 doi:10.20944/preprints201705.0156.v1

Article

The Normal Human Endometrium but not Myometrium Presents Menstrual Cycle-Dependent Fluctuations in the Immunoexpression of DNA Fragmentation Factor 40, DNA Fragmentation Factor 45, and B-cell Lymphoma 2 Protein.

Tomasz Banas[1*], Kazimierz Pitynski[1], Krzysztof Okon[2], Marcin Mikos[3], Joanna Bonior[4], and Artur Ludwin[1]

[1] Department of Gynecology and Oncology, Jagiellonian University Medical Krakow, Cracow, Poland
[2] Department of Pathomorphology, Jagiellonian University Medical College, Krakow, Poland
[3] Dietl Specialistic Hospital, Krakow, Poland
[4] Department of Medical Physiology, Jagiellonian University Medical College, Krakow, Poland
* Corresponding author: Tomasz Banas, MD, PhD, MPH, Department of Gynecology and Oncology; Jagiellonian University, Chair of Gynecology and Obstetrics, 21 Kopernika Street, 30-501 Cracow, Poland. E-mail: tbanas@mp.pl

Abstract: DNA fragmentation factors 40 and 45 (DFF40 and DFF45) and B-cell lymphoma 2 (Bcl-2) expression were evaluated in the normal human endometrium and myometrium. DFF40, DFF45, and Bcl-2 expression was assessed via immunohistochemistry in the proliferative, secretory, and atrophic endometrium and myometrium collected postmenopausally and premenopausally during the proliferative and secretory phases of the menstrual cycle. The endometrium showed significantly higher DFF40 and DFF45 expression than that in the uterine myometrium; compared to the stroma, endometrial glands showed the highest expression in pre- and postmenopausal specimens. Glandular expression of DFF45 was dependent on the menstrual cycle, reaching its highest level in the secretory endometrium. The glandular expression of DFF40 and DFF45 was significantly lower in postmenopausal specimens than that in premenopausal tissue. No cycle-dependent changes were reported for stromal or myometrial DFF40 or DFF45 expression. Compared to the endometrial stroma and myometrium, Bcl-2 showed the highest expression in the glandular proliferative endometrium and the lowest expression in the stromal secretory endometrium and myometrium during the secretory phase of the cycle. DFF45 and Bcl-2 showed menstrual cycle-dependent expression, which was limited to the glandular layer of the endometrium.

Keywords: DNA fragmentation factors 40; DFF40; DNA fragmentation factors 45; DFF45; b-cell lymphoma 2; bcl-2; endometrium; menstrual cycle; uterine myometrium.

1. Introduction

During the reproductive period, the human non-pregnant uterus undergoes hormonal-dependent cyclic changes that cease after the menopause. These fluctuations are predominantly observed in the human endometrium, a unique type of tissue that undergoes cyclic structural and functional modifications. Menstruation is the result of enhanced apoptosis that occurs at the end of every cycle without conception [1].

The apoptosis of endometrial cells begins shortly before menstruation, triggered by the activation of both the receptor and mitochondrial apoptotic pathways [2]. The secretory endometrium shows increased Fas and Fas ligand immunoreactivity as well as increased tumor necrosis factor α levels, the role of which is well established in the receptor-dependent apoptotic

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pathway [3,4]. The endometrial expression of B-cell lymphoma 2 (Bcl-2) protein, an anti-apoptotic mitochondrial protein, depends on the menstrual cycle phase and is upregulated during the proliferative phase of this cycle [5]. In our previous studies of the endometrial glands of the secretory endometrium, we observed the increased expression of the apoptosis-related DNA fragmentation factor 45 (DFF45) protein; unfortunately, we did not investigate DFF45 myometrial expression at that time [6,7].

DFF45 (also known as an inhibitor of caspase-activated DNase (ICAD)) acts as a chaperone of DNA fragmentation factor 40 (DFF40; caspase-activated DNase (CAD)) and serves as a substrate for active caspase-3 [8,9]. The activation of the apoptotic cascade results in DF45 cleavage via the activated caspase-3 (and possibly caspase-7), causing DFF40 release and oligonucleosomal DNA degradation, which manifests as the "DNA-laddering" effect [10]. The DFF40/DFF45 complex is localized within the nucleus. According to Widlak et al. [10], DFF45 is not a simple DFF40 inhibitor; rather, it acts as a DFF40 chaperone that is responsible for the proper folding of DFF40 to acquire its biological function. Abundant DFF40 and DFF45 expression has been observed in ovarian endometriomas, human endometrial pathology (including endometrial cancer), and other malignancies such as ovarian epithelial cancers, colon and esophageal cancers, as well as glioblastoma [11–16.].

In contrast to the DFF40/DFF45 complex, the Bcl-2 protein is a core mitochondrial anti-apoptotic factor that has been widely investigated with regard to the human reproductive tract, including benign endometrial and myometrial disorders, as well as malignancies [17–19]. Decreased Bcl-2 was confirmed in the human secretory endometrium and ovarian endometriosis [20]. The Bcl-2 protein, which is located predominantly in the mitochondrial inner membrane, blocks the recruitment of proapoptotic factors such as *Bax* and stabilizes the mitochondrial membranes, thereby blunting the intrinsic death signaling pathway [21]. Additionally, together with *Bax*, Bcl-2 is responsible for preventing cytochrome c from triggering caspase-9 activity [22].

The present study evaluated DFF40, DFF45, and Bcl-2 expression in the human physiological endometrium and myometrium with respect to menstrual cycle phases and menopausal status because the results may assist in the interpretation of their expression in pathological findings.

