy.

The study was conducted at Department of Pathology, Clinical Centre of Kragujevac, Serbia. The study was approved by Ethical Committee of Clinical Center of Kragujevac, Kragujevac, Serbia.

### **Haematoxylin-Eosin staining**

Tissue materials were fixed in formalin, embedded in paraffin, and 5- $\mu$ m sections were stained with haematoxylin-eosin [H&E], and further examined by immunohistochemistry [13]. All pictures are taken in original resolution with x200 zoom. A representative sample of the myoma without regressive changes is separated for immunohistochemical analysis.

### **Immunohistochemistry**

Paraffin-embedded tissue sections were fixed in 10% neutral buffered formalin and embedded in paraffin using standard pathological protocols. Immunohistochemistry was performed on a single representative block from each case or two (when the surrounding myometrium is not visible along with the myoma on the first block). Antigenic retrieval was processed by submerging the sample in 10mM citrate buffer (pH 6) or commercial buffer (10mM EDTA Buffer for Heat-Induced Epitope Retrieval (pH8), AP-9004-125, Thermo Scientific, Waltham, MA, USA) and microwaving for 20 minutes at 96°C. Primary monoclonal antibodies were directed against ER Ab11 (mouse: 1:500, MS-354-R7, Thermo Scientific, Waltham, MA, USA), ER beta antibody (mouse/human: 1:200 dilution, MA1-23217, Thermo Scientific, Waltham, MA, USA), VEGF (rabbit: 1:100 dilution, RB-9031-RQ, Thermo Scientific, Waltham, MA, USA), Ki-67 (rabbit: 1:100 dilution, RB-9106-R7, Thermo Scientific, Waltham, MA, USA), PRa Ab-8 (mouse: 1:25 dilution, MS-298-R7, Thermo Scientific, Waltham, MA, USA), CD105 (rabbit: 1:25 dilution, RB-9291-R7, Thermo Scientific, Waltham, MA, USA), and caspase-3 Ab-3 (human: 1:100 dilution, MS-1123-R7,

Thermo Scientific, Waltham, MA, USA). Tissue sections were incubated with appropriate primary antibody and commercial biotinylated secondary anti-immunoglobulin, at room temperature, according to the manufacturer's instructions (UltraVision LP Large Volume Detection System: HRP Polymer (Ready-To-Use), TL-125-HL, Thermo Scientific, Waltham, MA, USA). An evaluation of the immunohistochemical analysis was carried out by a semi-quantitative assessment of the expression of the examined markers, by scaling to the scales specific to each marker. All pictures are taken in original resolution with x200 zoom.

### **Expression of Estrogens, Progesterone, VEGF and Ki-67**

The expression of estrogen and progesterone receptors will be quantified based on the Allred score, i.e. by adding parameters that indicate percentage representation (from 0 to 5) and intensity of cell expression (from 1 to 3) [14]. The sum of these parameters will represent the values of the total score (from 0 to 8), where the values  $\geq 3$  was considered positive. The expression of VEGF, Ki-67 and caspase-3 was determined based on the percentage of immunoreactive cells. Based on this expression, groups with low (0-15%), moderate (16-30%) and high proliferative or apoptotic index (31-100%) were formed.

### **Expression of CD105**

Immunohistochemical analysis of the expression of endoglin (CD105), an assessment of the degree of angiogenesis will be performed. The right index of intensity of angiogenesis is the density of intra and peritumoral microcirculation or microvessel density. The analysis will be carried out quantitatively by counting blood vessels in zones with their highest density (hot spot areas). We used the recommendations given by Weidner on the magnitude of the field of vision and the counting method [15]. The focal points of the highest density of blood vessels were determined on a small microscopic enlargement (x40). Determination of the focus of the largest microvascular density were performed by two researchers independently, with no clinical and pathohistological data available. After that, the counting of individual blood vessels was performed at a mean microscopic enlargement (x200), which implies an area of  $0.739 \text{ mm}^2$ . The mean value of the results obtained by counting in 3 visible fields was the final result. When counting blood vessels in each "hot zone", the expression of individual

endothelial cells, and not just the lumen of a blood vessel with visible red blood cells, was calculated. After obtaining the data on the number of blood vessels for each case separately, the mean value of the 3 read fields were calculated, and then the median in relation to which all myomas were classified into two groups, those with low degree and those with a high degree of angiogenesis, accordingly whether the number of blood vessels is less than or equal to or greater than the value of the calculated median.

