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Evaluation of morphology, apoptosis, and cell proliferation of the uterus in postmenopausal women

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Summary. Background. The aim of this study was to evaluate the morphology (atrophy and fibrosis), apoptosis, and cell proliferation in the uterine wall. The research material came from postmenopausal women who had undergone hysterectomy due to uterine myomas or prolapse of the reproductive organ and were not taking menopausal hormone therapy (MTH).

Material and Methods. The collected material was divided into three groups. Group I (n=18) con-sisted of uterine sections taken 1 to 5 years after the last menstruation, Group II (n=17) 6 to 10 years after the last menstruation, and Group III (n=15) over 11 years after the last menstruation. To assess morphology and fibrosis, the uterine sections were subjected to hematoxylin and eosin (HE) staining and to Mallory's staining. In addition, we performed a histochemical examination to identify apopto-sis in endometrial and myometrial cells using the TUNEL method. An immunohistochemical analysis of endometrial and myometrial cells was also performed to detect the location of the proliferating cell nuclear antigen (PCNA).

Results. Differences in apoptosis were only found in the myometrium between Group I and Group III, and were strongest in Group I myometrial cells, and weakest in Group III. Neither the endome-trium nor the myometrium showed statistically significant differences in the overall percentage of PCNA(+) cells between groups.

Conclusion. Morphological changes in the endometrial and myometrial layers of postmenopausal uteri increased with time since the last menstruation.

Key words: Cell death, Proliferation, Morphological changes, Postmenopause

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Introduction

Demographic data show that about 25 million women worldwide go through menopause each year (Hill, 1996). The World Health Organization (WHO) defines menopause as the cessation of menstruation resulting from the loss of ovarian follicle function. Menopause can be considered from several perspectives: biological, according to which all the experiences of peri- and postmenopausal women, including typical symptoms, depend on hormonal changes taking place in their bodies; psychological (also called psychosocial), which emphasizes the role of stress and stressors as factors contributing to the manifestation and exacerbation of menopausal symptoms; socio-cultural or environmental, which takes into account the importance given to menopause in a given cultural circle. It refers to typical cultural views on menopause and the lifestyle characteristic of women (Posadzy-Małaczyńska, 2011). Most cancers of the reproductive organs, such as ovarian, endometrial, breast, and cervical cancer, are observed in perimenopausal and postmenopausal women (Dunneram et al., 2019).

Progressive apoptosis of ovarian follicles as well as increasing necrosis and fibrosis of ovarian stromal cells are the main causes of menopause (Brodowska, 2014; Takahashi and Johnson, 2015). The perimenopausal period is associated with a physiological decline in the levels of endogenous ovarian sex hormones. This process initiates a number of atrophic changes in the genitourinary system (Gałczyński et al., 2013; Delamater and Santoro, 2018). During menopause, structural changes in the ovary and uterus depend on multi-stage changes in the cyclicity and amount of peptides and steroid hormones produced. In the first stage of ovarian aging, we observe failure of the corpus luteum, followed by a complete lack of ovulation, resulting in a decrease in progesterone synthesis in the ovary (Gracia and

Freeman, 2018; Santoro et al., 2021). Progesterone deficiency, on the other hand, leads to the predominance of estrogen action in the body (relative hyperestrogenism), which is enhanced by a slight increase in estradiol synthesis in the ovary in response to increasing FSH levels (Gracia and Freeman, 2018; Santoro et al., 2021). Relative hyperestrogenism may cause acyclic heavy menstrual bleeding, uterine myomas, severe premenstrual syndrome, mastopathic breast changes, and endometrial hyperplasia. In the next stage of ovarian aging, the amount of estrogen produced by the ovaries is so low that the endometrium is not rebuilt and menstrual bleeding disappears (Brodowska et al., 2007; Santoro et al., 2021).

The most common clinical symptoms resulting from estrogen deficiency in postmenopausal women include: hot flashes, night sweats, sleep disorders, depressed mood, and atrophic changes in the genitourinary area (Santoro et al., 2021). Estrogen deficiency causes a decrease in the tone of the smooth and striated muscles of the urogenital diaphragm, atrophic changes in the connective tissue, a decrease in the thickness of the mucous membrane of the urethra and bladder, a decrease in collagen content, and changes in the connective tissue of the urogenital diaphragm. Additionally, as a result of these changes, dryness, irritation, inflammation, and dyspareunia may develop in the vaginal epithelium (Gracia and Freeman, 2018; Santoro et al., 2021).

In the postmenopausal period, the uterus undergoes atrophy. Its mucosa becomes thinner, which is caused by the transformation of the epithelium from cylindrical to cubic, and by the atrophy of the basal layer of cells along with the stroma. Atrophic changes are also observed in the mucous glands, which become short and narrow, and the amount of secretion decreases (Makuła et al., 2003).

Apoptosis is a key mechanism involved in the remodeling of the endometrium, leading to menstruation or preparing the endometrium for embryo implantation (Otsuki, 2001; Dahmoun et al., 2004; Lovely et al., 2005; Taniguchi et al., 2011; Boeddeker and Hess, 2015). In the final phase of the menstrual cycle, apoptosis increases. This means that apoptosis of cells in the endometrium is lowest in the proliferative phase, then increases in the secretory phase, and reaches its maximum in the menstrual phase (Vaskivuo et al., 2000; Harada et al., 2004). During the process of apoptosis, a number of changes take place in the cell in order to remove it safely. Such a cell separates from others, and due to dehydration and loss of electrolytes, it changes shape and shrinks.

The volume of the cytoplasm also changes, and the cell surface becomes undulating. Additional-ly, the cell nucleus is fragmented and apoptotic bodies are formed. By creating an insoluble envelope, the cell contents do not escape but are absorbed by neighboring cells or macrophages. Disturbances in the process of apoptosis may contribute to the development of congenital defects, that is, early in the life of the organism. This is because the correct formation of tissues and organs during embryo development (embryogenesis) depends on the process of cell elimination (Xavier, 2002). The pro-cess of removing unnecessary or body-threatening cells is carried out with the help of proteins and enzymes: caspases, which digest nuclear and cytoplasmic proteins; transglutaminases, which pro-duce apoptotic bodies; and endonucleases, which cleave the phosphodiester bond within polynucleo-tide chains.

A simplified scheme for activating the process of apoptosis in a cell is through the stimulation of external apoptotic factors (hypoxia, infection, toxins, lipopolysaccharides, irradiation), which cause the activation of pro-apoptotic proteins, such as Bax, which perforate mitochondrial membranes. From the damaged mitochondrion, cytochrome c (CC) is released, which together with caspase 9 and the Apaf-1 protein form the apoptosome complex (AS). The apoptosome activates a number of effector caspases (Casp 3/7) involved in the proteolysis of cell structural proteins (PS), DNA damage in the cell nucleus (JK), and other irreversible changes leading to cell death. Alternatively, apoptosis can be induced by the Fas/FasL system, which activates caspase 8 (Casp 8) via the death domain (DD). Caspase 8 triggers a cascade leading to the activation of effector caspases (Casp 3) (Harada et al., 2004; Bossowska et al., 2007). Under the influence of various factors, cells undergo programmed death or are directed to the path of survival. Endometrial stromal cells do not undergo apoptosis if the ovum is fertilized. During decidualization (formation of decidua) apoptosis is inhibited by human chorionic gonadotropin (hCG), although regulation of apoptosis also involves a complex set of events, with the stimulatory and inhibitory effects of many factors on programmed cell death (Szmidt et al., 2010). Excessive proliferation of endometrial cells is a pathological condition called endometrial hyperplasia. Endome-trial hypertrophy in women causes numerous symptoms, including abnormal uterine bleeding and impaired fertility. Endometrial hyperplasia is often defined as a precancerous lesion (Pintican et al., 2021). In the uterus, the proliferation of epithelial cells changes during the menstrual cycle and preg-nancy (Jabbour et al., 2006; Otify et al., 2015). Hormonal imbalance, resulting from an increase in estrogen levels accompanied by a lack or low levels of progestogens, causes excessive, irregular growth of the endometrial glands. The lack of progesterone prevents the endometrium from thinning properly, which results in its abnormal thickening, called endometrial hyperplasia. Untreated hyper-plasia may lead to the development of endometrial cancer, which is a common malignancy in meno-pausal and perimenopausal women (Armstrong et al., 2017; Sanderson et al., 2017; Talaulikar, 2022). However, if the hormonal balance is disturbed (estrogen levels increase), the endometrium will start to grow again. Additionally, due to the lack of hormones that inhibit growth and stimulate endometrial shedding, this process will be beyond the body's control (Sanderson et al., 2017; Talaulikar, 2022).