2. Materials and Methods

2.1. Case Selection

Archived, paraffin-embedded slides collected between 1 January, 2015 and 31 July, 2016, were retrospectively analyzed, and samples of normal human endometrium and myometrium were obtained for further immunohistochemical investigation. The study protocol was approved by the University Review Board. The endometrial specimens chosen for the analysis were collected during hysteroscopic procedures in patients with an initial diagnosis of endometrial polyps or hyperplasia who were excluded based on their hysteroscopy and histopathological results. Myometrial samples were acquired from women with persistent or recurrent nonmalignant cervical pathology who qualified for total hysterectomy as a final treatment. The pathology of the uterine corpus was not observed in any of the cases. Each patient contributed only one specimen. In addition, specimens from patients (1) who had a history of malignancy (including breast cancer treated with tamoxifen); (2) who smoked; (3) who suffered from polycystic ovarian syndrome; and (4) who were prescribed hormonal treatment (including hormonal contraception) within the past 5 years were not eligible for this study. Finally, 25 samples of normal human proliferative endometrium, 25 samples of normal human secretory endometrium, and 27 samples of normal human postmenopausal endometrium, along with 71 cases of normal human myometrium (24 collected during the proliferative phase, 25 collected during the secretory phase, and 27 collected from postmenopausal uterine specimens) were employed for further investigation. 'Menopause' was defined as the date of the final menstrual period, with no menses reported during the subsequent 12-month period. Menstrual cycle characteristics were based on patient self-reports.

 doi:10.20944/preprints201705.0156.v1

2.2. Immunohistochemistry

From each case, a representative tissue block was selected for immunohistochemical investigation and recut into serial sections of 3 mm thickness. Subsequently, the sections were deparaffinized in xylene and rehydrated using graded ethanol. Before the staining procedure, the slides were boiled in a target retrieval solution (DAKO, Carpinteria, CA, USA) for 15 min at 98 °C following the instructions of the manufacturer, incubated with 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity, and washed with TRIS-buffered saline (pH 7.5) for 10 min. The sections were incubated with diluted normal serum as a blocking solution for 30 min. After this treatment, the blocking solution was discarded. A Vectastain Universal Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) was used following the instructions of the manufacturer. A standard immunohistochemical technique was performed using a rabbit polyclonal antibody to DFF45 (Abcam, Cambridge, UK), a rabbit polyclonal antibody to DFF40 (Abcam, Cambridge, UK), and a monoclonal mouse anti-human antibody to Bcl-2 (Leica Microsystems GmbH, Leica Biosystems Nussloch GmbH). The tissues were fixed in 10% neutral buffered formaldehyde solution. The antibody to DFF45 was applied at a dilution of 1:100; the antibody to DFF40 was applied at a dilution of 1:50; and the antibody to Bcl-2 was applied at a dilution of 1:200. All antibodies were incubated at room temperature for 60 min. After the slides were incubated with 3,3′-diaminobenzidine for 5 min and counterstained with hematoxylin for 30 s, the enzymatic reactivity was visualized. Following the manufacturer's guidelines, a colon carcinoma sample for Bcl-2, a human breast carcinoma tissue for DFF45, and human ovary tissue sections for DFF40 were used as positive controls. For the negative control, the same specimens and methods were used, but the primary antibodies were omitted.

2.3. Immunohistochemical Scoring

Two board-certified histopathologists blindly evaluated the DFF45, DFF40, and Bcl-2 staining for each slide using 5 high-power fields (x40) of maximal staining intensity. Each tissue was scored based on the intensity of staining (0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining) and the number of stained cells (0, expression in up to 10% of the cells; 1+, expression in 10–50% of the cells; 2+, expression in 51–80% of the cells; and 3+, expression in more than 80% of the cells). The final immunoreactivity score was determined by multiplying the intensity scores by the extent of the positivity scores of the stained cells to provide a score that ranged from 0–12. A discrepancy between the observations occurred in 18 (2.74%) cases, and the samples were verified again to achieve a consensus. Therefore, K.O. and H.M-O. performed another evaluation of selected slides 2 weeks after the primary evaluation to prevent recall bias.

3. Statistical Analyses

The clinical features of the study groups were compared using a one-way analysis of variance (ANOVA) or a Kruskal–Wallis test, depending on the homogeneity of variance. Post hoc tests were used where appropriate. Differences in the immunohistochemical scoring between more than two groups of tissue specimens were evaluated using the Kruskal–Wallis test, and post hoc tests were used where appropriate when analyzed variables were discrete. To compare the immunohistochemical expression of the analyzed factors between the endometrial glands and stromal tissue, the non-parametric Wilcoxon paired test was used and U Mann-Whitney test was applied to compare scorings between endometrium and myometrium as these tissues were unrelated. Clinical features were shown as the mean ± standard deviation (SD). Data from the immunohistochemistry results were presented as the median ± standard error of the mean (SEM). A multiply step-wise regression was used to evaluate the associations between DFF40, DFF45, and Bcl-2 expression and clinical characteristics. To evaluate the intra-rater agreement of immunohistochemistry scoring, kappa statistics with a P value were applied. To randomize the patients who underwent evaluation for the intra-rater agreement, we used the research randomizer (www.randomizer.org), and 30 (20.40%) samples were randomly, separately, and distinctly chosen

 doi:10.20944/preprints201705.0156.v1

from a total of 142 samples regarding DFF40, DFF45, and Bcl-2 for observer 1 and observer 2. The Guidelines for Reporting Reliability and Agreement in Studies were used to verify these results [23]. A p-value of less than 0.05 was considered significant. All calculations were performed using STATISTICA version 12.0 (StatSoft, Inc. 2014. STATISTICA, version 12; www.statsoft.com).