All immunohistochemical staining were carried out with quality control and specificity of colouring, using positive and negative controls according to the UK National Quality Assessment for Immunocytochemistry. Microscopic tumor analysis and evaluation of marker expression were performed on a microscope of the Carl Zeiss, Axioscop 40 type. Preparations with representative fields were painted using three microscopic enlargements (x40 and x200) using a Canon PC 1089 camera.

### **Sampling**

Regarding the method of selecting a study sample from the entire population, all samples of the material archive will be potentially considered for inclusion. The criteria for the involvement of subjects in the study were a pathohistologically verified uterine leiomyomas disease and premenopausal or postmenopausal status. Excluding criteria for selecting subjects were: associated malignant diseases of the ovary and cervix, incomplete clinical data on menstrual status, use of oral contraceptives and other forms of hormonal therapy.

### **Statistical analysis**

Statistical processing of results will be performed using a commercial software package SPSS (version 17.0, SPSS Inc., Chicago, IL). In the analysis of the obtained results, descriptive statistics was first used to describe the general characteristics of the sample: absolute numbers and proportions (frequencies, percentages), median and variability measures (standard deviation), maximum and minimum. The regularity of the distribution was evaluated by Kolmogorov-Smirnov test. For the comparison of the mean values of the

variable two populations, the independent T test, Kruskal-Wallis and Mann-Whitney test were used, and for comparison of the mean variables of variable populations Analysis of variance. The dependence of two descriptive variables were carried out using the  $\chi$ -square test and the Fisher test, the dependence of two numerical variables using Pearson's and Spearman's correlation coefficient, while the influence of more variables on the binary variable were investigated using a multivariate binary logistic regression.

## **RESULTS**

### **H&E staining**

The standard light microscopic analysis is defined histological type of myoma, the mitotic index expressed through the number of mitotic figures on 10 fields of great enlargement, the presence of regressive changes (necrosis, hyaline and myxomatous degeneration, foci of hemorrhage and etc), the condition of the endometrium and surrounding myometrium (Figure 1A, B).

### **Expression of estrogens receptors alpha and beta in myoma and myometrium**

ER alpha neither ER beta showed statistically significant expression in myoma of premenopausal compared to postmenopausal women (Figures 2A, B and Figures 2E, F). Similarly, those parameters were not different among examined groups in myometrium samples (Figures 4A, B and Figures 4E, F).

### **Expression of progesterone receptor in myoma and myometrium**

Progesterone receptor was over expressed in myoma of premenopausal compared to myoma of postmenopausal women (Figure 2C and 2G). Also, statistically significant increased values of PRa were detected in myometrium of PreM compared to PostM women (Figure 4C and 4G).



### **Expression of VEGF in myoma and myometrium**

In myoma tissue VEGF was not significantly different among groups (Figure 1D and 1H). Similarly, same result was obtained in myometrium of PreM women compared to PostM women (Figure 4D and 4H).

### **Expression of CD105 in myoma and myometrium**

In myoma of PreM and PostM women CD105 didn't show any difference in its expression (Figure 3A and 3D). However, in myometrium CD105 was statistically significant increased in PreM women compared to PostM women (Figure 5A and 5B).

### ***Expression of Ki67 in myoma***

Ki67 was not significantly different among PreM and PostM women (Figure 3B and 3E).

### **Expression of Caspase-3 in myoma**

Expression of Caspase 3 was statistically significant increased in PostM women compared to PreM group (Figure 3C and 3F).

### **Correlation between all examined parameters in myoma tissue**

Expression of cas-3 in myoma tissue of all examined groups was in weak but statistically significant negative correlation with expression of ER $\beta$  in the same tissue (Figure 6A). Moreover, expression of cas-3 was in strong and significant negative correlation with PRa in myoma tissue of both PreM and PostM women (Figure 6B).