In postmenopausal women, with the cessation of ovarian hormonal function, the endometrium may undergo histological changes. After menopause, the thickening of the endometrium indicates high cell proliferation, which is also associated with inhibition of apoptosis (Ulin et al., 2020). In menopausal and perimenopausal women, the fibrotic process is manifested by the presence of myomas in the uterine wall. It is estimated that nearly 70% to 80% of women will develop benign tumors (myomas). Hormonal disorders associated with the perimenopausal period promote the formation of these benign lesions (Dutta and Talukdar, 2015). A decrease in steroid hormone synthesis in the ovary results in the failure of the corpus luteum in the ovary, followed by a complete lack of ovulation, which results in a decrease in progesterone synthesis in the ovary. In response to increasing FSH concentrations, we observe relative hyperestrogenism in the body, leading to uterine myomas (Sanderson et al., 2017). In contrast, menopause contributes to the regression of myomas, reducing their size and the symptoms they cause (Dutta and Talukdar, 2015). Uterine myomas are associated with their location, size, and number. Clinical signs include: heavy menstrual bleeding, bleeding between menstrual periods, prolonged menstrual periods, pressure or pain in the pelvis, a feeling of fullness in the lower abdomen, bloating, frequent urination, constipation (Bouchard, 2011; Sanderson et al., 2017).

In recent years, due to the increase in life expectancy, the problems of menopausal women have re-ceived increasing attention. Four reproductive organ cancers (breast, cervical, ovarian, and endome-trial) are among the top ten most common cancers among women worldwide. The increased risk of induction of endometrial cancer in postmenopausal women is probably associated with the influence of high concentrations of circulating pituitary gonadotropins in the absence of ovarian response in the form of increased production of steroid hormones. For this reason, it was important to answer the question of whether the time elapsed since menopause affects the structure and function of the uterus, and to evaluate the activity of apoptosis and proliferation within the mucosa and the muscular wall of the uterus depending on the time elapsed since menopause (Brodowska et al., 2007; Black et al., 2016).

The aim of our study was to evaluate the morphology, apoptosis, and proliferation of endometrial cells in postmenopausal women with regard to the time since the last menstruation.

Materials and methods

Material

The research material consisted of 50 paraffin blocks containing uterine body sections taken from post-menopausal women who had undergone hysterectomy due to uterine myomas or prolapse of the reproductive organ. The research material was obtained from Polish women (Caucasian) aged 44-70. The women were not on menopausal hormone therapy (MHT), which was confirmed by the results of serum FSH levels. The patients were not treated for cancer or endometrial hyperplasia and the patients declared that they had not had an abortion. The patient did not report postmenopausal bleeding. The criteria for exclusion from the study were: active cancer disease, alcoholism, thyroid diseases, and taking glucocorticosteroids. They were hospitalized in the gynecology wards of the Janusz Korczak Provincial Specialist Hospital in Słupsk, and the Independent Public Specialist Healthcare Center "Zdroje" in Szczecin between 2012-2013. The patients' uteri were divided into three groups according to the time since the last menstruation: Group I (n=1), up to 5 years; Group II (n=17), from 6 and 10 years; and Group III (n=15), over 11 years.

The study was approved by the Bioethical Commission of the Pomeranian Medical University in Szczecin (approval number: KB-0012/56/11/11). All participants gave their informed written consent to take part in the study.

Methods

Morphological analysis

Paraffin blocks (n=50) were assigned to groups depending on the time that had elapsed since the last menstrual period. Paraffin blocks with embedded uterine tissues were cut into 3-4 μm thick sections on a microtome (MIKROM, MH 325). They were then placed on basic slides, as well as on polylysine-coated slides. These sections included: the mucous, muscular, and serous membranes. Then, to assess uterine morphology, basic histological staining was performed using the HE (hematoxylin-eosin) method.

The thickness of the endometrium and myometrium of the uterine body was measured using the Image Scope viewer software tool panel (Aperio Technologies, Inc., Vista, CA, USA). In each preparation, six measurements of each of the examined structures (endometrium, myometrium) were made, excluding extreme values. From the obtained results, the mean and standard deviation of the thickness of the endometrium and myometrium were calculated (data not shown). Analysis of uterine fibrosis was performed using the Mallory method. To evaluate uterine morphology, 10 slides were selected from each group and histological staining was performed using the Mallory method. Fibrosis was assessed based on the changes occurring both under the surface epithelium and in the periglandular area. Sections of the uteri were deparaffinized and hydrated in a series of alcohols of decreasing concentration. The slides were then stained with 0.5% acid fuchsin for 5 minutes. In the next step, 1% phosphotungstic acid was spotted on the slides and the slides were incubated for 1

minute. To visualize collagen fibers, the slides were stained with Mallory's reagent (7 minutes). The slides were then dehydrated in a series of graded alcohols and finally xylene. The slides were sealed in a Histokit (Mar-Four, Łódź, Poland). The slides were evaluated under a light microscope (BX41, Olympus Optical Co., Ltd., Japan) using magnifications x50 and x200.

Cell apoptosis test (TUNEL method)

To determine apoptotic cells in the uteri of postmenopausal women, sections of the uteri were deparaffinized and hydrated in a series of alcohols of decreasing concentration. Antigenic determinants were exposed by proteinase K (10 µg/ml in 0.05 M Tris/HCl pH 7.6) digestion (Dako Denmark A/S, Glostrup, Denmark). Peroxidase Blocking Solution was used to block the activity of endogenous peroxidase. This step was performed in a humid chamber for 10 minutes. Next, the uterine sections were incubated with terminal deoxynucleotidyl transferase (TdT, Millipore, Billerica, MA, USA) for 60 minutes in a humid chamber at 37°C. The sections were then incubated with anti-digoxigenin antibodies in a humid chamber at room temperature for 30 minutes. The chromogen used to visualize the histochemical reaction was 3,3' Diaminobenzidine (DAB, Dako Denmark A/S, Glostrup, Denmark), and the contrast dye was Mayer's hematoxylin. Each step of the reaction was preceded by rinsing the slides with PBS wash buffer. The last stage of the reaction was dehydration of the slides and placing them in a Histokit. A negative control was also performed for the TUNEL reaction. The slides were evaluated under a light microscope (BX41, Olympus Optical Co., Ltd. Japan).