4. Results

4.1. Patients and Materials

The tissue samples included proliferative (n = 25), secretory (n = 25), and atrophic (n = 27) endometrium and myometrium collected during the proliferative (n = 24) and secretory (n = 20) phases of the menstrual cycle as well as postmenopausally (n = 21). The donors of the postmenopausal endometrial samples were significantly older than the women investigated during the proliferative ($p < 0.001$) and secretory ($p < 0.001$) phases of the menstrual cycle (Table 1). Similarly, the women who contributed postmenopausal uterine myometrium samples were significantly older than those who provided myometrium samples during the proliferative ($p < 0.001$) and secretory ($p < 0.001$) phases of the menstrual cycle; no significant differences in median age were observed between the postmenopausal donors of the endometrium and myometrium samples ($p = 0.919$; Table 1). Postmenopausal women more frequently reported irregular periods with a wider range in menstrual cycle duration than the other groups of premenopausal women; however, these differences were not significant (Table 1). These findings may be explained by the fact that postmenopausal patients predominantly reported that the last cycles occurring during the perimenopausal period were typically longer and irregular. No other differences in the demographic characteristics of the participants were observed.

Table 1. Clinical characteristics of tissue specimens of donors.

	Endometrium			Myometrium			p
	Proliferatory phase (n=25)	Secretory phase (n=26)	post-menopausal (n=27)	Proliferatory phase (n=24)	Secretory phase (n=20)	Post-menopausal (n=21)	
Mean age [years] (SD*) range	42.20 (±6,74) 27.00 – 52.00	45.36 (±6.41) 30.00 – 54.00	65.26 (±9.77) 50.00 – 84.00	43.75 (±1.57) 41.00 – 48.00	44.08 (±3.35) 38.00 - 49.00	65.05 (±10.49) 50.00 - 83.00	<0.001$ #
Mean BMI [kg/m²] (±SD*) range	25.15 (±3.67) 21.93 – 35.14	23.86 (±2.06) 21.45 – 30.85	24.60 (±4.18) 20.20 – 34.91	24.85 (±2.94) 20.38 – 31.91	23.82 (±4.16) 19.72 – 31.62	26.21 (±2.89) 22.50 – 33.62	0.195$
Mean age of first menstrual period [years] (±SD*) range	12.56 (±1.39) 11.00 – 16.00	12.96 (±1.54) 10.00 – 17.00	12.85 (±1.81) 12.00 – 18.00	12.42 (±1.53) 10.00 – 16.00	12.55 (±1.05) 10.00 – 14.00	12.19 (±1.27) 10.00 – 15.00	0.606$
Mean age of menopause [years] (±SD*) in postmenopausal women	not available	not available	51.33 (±2.08) 46.00 – 55.00	not available	not available	51.48 (±1.86) 47.00 – 55.00	0.806$$
Mean duration of menstrual cycle& [days] (±SD*) range	28.32 (±2.36) 24.00 – 33.00	28.00 (±1.91) 25.00 – 33.00	28.74 (±2.88) 22.00 – 37.00	27.67 (±1.46) 25.00 – 30.00	28.41 (±1.77) 26.00 – 32.00	28.33 (±2.67) 25.00 – 38.00	0.663$
Menstrual cycles& - regular - irregular	23 (92,00%) 2 (8,00%)	24 (92.31%) 2 (7.69%)	20 (74.07%) 7 (25.93%)	22 (91.67%) 2 (8.33%)	18 (90.00%) 2 (10.00%)	17 (80.95%) 4 (14.83%)	0.273**
Menstrual cycles& - painful - painless	3 (12,00%) 22 (88.00%)	4 (15.38%) 22 (84.62%)	3 (11.12%) 24 (88.88%)	2 (8.33%) 22 (91.67%)	1 (5.00%) 19 (95.00%)	2 (5.00%) 19 (95.00%)	0.902**
Mean duration of menstruation [days] (±SD*) range	4.12 (±0.97) 3.00 – 6.00	4.13 (±0.78) 3.00 – 6.00	4.44 (±1.01) 3.00 – 7.00	4.08 (±0.93) 3.00 – 6.00	4.25 (±1.12) 2.00 – 7.00	4.43 (±0.93) 3.00 – 6.00	0.628$

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Type of menstrual bleeding[&]							
- scant	2 (8.00%)	2 (7.69%)	3 (11.11%)	2 (8.34%)	1 (5.00%)	1 (4.77%)	
- normal	22 (88,00%)	22 (84.62%)	22 (81.48%)	20 (83.32%)	17 (85.00%)	18 (85.71%)	0.997[**]
- heavy	1 (4,00%)	2 (7.69%)	2 (7.41%)	2 (8.34%)	2 (10.00%)	2 (9.52%)	
Parity							
- nullipara	2 (8.00%)	3 (11.54%)	2 (7.41%)	2 (8.33%)	1 (5.00%)	2 (9.53%)	0.999[**]
- primipara	2 (8.00%)	2 (7.69%)	3 (11.11%)	3 (12.50%)	2 (10.00%)	2 (9.53%)	
- multipara	21 (84.00%)	21 (80.77%)	22 (81.48%)	19 (79.17%)	17 (85.00%)	17 (80.94%)	

*SD – standard deviation; [**] chi-square test; [$]ANOVA – One-Way Analysis of Variance; [$$] t-Student`s test [#]p value statistically significant; [##] statistically significant value; [###]NA – data not available; [&]for the last 24 months of reproductive age.