In strong correlation with expression of PRa was expression of CD105, VEGF and Ki67 as it is showed in Figures (Figures 6C, E and F). Moreover, expression of Ki67 was in

weak but statistically significant correlation with expression of ER $\beta$  (Figure 6D) while cas-3 was in negative correlation with ER $\beta$  (Figure 6A). Correlations between other examined parameters in tissue of myoma didn't show to be significant.

## DISCUSSION

Uterine leiomyoma is the most common benign tumor, despite its frequent manifestation the etiology and pathophysiology of this abnormality remain unknown. Extensive knowledge has accumulated on the role of hormones in the growth of leiomyomas because the occurrence of uterine leiomyomas during the fertile period and the regression after menopause indicate that gonadal steroids are central for development of these tumors [7, 16]. In the last decade, special attention was given to the role of estrogens and progesterone in the pathophysiology of leiomyomas. Uterine leiomyomas have been considered estrogen-dependent tumors, and this role was supported also by the finding that continuous gonadotropin-releasing hormone agonist (GnRHa) treatment, significantly decreases ovarian estrogen production, is as well associated with reduction in tumor size [17]. In order to achieve their effects, estrogens, act through the activation of estrogen receptors (ER alpha and ER beta). Both of these receptors exhibit DNA- and ligand-binding domain sequence conservation and they are encoded by two distinct genes, they also have different transcriptional activation domains as well as different tissue distribution [17]. In our study we showed that expressions of those receptors were not different in myoma of PreM compared to PostM women (Figure 1E, F). Nevertheless, expression of ER alpha and ER beta was not significantly different neither in myometrium of those women (Figure 3E, F). Similarly, Sakaguchi et al. showed that coordinated expression of ER alpha and ER beta might be necessary for normal estrogen action in myometrium [18, 19]. Also, it has been shown ER alpha is phosphorylated at a higher rate on serine in leiomyoma compared with surrounding myometrium, for that reason it is possible that phosphorylated ER $\alpha$  regulated by p44/42 MAPK, may have a role in development of uterine leiomyoma [20, 21].

In recent years the role of progesterone in uterine leiomyoma pathophysiology has become more established. As in case of ERs, nuclear progesterone receptors (PRs) work as ligand-activated transcription factors and there is two predominant isoforms of PR in humans: PRa and PRb [17]. In our study, PRa showed to be significantly unregulated in myoma of

PreM compared to myoma of PostM women which showed to be same in myometrium tissue (Figure 1G, 3G). Those finding correlate with fact that progesterone is cyclically elevated during the reproductive years, are significantly elevated during pregnancy, and are suppressed after menopause, however it is still very difficult to distinguishing the relative importance of estrogens versus progesterone [22, 23].

Since it is already historical fact that leiomyomas are dependent on angiogenesis for their growth and survival, to this date is also found that estrogens and progestins regulate the expression of several potent angiogenic factors, including vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) [24]. We found that in myoma VEGF was not significantly changed regardless menopausal status (Figure 2H), opposite VEGF was changed between groups in myometrium (Figure 4H). We found that VEGF is significantly increased in PreM compared to the PostM women. Similarly, Hague et al., according to menopausal status found that VEGF was significantly increased in PreM compared with PostM endometrium. With the premenopausal tissue exhibiting a significantly higher level of expression was found in the epithelium but not in the stroma or the blood vessels [25, 26].

Furthermore, one of the most commonly assessed angiogenesis markers is microvessel density which is determined on the bases of specific endothelial antigen expression (CD34, CD105) [27]. In the tumor we didn't notice any change between groups in expression of CD105 regarding menopausal status (Figure 3D), however CD105 was statistically significantly decreased in myometrium of PostM compared to PreM women (Figure 5B).

Moreover, regarding cell death, we evaluated two proteins known as markers involved in growth control of leiomyoma. There were no difference among two examined groups in expression of Ki67 (Figure 3E), however cas-3 was significantly increased in PostM compared to PreM women (Figure 3F). We notice that expression of Ki67 didn't follow trend of cas-3 expression, regarding that Plewka et al. showed that the apoptosis was not accompanied by proliferation. There were no immunolocalization of Ki-67 detected in leiomyomas manifesting apoptosis [7, 28].