Immunohistochemical analysis

To determine the location of the proliferating cell nuclear antigen (PCNA) in the uteri body of the postmenopausal women (n=50), sections of the uteri were deparaffinized and hydrated in a series of alcohols of decreasing concentration. The next step was to boil the uterine slides in a pH 9.0 buffer in a water bath at 96°C for 30 minutes. Peroxidase Blocking Solution was used to block endogenous peroxidase activity. This step was performed in a humid chamber at room temperature for 10 minutes. The sections were then incubated for 30 minutes in a humid chamber with a mouse monoclonal IgG antibody against PCNA, clone PC10 (Dako Denmark A/S, Glostrup, Denmark) diluted 1:200. The next step was to apply a horseradish peroxidase (HRP)-conjugated secondary antibody to the sections. The chromogen used to visualize the immunohistochemical reaction was DAB. Contrast staining was performed with Mayer's hematoxylin. Each step of the reaction was preceded by washing the slides in a wash buffer solution (PBS). The sections were dehydrated in a series of graded alcohols (from 70% alcohol to xylene) and sealed in a Histokit. A negative control for PCNA was performed on the sections. Immunohistochemical evaluation of the slides was also carried out under a light microscope (BX41, Olympus Optical Co., Ltd Japan).

Computer image analysis

The slides were scanned at 200x magnification and 0.25 µm resolution with a ScanScope AT2 scanner (Leica Microsystems, Germany). The obtained digital images were analyzed using the Image Scope software (version 11.2.0.780; Aperio Technologies, Inc., Vista, CA, USA). Quantitative analysis of cell apoptosis (TUNEL method) and PCNA marker location was performed in areas of comparable surface area (0.25 mm^2), cell nuclei showing a positive reaction (stained brown) were counted, and their percentage in relation to all counted nuclei was determined using the Nuclear v.9 algorithm (version 11.2.0.780; Aperio Technologies, Inc., Vista, CA, USA) for computer analysis. The areas of analysis were also manually determined. The reproducibility of histochemical and immunohistochemical reaction results was obtained by analyzing 30 randomly selected comparable surface areas in the endometrium and myometrium of the uterine sections. The mean surface areas were analyzed in each group to obtain repeatable results. Analyzes were performed for all patients.

Statistical analysis

The SPSS Statistics version 17.0 software (StatSoft, Krakow, Poland) was used to perform statistical analysis of the data. Basic statistics, such as arithmetic mean ($\bar{X}$) standard deviation (SD), and minimum (Min) and maximum (Max) values were calculated. For distributions deviating from the normal distribution for independent samples, the Mann-Whitney U test was used and the Kruskal-Wallis test was used to compare groups. Correlations between parameters were assessed using Spearman's rho correlation coefficient. A correlation coefficient of 0.0-0.2 was assumed as a lack of correlation, 0.2-0.4 weak correlation, 0.4-0.6 moderate correlation, 0.6-0.8 strong correlation, 0.8-1.0 very strong correlation. The level of significance was set at $p \leq 0.05$.

Results

The morphology of the uterine wall was evaluated using uterine sections collected from postmenopausal women with regard to the time elapsed since the last menstruation. Changes included physiological atrophy of the organ (involution), the ectopic location of the endometrium within the myometrium (internal endometriosis), and progressive fibrosis. We also compared the percentage of apoptotic cells in the endometrium and myometrium according to the time since menopause. The immunohistochemical analysis-evaluation of PCNA expression-allowed us to assess the

proliferative capability in the endometrium and myometrium of postmenopausal women depending on the time since the last menstruation.

Morphological structure of postmenopausal uteri stained with HE

Group I consisted of uterine sections collected from women up to 5 years after the last menstruation. Microscopic observation did not reveal any differences in the structure of the endometrium between postmenopausal and reproductive-age women (Teresiński et al., 2019; Ulin et al., 2020). Uteri were covered with a simple cylindrical epithelium, with visible epithelial inclusions formed by glandular ducts. The shape of epithelial cells, the size of cell nuclei, and the amount of cytoplasm were normal. So was the structure of connective tissue stromal cells. Composed of normal smooth myocytes, the myometrium was formed by interlacing bundles of smooth muscle cells. Imaging of the myometrium showed cross-sections of morphologically unchanged blood vessels. There were no signs of mitotic activity or a neoplastic process in the uterine wall (Fig. 1).

Group II consisted of uterine sections collected from women 6 to 10 years after menopause. In the microscopic image, slight signs of organ involution were observed in the uterine wall. The mucosa was covered with a simple cylindrical epithelium without significant changes. The epithelium penetrated the mucosa (stroma) forming glandular ducts. In some slides, the glandular ducts were changed and dilated (enlarged). The simple cylindrical epithelium lining the glandular ducts evolved into a simple cubic epithelium (metaplasia). The myocytes forming the myometrial layer had spindle-shaped, blunt-ended nuclei. In the slides, there were no signs of mitotic activity in these cells (Fig. 1).

Group III consisted of uterine sections collected from women over 11 years after menopause. A large area of the mucosa was covered with a simple cubic epithelium. This epithelium penetrated the stroma. Histological signs of organ involution were changes in the shape of epithelial cells from cylindrical to cubic, visible as a characteristic flattening of the tubular epithelium. The microscopic image of histological slides showed cystic dilatation of glandular ducts in the connective tissue mucosa. In the uteri of Group III women, morphological changes were also found in myocytes in the myometrial layer - myocyte cell nuclei were small, and the amount of cytoplasm was reduced (Fig. 1). No signs of cell division or other changes indicating an ongoing pathological process were identified in the slides.

The process of myometrial fibrosis was observed in all study groups. It was especially apparent on the serous membrane side, while on the endometrium side, fibrosis was very faint. There were no significant differences in the process of myometrial fibrosis between groups (Fig. 2).

Evaluation of apoptosis in uterine cells depending on the time since the last menstruation

In all groups, TUNEL(+) cell percentages and their location were assessed by computer image analysis, in both the endometrium and myometrium of the uterine wall. In the endometrium, there were no statistically significant differences in the histochemical reaction of TUNEL(+) (p=0.942) and TUNEL(-) (p=0.942) cells between groups. It is noteworthy, however, that the percentage of cells showing apoptosis was highest in Group II (Tables 1, 2, Fig. 3).

In the myometrium, there were statistically significant differences between all groups regarding the histochemical reaction of TUNEL(+) cells. Statistically significant differences were found between all groups (Group I vs. Group II vs. Group III) in the total percentages of TUNEL(+) cells (29.60 vs. 29.62 vs. 13.03) (p=0.048) and TUNEL(-) cells (70.37 vs. 70.41 vs. 86.97) (p=0.050) (Table 1). Groups I and III statistically significantly differed (p=0.029) in terms of the total number of apoptotic cells in the myometrium, which indicated a decrease in the apoptotic process with time from the last menstruation (Fig. 3).

The lack of significant differences in the percentage of cells undergoing apoptosis in the endometrium indicates hormonal changes in postmenopausal women who have significant estrogen deficiency. Therefore, if women do not have a menstrual cycle, the endometrium does not undergo cyclical changes and intensive growth of the mucous membrane, differentiation, exfoliation,

Table 1. Percentages of apoptotic cells in the endometrium and myometrium of postmenopausal women.