4.2. DFF40 and DFF45 Expression

DFF40 immunoreactivity was observed in all the endometrial glandular epithelium samples (n = 77/77), 97.40% (n = 75/77) of the endometrial stroma, and 84.23% (n = 58/65) of the uterine myometrium samples presenting predominately nuclear expression (Figures 1). A comparable median DFF40 expression was observed in the endometrial glandular epithelium during both the proliferative and secretory phases of the menstrual cycle, which was significantly higher compared to its glandular expression in atrophic endometrium (Table 2, Figure 2). No differences in the median DFF40 expression were observed in the endometrial stroma or uterine myometrium with respect to menstrual cycle phases or menopausal status (Table 2, Figure 2). During the proliferative phase of the menstrual cycle, the endometrial glandular epithelium showed significantly higher median DFF40 expression than the endometrial stroma ($p < 0.001$) and uterine myometrium ($p < 0.001$; Table 2). In addition, in the proliferatory phase of the menstrual cycle, endometrial stroma showed significantly higher DFF40 expression compared to uterine myometrium ($p < 0.001$; Table 2). A similar pattern of median DFF40 expression was observed in the secretory phase of the menstrual cycle, with its significantly higher expression observed in the endometrial glandular epithelium than that in the endometrial stroma ($p = 0.002$), and uterine myometrium ($p < 0.001$) that was significantly lower if compared to the endometrial stroma ($p < 0.001$; Table 2). In the postmenopausal samples, no significant differences were observed in the median DFF40 expression between the endometrial glandular epithelium and the endometrial stroma, whereas a significantly lower median DFF40 expression was observed in the uterine myometrium compared with that in both the endometrial glandular epithelium ($p < 0.001$) and endometrial stroma ($p = 0.001$; Table 2).

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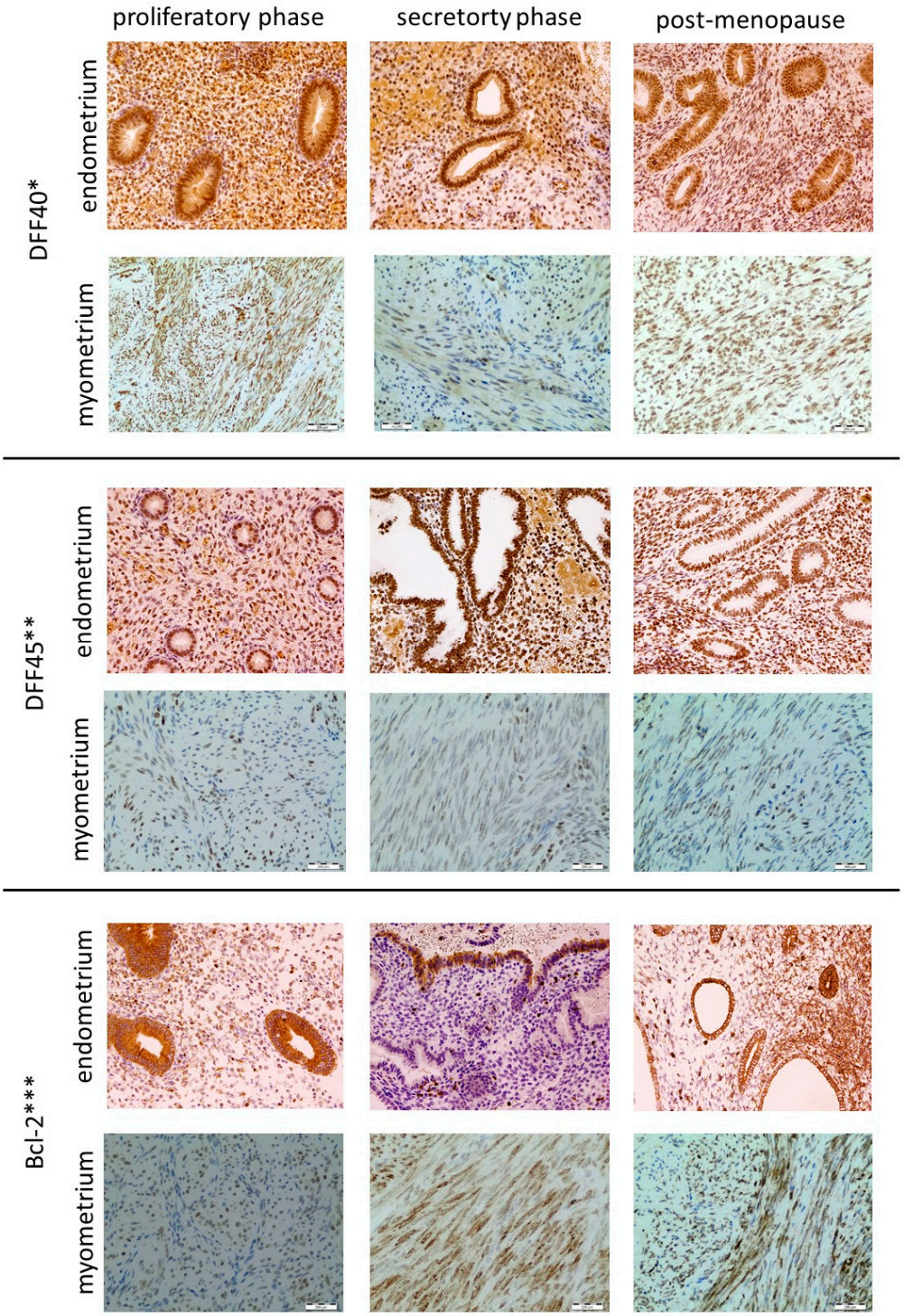

Figure 1. Expression of DFF40*, DFF45**, and Bcl-2*** (x200) in normal human endometrium and myometrium in the proliferatory, secretory phase of menstrual cycle and after the menopause.