Additionally, as we can see from Figure 6 that in myoma cells PR $\alpha$  is in positive correlation with VEGF, CD105 and Ki67 (Figure 6C, E and F), and in negative correlation with cas-3 (Figure 6B). On the other hand, ER $\beta$  was in negative correlation with cas-3 and positive correlation with Ki67 (Figure 6A, D).

## CONCLUSION

Although, ER $\beta$  have effect on cell proliferation and apoptosis, through all data more relevant seems to be PRa. According to our data, PRa had higher influence on apoptosis and cell growth then estrogen receptors. Since PRa was increased in PreM in both myoma and myometrium, probably this expression led further to lower expression of apoptotic marker, increased cell proliferation and angiogenesis in PreM women.

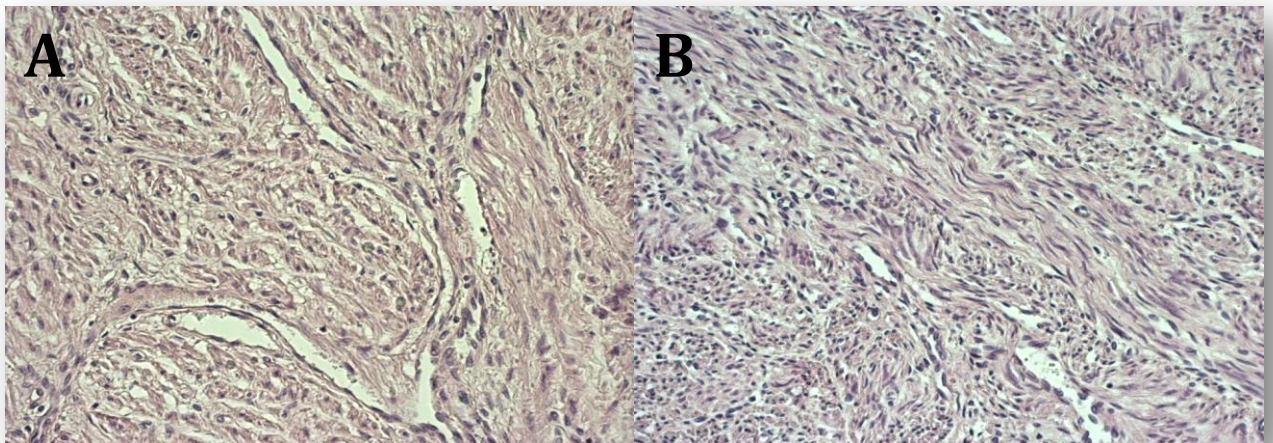
Further studies need to be conducted in order to better understand the mechanisms associated with progesterone driven according to our data, in order to develop more efficient therapies.