Percentage of cells	min-max; Mean ± SD			*p*
	Group I	Group II	Group III	
Endometrium				
TUNEL(+) cells	1.92-42.62; 26.39±15.60	7.56-60.95; 31.83±21.27	13.43-56.13; 27.46±19.95	0.942
TUNEL(-) cells	57.38-98.08; 73.61±15.60	39.05-92.44; 68.17±21.27	43.87-86.57; 72.54±19.95	0.942
Myometrium				
TUNEL(+) cells	7.21-51.92; 29.60±13.91	11.03-55.19; 29.62±15.48	3.79-24.09; 13.03±8.42	0.048*
TUNEL(-) cells	48.08-92.79; 70.37±13.92	44.81-88.97; 70.41±15.33	75.91-96.21; 86.97±8.42	0.050*

Min, minimum; Max, maximum; SD, standard deviation; p, statistical significance; *, statistically significant parameter.

and regeneration. In the myometrium, the process of apoptosis is the weakest in Group III, i.e., in patients over 11 years after menopause. This may indicate the blocking of apoptosis as a result of increased expression of molecular factors, e.g., BCL2 protein. The obtained results may indicate a disruption of the apoptosis process in the uterine myometrium depending on the time elapsed since the last menstrual period, which may contribute to the formation of, for example, uterine fibroids.

Evaluation of proliferation in uterine cells depending on the time since the last menstruation

Immunoexpression of PCNA(+) cells was found in both the endometrium and myometrium in all study groups. The percentage of PCNA(+) cells depending on their location was assessed by computer image analysis.

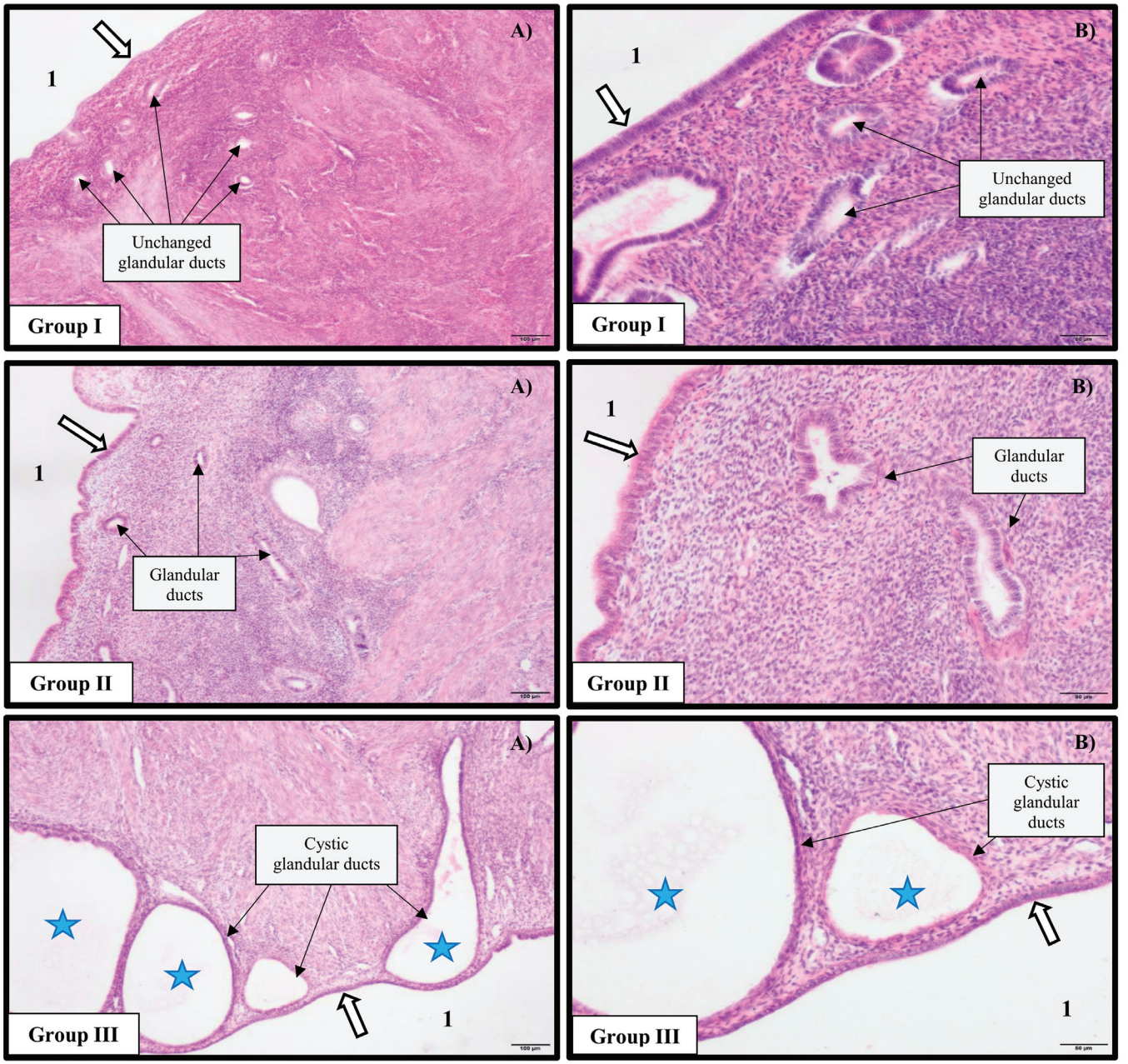

Fig. 1. HE staining of uterine sections in the study groups. 1: Lumen of the uterine cavity. White arrow: The endometrial epithelium in Groups I and II is a simple columnar epithelium; in Group III it is a simple cubic epithelium; in Groups I and II unchanged tubuli lined with a simple columnar epithelium; in Group III extended cystic tubes lined simple columnar epithelium. Blue star: Lumen of widened tubuli. A, x 40; B, x 200.

Neither the endometrium nor the myometrium showed statistically significant differences in the percentage of PCNA(+) cells between groups, p=0.809 and p=0.737, respectively. Similarly, no differences were noticed in the percentage of PCNA(-) cells in the endometrium (p=0.853) and myometrium (p=0.822) between all three groups (Tables 3, 4). In both the endometrium and myometrium, there was a noticeable trend toward a weakening in cell proliferation (Fig. 4).

The research results indicate that, after the onset of menopause and the decline in hormones, there is no significant decrease in cell proliferation in the endometrium or myometrium with the time since the last menstrual period. This may mean that postmenopausal hormonal changes no longer have such a strong impact on the proliferation process as during the menstrual cycle, but they do not weaken this process. However, the observed trend of decreased proliferation in the layers of