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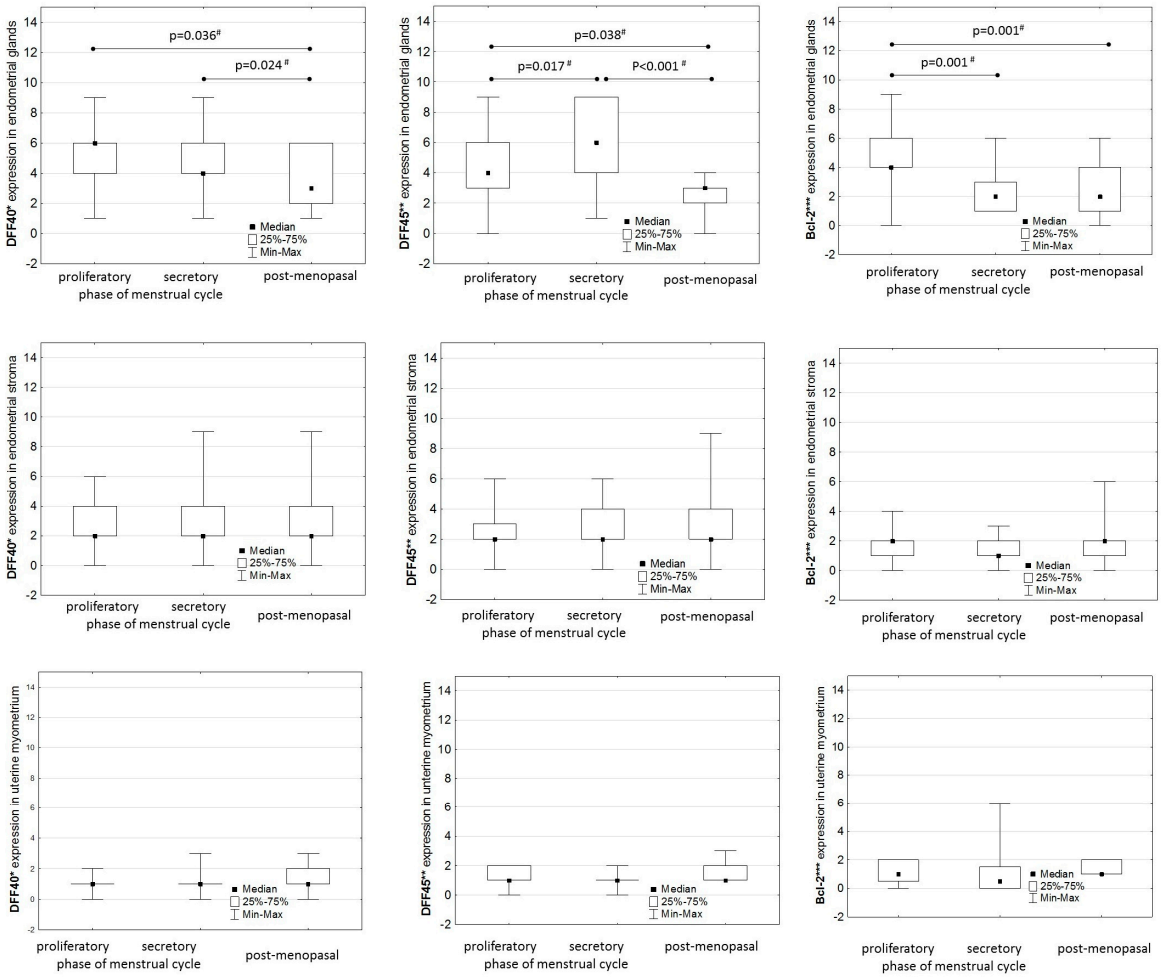

192

193 **Figure 2.** Median DFF40*, DFF45**, and Bcl-2*** expression in the normal endometrial glands, normal
194 endometrial stroma, and normal uterine myometrium in respect to phases of the menstrual cycle and
195 menopausal status. *DNA fragmentation factor 40; **DNA fragmentation factor 44; *** B-cell
196 lymphoma 2; #p-value statistically significant.

197 **Table 2.** Immunoexpression of DFF40*; DFF45**, and Bcl-2*** in the normal human endometrium and
198 normal human uterine myometrium in different phases of the menstrual cycle and postmenopausally.
199 *DNA fragmentation factor 40; ** DNA fragmentation factor 45; *** B-cell lymphoma 2.

		Menstrual cycle phasae		Post-menopausal
		proliferatory	secretory	
DFF40	Median glandular expression in endometrium (IQR) and min.-max.	6.00 (2.00) 1,00 - 9,00	6.00 (2.00) 3.00 – 9.00	3.00 (4.00) 1.00 – 6.00
	Median stromal expression in endometrium (IQR) and min.-max	2.0 (2.00) 0,00 - 6,00	2.00 (2.00) 0.00 – 9.00	2.00 (2.00) 1.00 – 9.00
	Median expression in uterine myiometrium (IQR) and min.-max	1.00 (1.50) 0.00 – 4.00	1.00 (1.00) 0.00 – 3.00	1.00 (1.00) 0.00 – 3.00
DFF45	Median glandular expression in endometrium (IQR) and min.-max.	4.00 (1.00) 0.00 – 6.00	6.00 (2.00) 1.00 – 9.00	3.00 (1.00) 0.00 – 4.00
	Median stromal expression in endometrium (IQR) and min.-max	2.00 (2.00) 0.00 – 6.00	2.00 (1.00) 0.00 – 6.00	2.00 (1.00) 0.00 – 4.00

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	Median expression in uterine myiometrium (IQR) and min.-max	1.00 (1.00) 0.00 – 2.00	1.00 (0.00) 0.00 – 2.00	1.00 (0.00) 0.00 – 2.00
Bcl-2	Median glandular expression in endometrium (IQR) and min.-max.	4.00 (3.00) 1.00 – 9.00	2.00 (2.00) 1.00 – 6.00	2.00 (3.00) 0.00 – 6.00
	Median stromal expression in endometrium (IQR) and min.-max	2.00 (1.00) 0.00 – 4.00	1.00 (1.00) 0.00 – 3.00	2.00 (1.00) 0.00 – 6.00
	Median expression in uterine myiometrium (IQR) and min.-max	1.00 (1.50) 0.00 – 4.00	0.50 (1.50) 0.00 – 6.00	1.00 (1.00) 1.00 – 2.00