**Conflict of interest:** None declared

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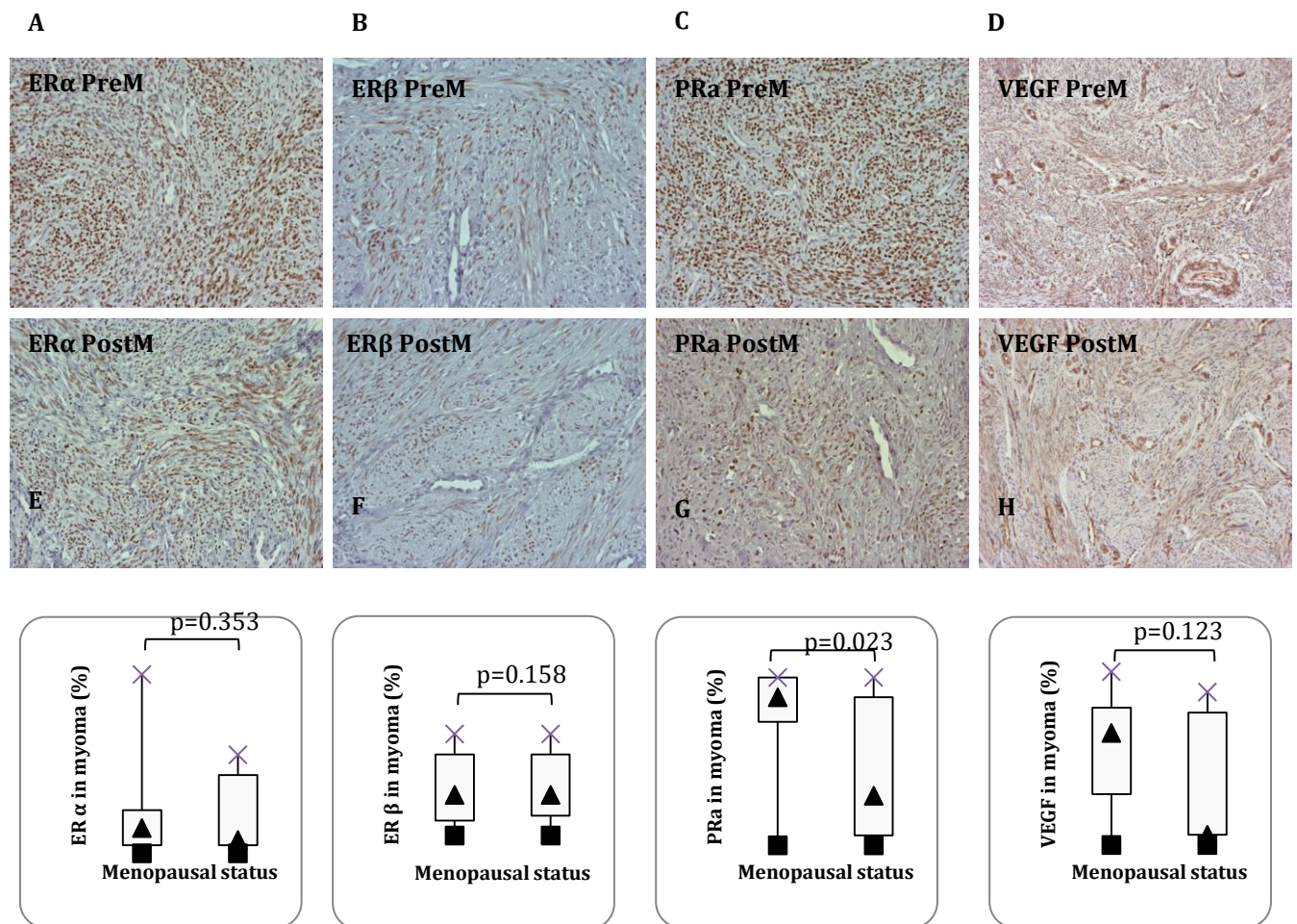
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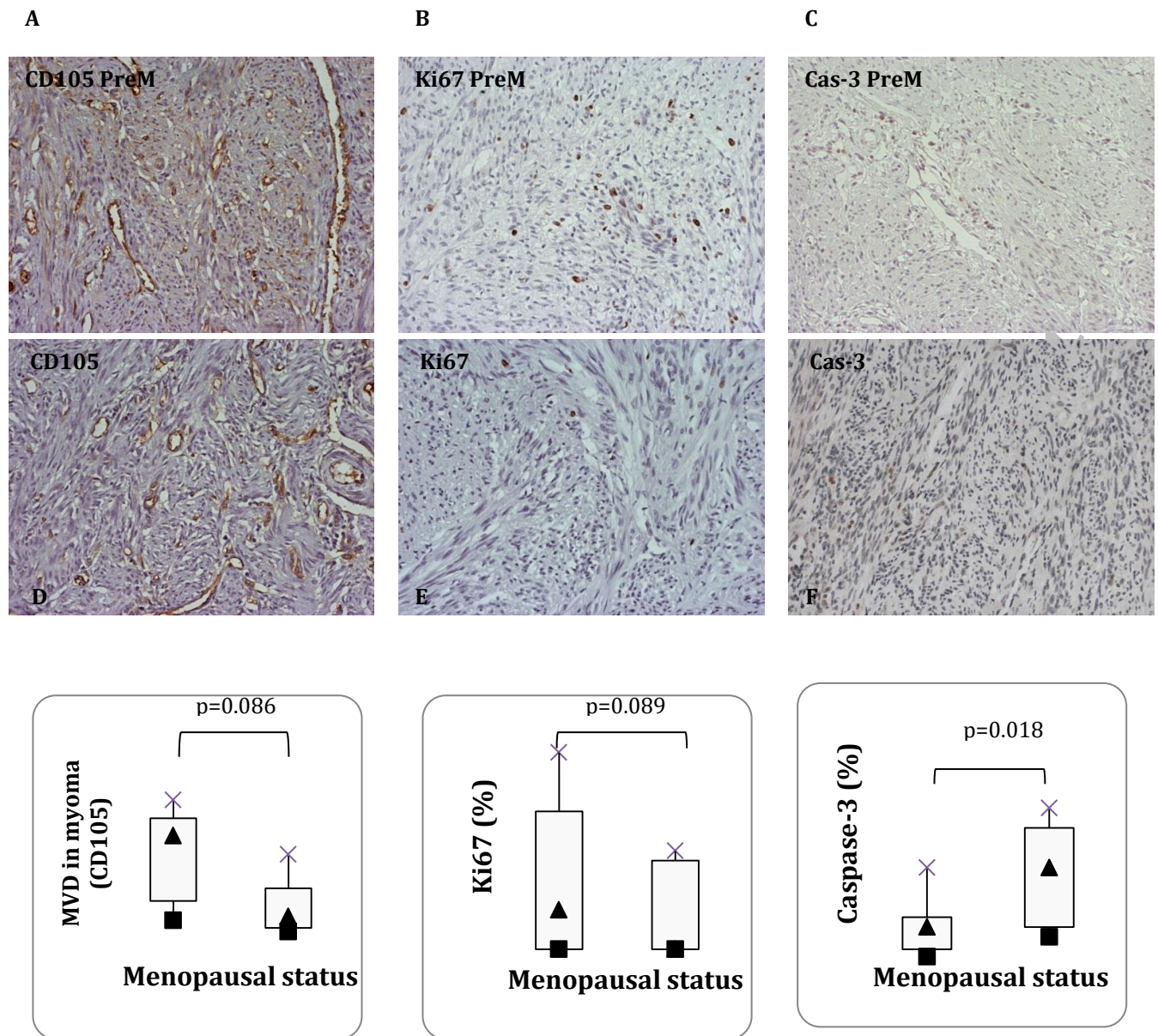
**Figure 1.** A: Representative image of human myoma (H&E, original magnification:  $\times 200$ ); B: representative image of human myometrium (H&E, original magnification:  $\times 200$ )