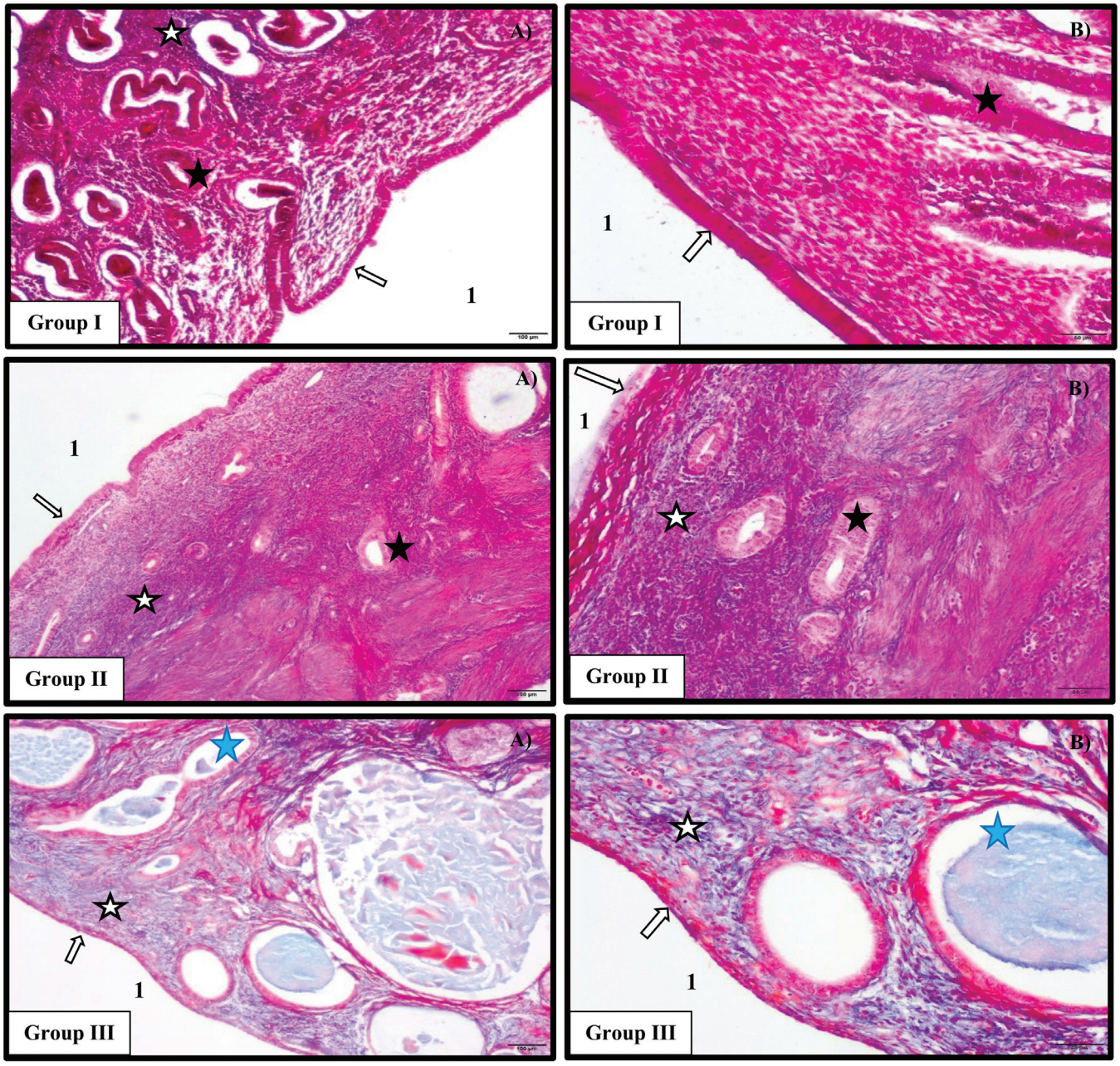

Fig. 2. Endometrial fibrosis. Visible progressive fibrosis in the study groups. 1: Lumen of the uterine cavity. Blue star: Fibrosis. White arrow: Endometrial epithelium. White star: Lumen of widened tubuli. Black star: Unchanged tubuli. Mallory staining: collagen fibers - stained blue, connective tissue stromal cells, epithelial cells, smooth myocytes - dark pink. A, x 40; B, x 200.

the uterine corpus may indicate the progress of organ atrophy. If there was no downward trend in the proliferation process, it is quite likely that women could develop endometrial hyperplasia or even cancer.

Correlation between apoptosis and proliferation in the uterine endometrium and myometrium, depending on the time since the last menstruation.

We analyzed the correlation between the number of cells undergoing apoptosis and the number of proliferating cells in the endometrium and myometrium, depending on the time since the last menstruation (Table 5). Among all analyzed preparations, the number of TUNEL(+) cells in the endometrium was shown to positively correlate with the number of TUNEL(+) cells in the myometrium (R=0.621, p=0.005). A positive correlation was also demonstrated between the number of TUNEL(+) cells and the number of PCNA(+) proliferating cells in the endometrium (R=0.639, p=0.004). In addition, the number of TUNEL(+) cells in the myometrium positively correlated with the number of PCNA(+) cells in the endometrium (R=0.628, p=0.004). Furthermore, the number of PCNA(+) cells in the endometrium positively correlated with the number of PCNA(+) cells in the myometrium (R=0.747, p<0.001).

In Group I, the number of TUNEL(+) cells in the endometrium correlated with the number of TUNEL(+) cells in the myometrium (R=0.857, p=0.007) and with the number of PCNA(+) cells in the endometrium (R=0.905, p=0.002). Correlations were also found between PCNA(+) cells in the endometrium, TUNEL(+) cells in the myometrium (R=0.714, p=0.047), and PCNA(+) cells in the myometrium (R=0.762, p=0.028). In Group I, proliferation in both the endometrium and myometrium occurs at the highest level. The obtained analysis results indicate that along with the proliferation of endometrial cells, the process of apoptosis occurs.

In Group II, the number of TUNEL(+) cells in the myometrium positively correlated with the number of PCNA(+) cells in the endometrium (R=0.943, p=0.005), and PCNA(+) cells in the myometrium (R=0.829, p=0.042). Positive correlations were also shown between PCNA(+) cells in the endometrium and the number of PCNA(+) cells in the myometrium (R=0.821, p=0.023).

In Group III, no correlations were found between the above-mentioned parameters. This indicates reduced sensitivity of cells to signals initiating cell proliferation and apoptosis, which is associated with progressive organ atrophy.

The analyzed values of significant correlations are positive, which means that as the percentage of TUNEL(+) cells in the endometrium or myometrium decreases, the percentage of PCNA(+) cells will also decrease. This indicates that both processes are interdependent or subject to the same changes depending on the time that has passed since the last menstrual period. The processes of apoptosis and proliferation are interdependent in the uterus of women up to 10 years after meno-pause, however, no such relationship is observed beyond 11 years after menopause.

Table 2. Statistical relationship between the percentage of apoptotic cells in the endometrium and myometrium and the time since the last menstruation.

Percentage of TUNEL(+) cells	*p*-value		
	Group I vs. II	Group I vs. III	Group I vs. III
Endometrium	0.772	0.932	0.925
Myometrium	0.096	0.029*	0.054

TUNEL(+) cells, cells undergoing apoptosis in the endometrium and myometrium; *, statistically significant parameter.

Discussion

There are many scientific publications on the structure of the female uterus but most concern the reproductive period. Comparative studies mainly focus on the changes that occur in the endometrium and myometrium of the uteri of pre- and postmenopausal women. A study by Dutta and Talukdar (2015) presented differences in the morphology of the endometrium of pre- and postmenopausal women. So far, changes in the uterine layers depending on the time since the last menstruation have not been described in detail (Teresiński et al., 2019; Ulin et al., 2020).

Table 3. The percentages of cells showing expression of PCNA(+) in the endometrium and myometrium in particular groups.

Percentage of cells	min-max; Mean ± SD			*p*
	Group I	Group II	Group III	
Endometrium				
PCNA(+) cells	22.13-86.09; 50.55±18.22	13.90-78.94; 45.38±23.79	1320.10-73.04; 42.60±20.49	0.809
PCNA(-) cells	13.91-77.87; 50.43±18.55	21.06-86.10; 54.62±23.79	26.96-79.90; 57.40±20.49	0.853
Myometrium				
PCNA(+) cells	14.32-75.04; 40.01±21.03	9.17-62.10; 34.85±62.10	14.71-58.11; 31.05±15.78	0.737
PCNA(-) cells	24.96-85.68; 61.73±20.15	37.90-90.83; 65.15±19.83	41.89-85.29; 69.31±15.46	0.822

PCNA(+), proliferating cell nuclear antigen; Min, minimum; Max, maximum; SD, standard deviation; *p*, statistical significance.