Nuclear DFF45 immunoreactivity was confirmed in 97.40% (n = 75/77) of the endometrial glandular epithelium, 93.51% (n = 72/77) of the endometrial stroma, and 84.62% (n = 55/65) of the uterine myometrium specimens (Figure 1). The median DFF45 expression in the endometrial glandular epithelium was significantly higher during the secretory phase of the menstrual cycle than during the proliferative phase and if compared with postmenopausal samples (Table 2, Figure 2). No differences in DFF45 scoring were observed in endometria stroma nor uterine myometrium with respect to menstrual cycle phases or menopausal status (Table 2, Figure 2). During the proliferative phase of the menstrual cycle, the glandular epithelium showed significantly higher median DFF45 expression than the endometrial stroma ($p = 0.012$) and uterine myometrium ($p < 0.001$). In addition, significant differences were found in the median DFF45 expression between the endometrial stroma and uterine myometrium ($p < 0.001$; Table 2). During the secretory phase of the menstrual cycle, the median DFF45 expression was also significantly higher in the endometrial glandular epithelium than that in the endometrial stroma ($p < 0.001$) and uterine myometrium ($p < 0.001$). In addition, the uterine myometrium showed a significantly lower median DFF45 expression than that in the secretory endometrial stroma ($p < 0.001$; Table 2). The postmenopausal specimens also showed significantly higher median DFF45 expression in the endometrial glandular epithelium than that in the endometrial stroma ($p = 0.014$) and uterine myometrium ($p = 0.003$), whereas no significant difference was found between the endometrial stroma and uterine myometrium with regard to median DFF45 expression (Table 2).

Glandular endometrium showed significantly higher DFF40 expression compared to DFF45 in the proliferatory phase of the menstrual cycle and postmenopausally ($p = 0.003$ and $p = 0.004$, respectively; Table 2) but not in the secretory phase. However, a positive and significant correlation between DFF40 and DFF45 expression in endometrial glands in proliferative ($R = 0.510$; $p = 0.009$), secretory ($R = 0.696$; $p = 0.001$), and postmenopausal ($R = 0.446$; $p = 0.018$) samples was confirmed.

In endometrial stroma, DFF40 showed comparable expression to DFF45 in the proliferatory, secretory, and postmenopausal endometrium. The association between DFF40 and DFF45 in endometrial stroma was sustained, showing positive significant correlation in the proliferatory ($R = 0.640$; $p < 0.001$) and secretory ($R = 0.542$; $p = 0.003$) phases of the menstrual cycle, as well as postmenopausally ($R = 0.571$; $p = 0.002$). Uterine myometrium showed comparable DFF40 and DFF45 expression irrespective of the phase of the menstrual cycle (proliferatory and secretory, respectively) and postmenopausally. Similarly, as in endometrium DFF40 and DFF45, immunoexpression was associated in uterine myometrium showing a positive significant correlation in the secretory and proliferatory phase of the menstrual cycle and postmenopausally.

4.3. Bcl-2 Expression

Cytoplasmic Bcl-2 expression was observed in 87.01% (n = 67/77) of the endometrial glandular epithelium, 89.61% (n = 69/77) of the endometrial stroma (Figure 1), and 75.38% (n = 49/65) of the uterine myometrium specimens (Figure 5). The highest median Bcl-2 expression was observed with regard to the glandular layer of the proliferative endometrium, and it differed significantly from the median Bcl-2 expression observed in the stromal layer of the endometrium ($p < 0.001$) and uterine myometrium ($p < 0.001$) derived during the proliferative phase of the menstrual cycle. In addition, the median DFF40 scoring in the stromal layer of the proliferatory endometrium was significantly higher if compared to uterine myometrium from the proliferatory phase of the menstrual cycle ($p =$

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0.038; Table 2). Uterine myometrium in the secretory phase of menstrual cycle showed significantly lower median Bcl-2 expression if compared to the glandular layer of the secretory endometrium (p = 0.001) and its stromal layer (p = 0.022), while there were no differences in the median Bcl-2 scoring between the latter two (Table 2). No differences in the median Bcl-2 expression were found among the endometrial glandular epithelium, endometrial stroma, or uterine myometrium in the postmenopausal samples (Table 2). Subsequently, only the expression of Bcl-2 in the endometrial glandular epithelium was dependent on the menstrual cycle phases, showing the highest median expression during the proliferative phase compared with that in the secretory phase ($p < 0.001$) and postmenopausal stage ($p<0.001$; Figure 4).

In the glandular endometrium, there was no correlation probed between Bcl-2 and DFF40 or Bcl-2 and DFF45 in the proliferatory and secretory phases of the menstrual cycle, nor in the postmenopausal samples. Similarly, the endometrial stroma did not show any associations between Bcl-2 and DFF40 or Bcl-2 and DFF45 in the proliferatory, secretory phase of menstrual cycle, nor after the menopause. Subsequently, in the uterine myometrium no associations between Bcl-2 and DFF40 or Bcl-2 and DFF45 were observed, regardless of the phase of the menstrual cycle and menopausal status.

4.4. Association of Clinical Features and DFF40, DFF45, and Bcl-2 Endometrial and Myometrial Expression

The expression of DFF40 in endometrial glands shows no association with age (P = 0.957), age at menarche (P = 0.965) and at menopause (P = 0.807), BMI (P = 0.315), length of menstrual cycle (P = 0.437) and menses (P = 0.882), parity (P = 0.613), or the phase of the menstrual cycle (P = 0.394), while the presence of the menopause was associated negatively with DFF40 expression in endometrial glands (P = 0.006). Furthermore, stromal DFF40 expression did not show any association with age (P = 0.918), age at menarche (P = 0.267) and at menopause (P = 0.199), BMI (P = 0.824), length of the menstrual cycle (P = 0.556) and menses (P = 0.661), parity (P = 0.613), menopausal status (P = 0.918), and the phase of the menstrual cycle (P = 0.302).