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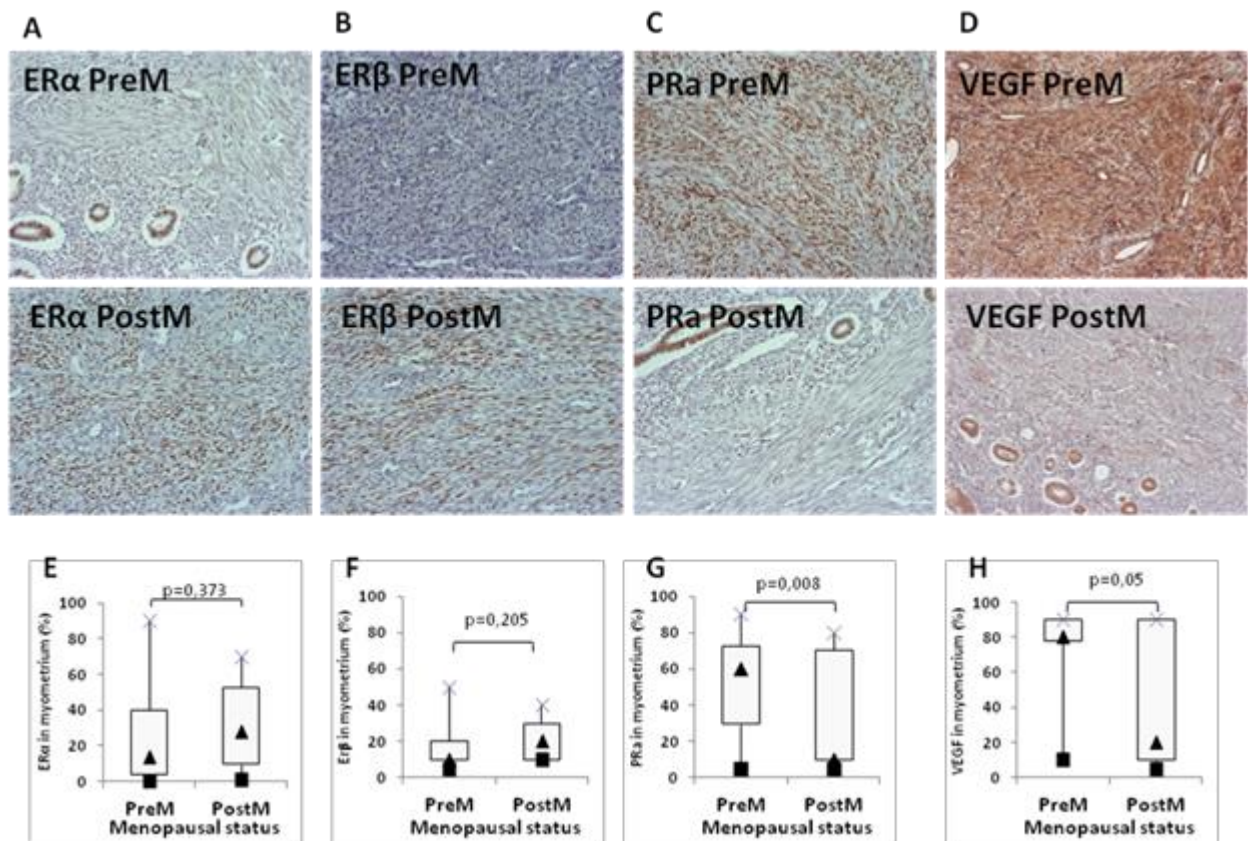