Changes that occur in the uterus after menopause mainly affect its body. In the postmenopausal period, we observe endometrial atrophy, which is often accompanied by the transformation of glan-dular epithelial cells from cylindrical to low-cylindrical or cubic (Teresiński et al., 2019). It has also been found (Dutta and Talukdar, 2015) that the postmenopausal endometrium consists of only the basal layer (stratum basalis), and it does not show any signs of proliferative or secretory activity.

Otify et al. (2015) reported that the histological picture of the endometrium depended on hor-mone levels in the female body just before the onset of menopause. If the last menstrual cycle ended with poor proliferation or secretion, endometrial atrophy manifested as narrow glands lined with atrophic epithelium.

According to Dutta and Talukdar (2015), the myometrium becomes thinner in the postmeno-pausal period. The myometrial stroma is made up of long, slender myocytes, arranged in hard-to-define layers. The outer and innermost layers of the myometrium are thin and composed of myocytes arranged longitudinally and transversely to each other.

Our analysis of the morphology of the uterine wall showed no significant differences between the endometria of women up to five years after menopause and those of healthy reproductive-age women. In Group I, the uterine endometrial layers had numerous glandular ducts lined with a simple cylindrical epithelium. Connective tissue stromal cells surrounding glandular ducts were typically shaped and not adjacent to each other. Boon et al. (1999) studied the uterine layers of perimenopausal women. They examined 142 uteri without signs of hyperplasia. The endometria were classified as abnormal secretory (41.5%), with impaired proliferation (2%), and mixed-secretory or non-secretory (14.1%). These authors concluded that histological classification of the endometrium used for cyclic changes in healthy women should not always be used to describe changes occurring in the endome-trium in the perimenopausal period (Boon et al., 1999). It should also be noted that the morphologi-cal picture of the endometrium in perimenopausal women is neither homogeneous nor characteristic. This was confirmed by Deeba and Khan (2016) and Forae and Aligbe (2013) who assessed histo-logical changes in the endometrium of postmenopausal women diagnosed with abnormal vaginal bleeding. In the study by Deeba and Khan (2016), 27 out of 110 women (24.5%) were diagnosed with endometrial atrophy; however, the patients were not classified according to the time since the last menstruation.

In our study, signs of organ involution were observed in women who were 6-10 years post-menopausal (Group II). The endometrium was thinner and covered with a simple cylindrical epithe-lium. In only a few slides, glandular ducts were dilated and covered with cubic epithelium. Our ob-servations are in line with those reported by Noci et al. (1996), who analyzed the uteri of 28 post-menopausal women, finding atrophic changes in 12 and a hyper-trophic endometrium in 16. There were spindle-shaped nuclei in the muscle cells of the myometrial layer.

In Group III (over 11 years since the last menstruation), we observed more advanced uterine changes, indicating organ involution. The endometrium was thinner than in Groups I and II. As a result of endometrial changes, epithelial cells transformed from cylindrical to cubic, which was visi-ble as a characteristic flattening of the epithelium. Additionally, cystically dilated glandular ducts lined with a simple cubic epithelium were visible in the connective tissue mucosa. Changes were also ob-served in myometrial myocyte cell nuclei and the amount of cytoplasm was significantly reduced.

Table 4. Statistical relationship between the percentage of apoptotic cells in the endometrium and myometrium and the time since the last menstruation.

Percentage of PCNA(+) cells	*p*-value		
	Group I vs. II	Group I vs. III	Group II vs. III
Endometrium	0.772	0.561	0.830
Myometrium	0.736	0.459	0.772

PCNA(+), proliferating cell nuclear antigen.

Table 5. Correlations between TUNEL(+) and PCNA(+) cells in uteri of postmenopausal women.

Location of cells showing positive immunohistochemical reaction		Whole study sample		Group I		Group II		Group III	
		R	*p*	R	*p*	R	*p*	R	*p*
TUNEL(+) endometri-um	TUNEL(+) myometrium	0.621	**0.005***	0.857	**0.007***	0.750	0.052	0.400	0.600
TUNEL(+) endometri-um	PCNA(+) endometri-um	0.639	**0.004***	0.905	**0.002***	1.000	0.826	-0.600	0.400
TUNEL(+) endometri-um	PCNA(+) myometrium	0.323	0.191	0.476	0.233	0.714	0.111	-0.600	0.400
TUNEL(+) myometrium	PCNA(+) endometri-um	0.628	**0.004***	0.714	**0.047***	0.943	0.005*	0.300	0.624
TUNEL(+) myometrium	PCNA(+) myometrium	0.388	0.082	0.033	0.932	0.829	**0.042***	0.257	0.623
PCNA(+) endometri-um	PCNA(+) myometrium	0.747	**<0.001***	0.762	**0.028***	0.821	0.023*	0.657	0.156

TUNEL(+), cells undergoing apoptosis; PCNA(+), proliferating cell nuclear antigen; R, correlation coefficient; *p*, statistical significance; *, statistically significant parameter.