Glandular expression of DFF45 was dependent on the phase of the menstrual cycle (P = 0.002) and menopausal status (P = 0.005), but was not associated with age (P = 0.840), age at menarche (P = 0.627) and at menopause (P = 0.973), BMI (P = 0.721), lengths of the menstrual cycle (P = 0.408) and menses (P = 0.719), or parity (P = 0.482). Stromal expression of DFF45 in the endometrium was independent from age (P = 0.689), age at menarche (0.643) and at menopause (0.707), BMI (P = 0.289), length of menses (P = 0.327), and parity (P = 0.972), but was correlated inversely with the length of the menstrual cycle (P = 0.019).

Myometrial DFF40 and DFF45 expression was independent from age (P = 0.731 and P = 0.911, respectively), age at menarche (P = 0.978 and P = 0.921, respectively), BMI (P = 0.326 and P = 0.881, respectively), lengths of menstrual cycle (P = 0.558 and P = 0.335, respectively) and menses (P = 0.160 and P = 0.932, respectively), parity (P = 0.293 and P = 0.721, respectively), menstrual cycle phase (P = 0.622 and P = 0.296, respectively) and menopausal status (P = 0.731 and P = 0.262, respectively), while both DFF40 and DFF45 myometrial expression negatively correlated with the age at menarche (P = 0.005 and P = 0.032, respectively).

No association between endometrial glandular Bcl-2 expression and age (P = 0.488), age at menarche (P = 0.543) and at menopause (P = 0.062), BMI (P = 0.581), menstrual cycle (P = 873) and menses lengths (P = 0.580), parity (P = 0.909), nor menopausal status (P = 0.546) were proved, while in premenopausal women these expressions were dependent on the menstrual cycle phase ($P < 0.001$). Stromal endometrial expression of Bcl-2 was independent from age (P= 0.846), age at menarche (P = 0.682) and at menopause (P = 0.057), BMI (P = 0.075), menstruation length (P = 0.242), parity (P = 0.535), menopausal status (P = 0.591), and the phase of the menstrual cycle (P = 0.491), but was negatively associated with the length of the menstrual cycle (P = 0.049). In contrast to the above, myometrial Bcl-2 expression was independent from age (P= 0.278), age at menarche (P = 0.415) and at menopause (P = 0.853), BMI (P = 0.198), lengths of the menstrual cycle (P = 0.569) and menses (P = 0.561), parity (P = 0.442), the phase of the menstrual cycle (P = 0.701), and menopause (P = 0.794).

 doi:10.20944/preprints201705.0156.v1

4.5. Validation of the Intra-Rater Reliability for Immunohistochemistry Scoring

An almost perfect intra-rater agreement was confirmed with regard to the immunoscoring of DFF40, DFF45, and Bcl-2 expression. The following values were noted:

A) The intra-rater agreement of the first investigator (i.e., investigator 1 vs. 1) for the assessment of DFF40 is as follows: $\kappa = 1.0$ ($p < 0.001$); DFF45: $\kappa = 0.95$ ($p < 0.001$); and Bcl-2: $\kappa = 0.96$ ($p < 0.001$)

B) The intra-rater agreement of the second investigator (i.e., investigator 2 vs. 2) for the assessment of DFF40 is as follows: $\kappa = 1.0$ ($p < 0.001$); DFF45: $\kappa = 1.0$ ($p < 0.001$); and Bcl-2: $\kappa = 0.96$ ($p < 0.001$)

5. Discussion

To the best of our knowledge, this study marks the first comprehensive report of the DFF40, DFF45, and Bcl-2 expression in the human uterus under physiological conditions. Our results provide evidence that menstrual cycle-dependent changes in the expression of DFF40, DFF45, and Bcl-2 are present predominantly in the endometrial glandular epithelium, whereas the expression of these proteins remained stable throughout the menstrual cycle and after menopause in the uterine myometrium. These results are consistent with our previous findings that show changes in DFF45 expression in the human endometrium with respect to the phases of the menstrual cycle and the lack of such alterations during ovarian endometriosis [6,7,11].

DFF40 is a major apoptotic nuclease responsible for the final DNA fragmentation in apoptosis, and its high and comparable expression was observed in the endometrial glandular epithelium during both the proliferative and secretory phases of the menstrual cycle. Therefore, we assume that the tissues that show a high DFF40 expression are potentially susceptible to apoptosis. According to our results, the endometrial glandular epithelium showed higher potential receptivity to apoptosis than the endometrial stroma and uterine myometrium. This endometrium glandular epithelium receptivity likely remains constant throughout the menstrual cycle and decreases after the menopause. These findings are consistent with those of Matsumoto et al. [24], who observed that apoptosis most frequently appeared in the epithelial endometrial cells and was enforced during the mid- to late-secretory phases compared with the proliferative phases of the menstrual cycle. However, it must be noted that DFF40 alone is not enough to execute DNA fragmentation because when DFF40 is in the nucleus, it remains bound to DFF45, which inhibits the activity of DFF40. DFF40 is released from the DFF40/DFF45 complex upon DFF45 cleavage via active caspase-3. Conversely, DFF45 plays the dual role of DFF40 inhibitor and chaperone. The expression of DFF40 in the absence of co-expressed DFF45 results in the generation of inactive DFF40 aggregates [25,26]. In our study, the endometrial glandular epithelium showed the highest DFF45 expression during the secretory phase of the menstrual cycle compared with that during the proliferative phase and postmenopausally. Therefore, we assume that increased DFF45 expression in the endometrial glandular epithelium during the secretory phase of the menstrual cycle plays an important role in executing apoptosis via DFF40 because its expression remains constant throughout both the proliferative and secretory phases. Moreover, decreased DFF45 expression during the proliferative phase of the menstrual cycle potentially prevents endometrial glandular cells from undergoing apoptosis. Regression analysis proved that DFF40 expression was independent from most of the clinical features in both the glandular and stromal layer of the endometrium and confirmed that the menopausal status was the only characteristic significantly influencing DFF40 expression in endometrial glands. Similarly, DFF45 endometrial glandular expression was dependent only on the menstrual cycle phase and menopausal status but not on the other clinical features. Interestingly, stromal DFF45 expression was found to correlate negatively with menstrual cycle lengths and was independent from other clinical features. This observation needs further investigation. In contrast, the endometrium uterine myometrium showed independent DFF40 and DFF45 expression from all the analyzed clinical features.