**Figure 2.** A–D: Immunohistochemical expression of ER $\alpha$ , ER $\beta$ , PR $\alpha$  and VEGF (original magnifications:  $\times 200$ ) in myoma tissue of PreM and PostM women, representative tissue sections; E–H: percentage of expression of ER $\alpha$ , ER $\beta$ , PR $\alpha$  and VEGF and statistical difference in percentage of myoma cells between PreM and PostM women;  $\blacktriangle$  – median;  $\blacksquare$  – minimum; X – maximum



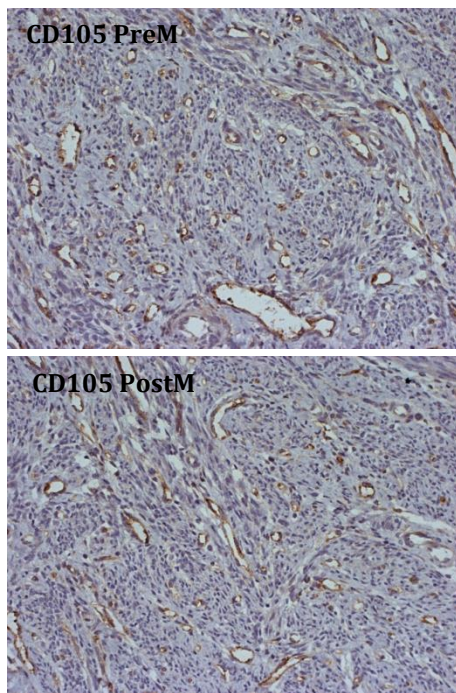


**Figure 3.** A–C: Immunohistochemical expression of CD105, Ki67 and Cas-3 (original magnifications:  $\times 200$ ) in myoma tissue of PreM and PostM women, representative tissue sections; D–F: percentage of expression of CD105, Ki67, and Cas-3 and statistical difference in percentage of myoma cells between PreM and PostM women;  $\blacktriangle$  – median;  $\blacksquare$  – minimum; X – maximum

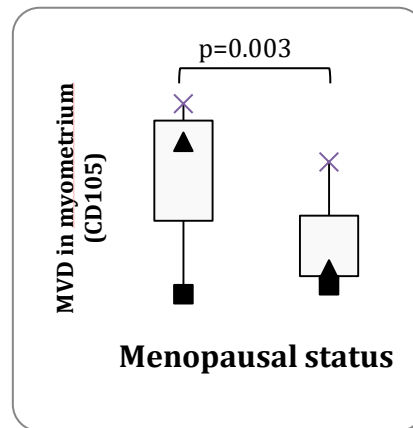


**Figure 4.** A-D: Immunohistochemical expression of ER $\alpha$ , ER $\beta$ , PRa and VEGF (original magnifications:  $\times 200$ ) in myometrium tissue of PreM and PostM women, representative tissue sections; E-H: percentage of expression of ER $\alpha$ , ER $\beta$ , PRa, and VEGF and statistical difference in percentage of myometrium cells between PreM and PostM women; ▲ – median; ■ – minimum; X – maximum