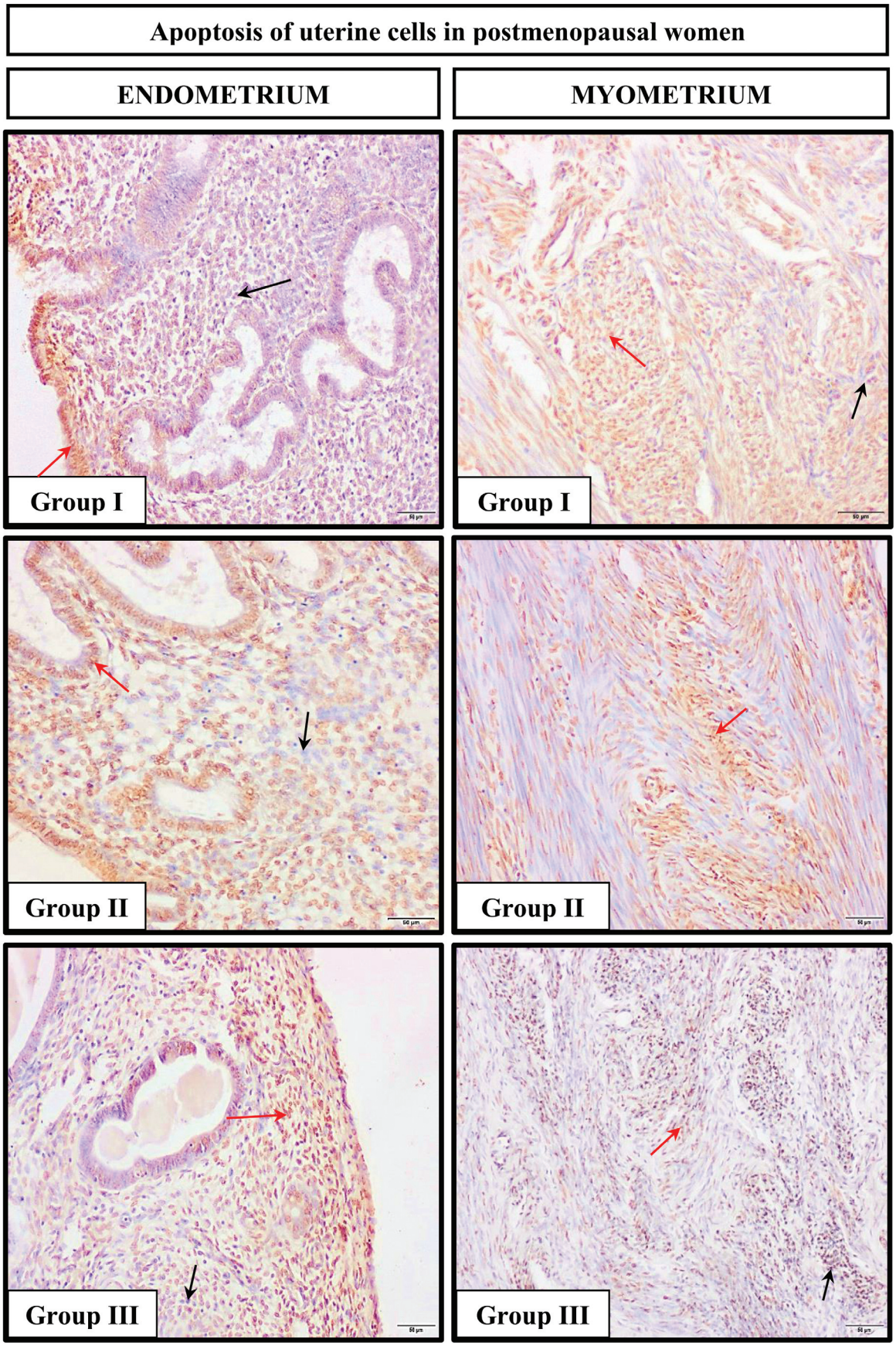

Fig. 3. Location of the apoptosis marker in endometrial and myometrial cells of the endometrium of postmenopausal women (TUNEL reaction). Cell nuclei showing the TUNEL(+) reaction stained brown. TUNEL(+) cells: Red arrow; TUNEL(-) cells: Black arrow). x 100.

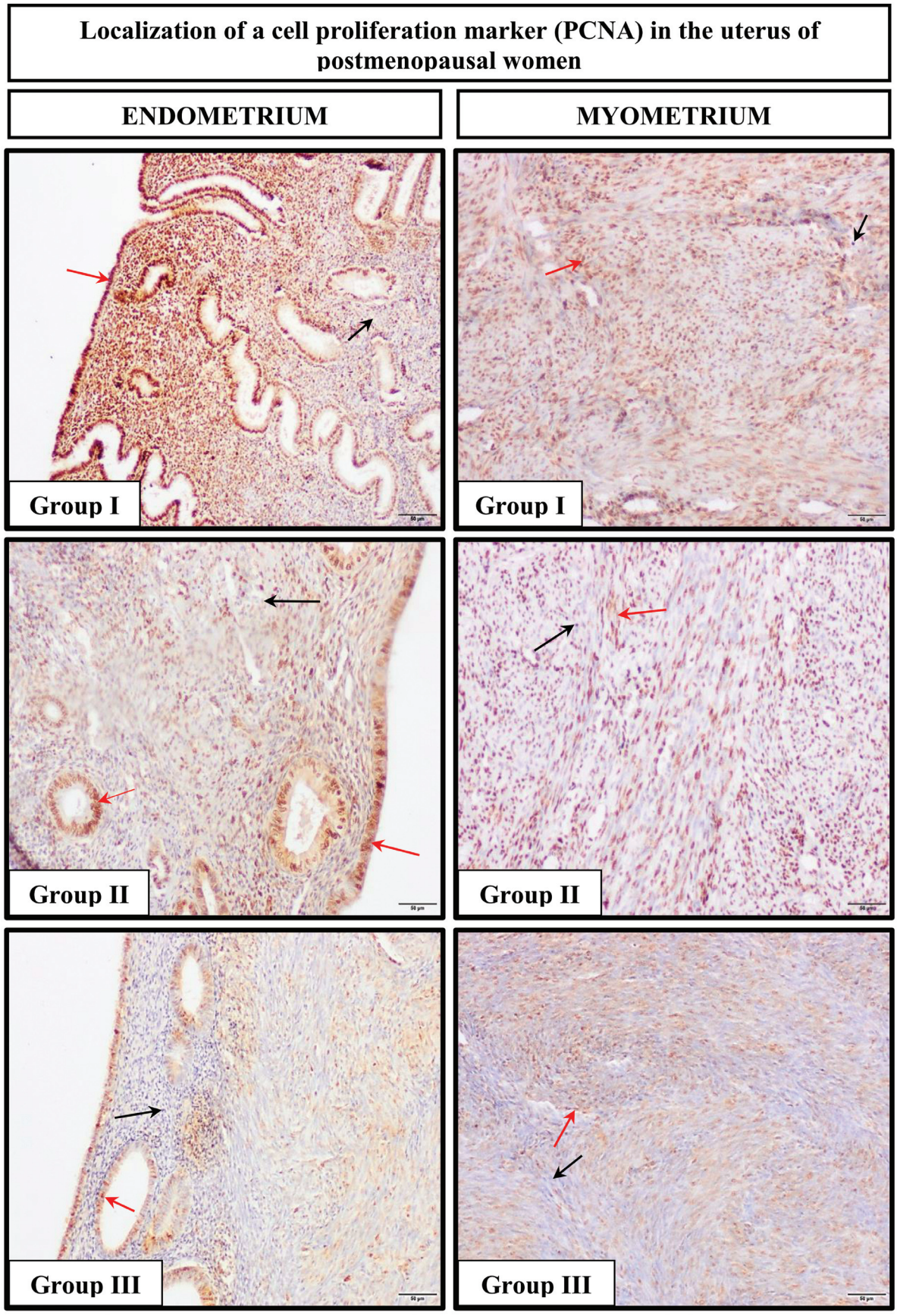

Fig. 4. Localization of the nuclear marker of proliferation (PCNA) in endometrial and myometrial cells of the uterine body. Cell nuclei showing the PCNA(+) reaction stained brown. PCNA(+) cells: Red arrow; PCNA(-) cells: Black arrow). x 100.

The morphological changes observed in women more than 11 years after the last menstruation were similar to those reported by other author (Teresiński et al., 2019).

Histological findings by other authors (Sivridis and Giatromanolaki, 2004) also indicated fea-tures of uterine involution in women who had undergone menopause. Fibrosis and atrophy were noted in both the endo-metrium and myometrium of postmenopausal women, as well as pathological changes in epithelial and stromal cells. Fibrosis was found in the endometrial stroma due to an in-crease in the number of collagen fibers. The increasing fibrosis was accompanied by a decrease in the number of endometrial secretory glands (Otify et al., 2015).

In our study, with the time since the last menstruation, progressive fibrosis of uterine tissues was observed in both the endometrium and the myometrium, as confirmed by Mallory's triple stain (collagen fibers turned blue). This process was weakest in Group I and more pronounced in Group III. The greatest number of stained collagen fibers was visible in Group III, where fibrosis was most visible in the superficial layers of the endometrium. In the myometrium, on the other hand, there were no differences in the stage of fibrosis between groups, which seems to indicate that the time since the last menstruation did not affect the fibrotic process in this uterine layer. Our findings sup-port those reported by Teresiński et al. (2019), who used a different staining method (Masson's Tri-chome) to identify fibrosis in the uteri of postmenopausal women. The results of our studies (Table 6) indicate progressive changes related to fibrosis and organ atrophy, which is consistent with the stud-ies of other authors (Noci et al., 1996; Sivridis and Giatromanolaki, 2004; Teresiński et al., 2019) that have been conducted to date.

Apoptosis plays a key role in maintaining tissue homeostasis and promotes the process of re-moving excess or damaged cells. Studies show that it helps to maintain homeostasis during the men-strual cycle. This is achieved by eliminating senescent cells from the functional layer of the endome-trium during the secretory and menstrual phases of the menstrual cycle. According to Harada et al. (2004), disruption of the apoptotic process is one of the causes of endometriosis in women, which is an estrogen-dependent disease. One way to treat it is to lower the concentration of estrogen to those levels observed in postmenopausal women (Harada et al., 2004). So far, studies of apoptosis in perimenopausal and postmenopausal women have mainly focused on the treatment of uterine myo-mas. Antiproliferative and proapoptotic treatment significantly reduces the size of myomas in the myometrium by increasing apoptosis and reducing proliferation (Palomba et al., 2005).