The current study also confirmed the significant decrease in Bcl-2 expression that occurs predominantly during the secretory phase of the menstrual cycle compared with that during the proliferative phase in the endometrial glandular epithelium. Bcl-2 glandular endometrial expression was dependent on the menstrual cycle phase but no other clinical characteristics, while its stromal

 doi:10.20944/preprints201705.0156.v1

expression, similar to DFF45, negatively correlated with the length of the menstrual cycle. These results are consistent with those of Otsuky et al. [27], who reported that glandular endometrial cells express Bcl-2 during the proliferative phase of the menstrual cycle through the early secretory phases (but not during the late secretory phase). Furthermore, these authors found that the disappearance of Bcl-2 expression was correlated with the appearance of apoptosis. In addition, Li et al. [28] postulated that the binding of c-Jun to estrogen receptor α regulates the proliferative phase-specific expression of the Bcl-2 gene in glandular endometrial cells. The high Bcl-2 expression and low DFF45 expression that occurs during the proliferative phase of the menstrual cycle may prevent apoptosis, even when the level of DFF40 remains consistently high, while the decreased Bcl-2 expression and increased DFF45 that occurs during the secretory phase of the menstrual cycle may enhance apoptosis.

Although our outcomes are consistent with the data obtained by other studies that investigated DFF45 and Bcl-2 expression in the human endometrium as well as our previous results, the present study has limitations. First, immunostaining was employed as the only study technique, and this semi-quantitative method does not allow us to directly compare the DFF40 and DFF45 levels with each other. Although in the stromal layer of endometrium and in uterine myometrium we observed 1:1 of median DFF40 and DFF45 expression, this however is not a stochiometric ratio and the result must be interpreted with caution. It can be extremely difficult to evaluate the DFF40 and DFF45 load separately in the glandular and the stroma layer of the endometrium using quantitative methods, therefore we were unable to confirm the thesis of Widlak et al., who postulated that DFF40 and DFF45 are present in a 1:1 nuclear stoichiometric expression [10]. This condition is mandatory for the proper execution of apoptosis, whereas alterations to this proportion can lead to abundant apoptosis and promote cell death [10]. As the roles that the DFF40/DFF45 complex and Bcl-2 play in apoptosis have already been explained, this goal was not the aim of our study. In addition, we realize that an immunostaining analysis is subjective. Therefore, two pathologists with wide-ranging and well-documented experiences in gynecology evaluated each sample, and a discrepancy occurred only in 2.74% of cases. These differences were reevaluated thereafter to achieve a final consensus. This methodology is widely accepted and employed by many other studies regarding the expression of DFF45 and Bcl-2 as well as other proteins in endometrial specimens and also in endometrial microvessels density evaluation [6–7,11–15, 19–20,29–30]. As complete inter-rater agreement was achieved by creating a consensus in ambiguous cases, the intra-rater disparity was the only potential bias in our sample assessment. This disparity showed an almost perfect correlation; therefore, intra-rater bias can be safely excluded. Immunostaining was selected as an investigation method for the following reasons: first, this approach allows our findings to be compared with previously published results; second, because immunochemistry is a widely-used method in pathomorphological laboratories this technique can be easily implemented and performed if our assessments of DFF40, DFF45, or Bcl-2 reach clinical applications. We also considered using an automatic assessment of digitalized whole slide images instead of pathologist-performed immunoscoring. An exact discernment between the endometrial glandular epithelium and stroma was required, which could be best provided by pathologists. Moreover, we did not find any description of this method regarding the immunoscoring of the DFF40/DFF45 complex. Thus, to avoid the potential bias caused by implementation of a new methodology, we decided to abandon this method.

The reliable histopathological classification of the specimens remains the core strength of our study. The paraffin-embedded slides were properly stored and well-prepared, which allowed for repeated immunostaining to be achieved for each sample, providing a brief period of retrospective analysis.

6. Conclusions

The current study provides important evidence regarding menstrual cycle-dependent changes in the expression of DFF40/DFF45 and Bcl-2 in the normal human endometrium, especially in the glandular layer, and shows their levels are stable in the normal uterine myometrium. This

 doi:10.20944/preprints201705.0156.v1

comprehensive evaluation provides a better understanding of other findings concerning DFF45 and Bcl-2 expression in female genital tract pathologies, including malignancies.

Acknowledgments: The authors thank Dr. H. Molak-Olczak, who independently and blindly analyzed the immunoexpression of the investigated proteins as a second specialist in histopathology.

Author Contributions: TB was the chief investigator who designed the study, selected the cases, performed data analysis, and drafted the manuscript. KP and AL participated in the specimen evaluation and the selection of eligible cases. KP, MM, and JB performed the statistical analysis and critically reviewed the manuscript. KO performed the protein immunoexpression assessment. All the authors accepted the manuscript.

Conflicts of Interest: None declared.

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