A

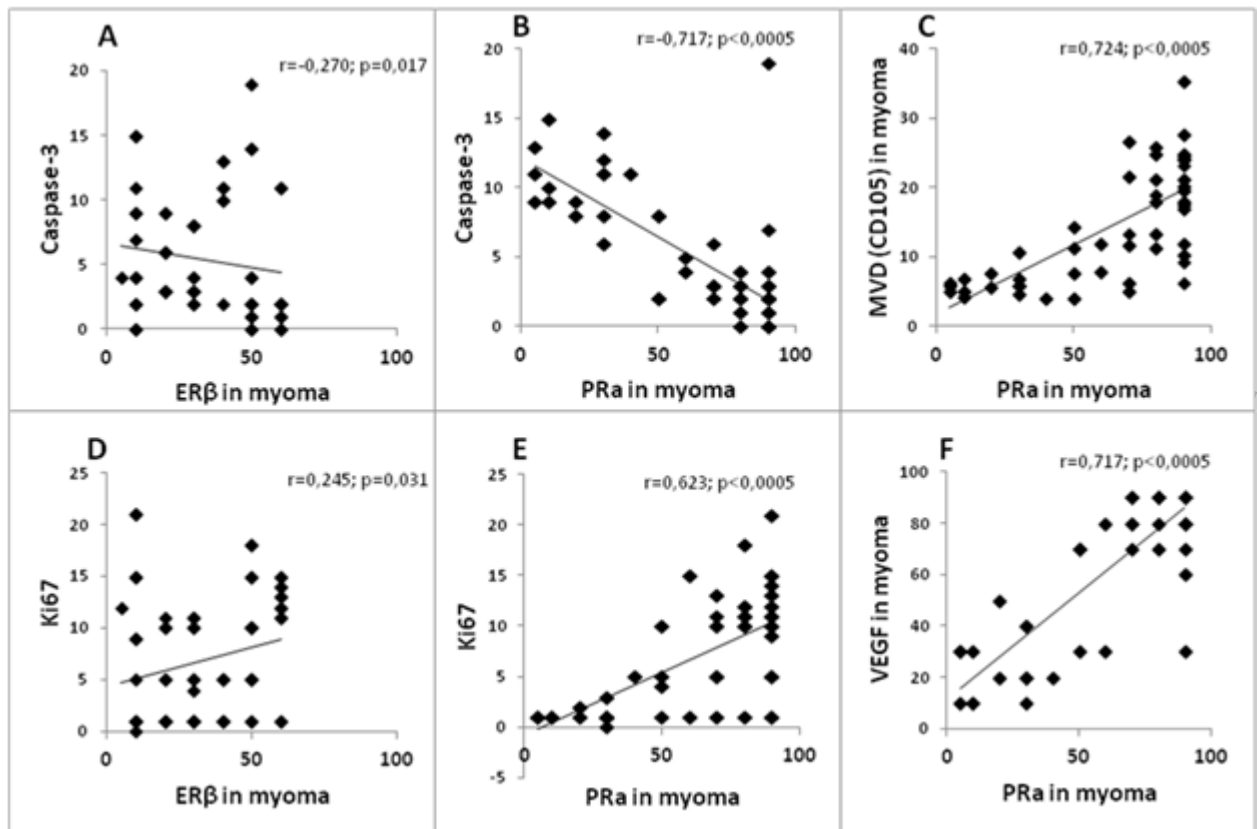


B



**Figure 5.** A: Immunohistochemical expression of CD105 (original magnification:  $\times 200$ ) in myometrium tissue of PreM and PostM women, representative tissue sections; B: percentage of expression of CD105 and statistical difference in percentage of myometrium cells between PreM and PostM women;

▲ – median; ■ – minimum; X – maximum



**Figure 6.** Correlation between all examined parameters in myoma tissue

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