In our study, there were no significant differences in the intensity of endometrial cell apoptosis depending on the time since the last menstruation. However, the total number of TUNEL(+) cells undergoing apoptosis in the myometrium differed between Groups I and III, as assessed by com-puter image analysis. Apoptosis in the myometrium was stronger in Group I than in Group III, which indicates that the process of removing redundant or damaged myometrial cells proceeds nor-mally up to five years after menopause. Cell apoptosis was weaker in Group III, which may suggest that it depends on the levels of female steroid hormones. The weakening process of apoptosis in the myometrium is associated with a weakening of the cell's response to factors initiating the apoptosis process. Clinically, the

Table 6. Morphological changes and fibrosis in the endometrium and myometrium of the uterine body, according to the time since the last menstrual period.

Group	ENDOMETRIUM		
	Endometrial epithelium	Tubuli	Fibrosis
I	Simple columnar epithelium	Numerous Lined with simple co-lumnar epithelium	In deeper layers, Poorly visualized with Mallory staining
II	Simple columnar epithelium	Numerous, sometimes cystically dilated with cubic epithelium	Throughout the entire thickness of the endome-trium Well visualized with Mallory staining
III	Simple cubic epithelium	Few, cystically dilated with cubic epithelium trans-forming into squamous epithelium	In the superficial layers of the endometrium, just be-low the epithelium Very well visualized with Mallory staining
Group	MYOMETRIUM		
	Myocytes	Blood vessels	Fibrosis
I	Acidophilic cytoplasm Clavate-shaped cell nuclei Large cells	No changes in the structure of vessel walls	Observed from the serosal surface, no differences between the studied groups
II	Acidophilic cytoplasm Spindle-shaped cell nuclei Medium-sized cells	Thickening of vessel walls	
III	Acidophilic cytoplasm Spindle-shaped cell nuclei Medium-sized cells	Significant thickening of the vessel walls	

weakening process of apoptosis is unfavorable for changes taking place in the myometrium depending on the time that has passed since the last menstrual period. As a result of a decrease in hormonal activity and a weakening process of apoptosis, uterine fibroids may develop.

In healthy women, the size of the uterus decreases with the time since the last menstruation, and the endometrium should not be more than 5 mm thick (3.6 mm on average). As stated by Merz et al. (1996), the same thing happens to leiomyomas. Wu et al. (2000), who analyzed cell pro-liferation (Ki-67 antibody) and cell apoptosis (TUNEL method) in the uteri of postmenopausal women, found that both the expression of Ki-67 protein and the rate of TUNEL(+) cells in both leiomyomas and the myometrium were low. The results obtained by these authors indicated that ster-oid sex hormones, whose concentrations decrease with age, affect the mechanisms that take place in uterine tissues through the processes of proliferation and apoptosis. Another study by the same au-thors (Wu et al., 2002) also implied that apoptosis in the postmenopausal myometrium decreases, which is manifested by a decline in the expression of anti-apoptotic proteins (Bcl-2), contributing to the development of benign smooth muscle tumors (leiomyomas). This is consistent with our findings. Banas et al. (2018) investigated the expression of apoptotic proteins (the DFF40/DFF45 protein complex and the Bcl-2 protein) in the endometrium and myometrium in the proliferative and secretory phases during the menstrual cycle and after menopause. As they reported, the DFF45 pro-tein involved in the activation of caspase 3, and thus the process of apoptosis, is dependent on the menstrual cycle. The expression of this protein was highest in the endometrium during the secretory phase. Moreover, the expression of the DFF40 and DFF45 proteins was significantly lower after menopause. Our results are in line with those described above, showing that after menopause apop-tosis in the myometrium declines, and this downward trend continues over time (a lower total num-ber of TUNEL (+) cells in Group III).

In the physiological endometrium, estrogen and progesterone regulate the balance between pro-liferation and apoptosis (Otify et al., 2015). Otify et al. (2015) claimed that the endometria of post-menopausal women contain progesterone receptors, whose clusters are located in the vicinity of polyps, which indicates an imbalance between the processes of proliferation and apoptosis, and leads to pathological changes. They also found that an imbalance between cell proliferation and apoptosis is the cause of uterine myomas. Scientific research and clinical evidence show that the detection of PCNA may be useful in the analysis of cellular proliferation in the uterus, as confirmed by the stud-ies of Sánchez-Rosales et al. (2004) and Amitkumar et al. (2017). PCNA is a protein located in the nuclei of proliferating cells. In the cell cycle, the amount of PCNA increases during the G1 phase, then reaches a lower level during the S phase, and decreases during the G2 phase. This protein is a cofactor in many cellular processes, it is involved in DNA repair and replication, regulation of the cell cycle, and post-replication processes (Gasińska and Biesaga, 2010).

Zasławski et al. (2001) studied the expression of estrogen receptors (ER) and progesterone re-ceptors (PR) as well as proliferating antigens, PCNA and Ki-67, in uterine myomas concerning the menstrual cycle and in postmenopausal women. Their findings showed that the expression of the steroid hormones tested and proliferating antigens do not change during the cycle and after meno-pause.

In our study, the immunoexpression of PCNA(+) was observed both in the endometrium and myometrium in all groups of women. We did not note any statistically significant differences in in-tensity between groups. Our results correspond with those reported by Zasławski et al. (2001). It is noteworthy, however, that in both the endometrium and the myometrium, the highest percentage of PCNA(+) cells was found in Group I (up to five years after the last menstruation). The proliferation index was also analyzed by Noci et al. (1996), based on the PCNA expression. They proved that in postmenopausal women, the atrophic endometrium showed moderate, weak cell proliferation rated at 1+. No statistically significant differences were observed in the proliferation index between atrophic and hyperplastic endometrium, which may also partially confirm our findings. Our study did not demonstrate any statistically significant differences in the total percentage of PCNA(+) cells in the endometrium and myometrium between the groups of postmenopausal women. However, what is worth emphasizing, the process of uterine cell proliferation tended to be noticeably weaker with time since the last menstruation. The lack of a significant decrease in the proliferation process in the en-dometrium and myometrium is an unfavorable phenomenon due to the possibility of developing en-dometrial hyperplasia or endometrial cancer.

Conclusion

Morphological changes (atrophy and fibrosis) in the endometrial and myometrial layers of postmen-opausal uteri increased with the time since the last menstruation. These are already clearly noticeable upon histological examination in the period of 6-10 years from the last menstruation. Immunoex-pression of PCNA(+) was found in both endometrial and myometrial cells of the uteri of postmeno-pausal women, and a trend toward the weakening of cell proliferation with time was noted. The time since the last menstruation had no impact on the process of apoptosis in endometrial cells. The pro-cess of programmed cell death observed in the myometrium varied between the groups, it was weakest in the uteri of women over 11 years after menopause.

Therefore, it is very important to diagnose and monitor postmenopausal women and check how the

cessation of ovarian hormonal activity affects histological changes in the endometrium and myometrium. This is also important in the context of hormone therapy with estrogen in women with high endometrial proliferation. Systematic monitoring of women during menopause will help prevent the onset of adverse clinical conditions and the development of ovarian, endometrial, and breast cancers.

Declaration of Figures Authenticity. All figures submitted have been created by the authors who confirm that the images are original with no duplication and have not been previously published in whole or in part.

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