Immunoexpression of DNA fragmentation factor 40, DNA fragmentation factor 45, and B-cell lymphoma 2 protein in normal human endometrium and uterine myometrium depends on menstrual cycle phase and menopausal status

Tomasz Banas[1], Kazimierz Pitynski[1], Krzysztof Okon[2], Marcin Mikos[3], Aleksandra I. Czerw[4], Andrzej Deptała[5], Artur Ludwin[1]

[1]Department of Gynecology and Oncology, Jagiellonian University Medical College, Krakow, Poland
[2]Department of Pathomorphology, Jagiellonian University Medical College, Krakow, Poland
[3]Dietl Specialist Hospital, Krakow, Poland
[4]Department of Public Health, Medical University of Warsaw, Warsaw, Poland
[5]Department of Cancer Prevention, Medical University of Warsaw, Warsaw, Poland

Corresponding author:
Aleksandra I. Czerw PhD
Department of Public Health
Medical University
of Warsaw
61 Zwirki Wigury St
08-456 Warsaw, Poland
Phone: +48 501 176 370
E-mail: ola_czerw@wp.pl

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Abstract

Introduction: DNA fragmentation factors 40 and 45 (DFF40 and DFF45) are final executors of apoptosis, and B-cell lymphoma 2 (Bcl-2) is a well-recognized apoptosis inhibitor. We aimed to evaluate DFF40, DFF45 and Bcl-2 immunoexpression in the normal human endometrium with respect to the glandular and stromal layer and in uterine myometrium.

Material and methods: DFF40, DFF45, and Bcl-2 expression was assessed via immunohistochemistry in the endometrium and myometrium collected postmenopausally and premenopausally during the proliferative and secretory phases of the menstrual cycle.

Results: Compared to the myometrium and stroma, endometrial glands showed the highest DFF40 and DFF45 expression in pre- and postmenopausal specimens. DFF45, but not DFF40, glandular expression dependent on menstrual cycle phase and DFF40 and DFF45 scoring was significantly lower in postmenopausal specimens. Significantly higher Bcl-2 expression was observed in proliferative glandular endometrium compared to secretory and postmenopausal specimens. No cycle- or menopause-dependent changes were reported for stromal or myometrial DFF40, DFF45 or Bcl-2 expression. DFF40, DFF45 and Bcl-2 expression was independent of age, age at menarche and menopause, BMI, menstrual cycle and menses lengths, parity and gravidity.

Conclusions: The 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 that their levels are stable in the normal uterine myometrium.

Key words: endometrium, Bcl-2, menstrual cycle, DNA fragmentation factors 40, DFF40, DNA fragmentation factor 45, DFF45, B-cell lymphoma 2, uterine myometrium.

Introduction

During the reproductive period, the human non-pregnant uterus undergoes hormonal-dependent cyclic changes, and menstruation is the result of enhanced apoptosis [1]. Abnormal uterine bleeding, although non-pathognomonic, may be the first manifestation of endometrial pathology including malignancies [2]. In our previous studies we observed increased expression of apoptosis-related DNA fragmentation factor 45 (DFF45) in the endometrial glands of the secretory endometrium; unfortunately, we did not investigate myometrial expression of DFF45 and other apoptosis-related factors at that time [3, 4].

DFF45 acts as a chaperone of DNA fragmentation factor 40 (DFF40) and serves as a substrate for caspase-3 [5, 6]. The activation of the apoptotic cascade results in DF45 cleavage via the activated caspase-3, causing DFF40 release and oligonucleosomal DNA degradation [7]. According to Widlak *et al.*, 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 [7]. Abundant DFF40 and DFF45 expression has been observed in ovarian endometriomas, endometrial cancer and other malignancies such as ovarian epithelial cancers, colon and esophageal cancers, as well as glioblastoma [8–13].

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 [14–16]. Decreased Bcl-2 was confirmed in the human secretory endometrium, ovarian endometriosis, human stromal cell lines (ThESC, ATCC, 4003) treated with raloxifene and also in the lamina propria lymphocytes of intestinal tissues from patients with Crohn's disease [16–18]. 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 [19]. Additionally, together with Bcl-XL, Bcl-2 is responsible for preventing cytochrome c from triggering caspase-9 activity [17].

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.

Material and methods

Case selection

This retrospective study of paraffin-embedded slides was approved by the Jagiellonian University Review Board. The endometrial specimens were collected during hysteroscopic procedures in patients with an initial diagnosis of uterine malformation (n = 21), endometrial polyps (n = 47) or submucosal myoma (n = 9) that finally were excluded based on their hysteroscopy and histopathological results. Myometrial samples were acquired from women with persistent cervical intraepithelial neoplasia (CIN) (19 cases of CIN2 and 17 cases of CIN3) observed over a 12-month follow-up after cervical conisation) or recurrent (21 cases of CIN2 and 8 cases of CIN3) premalignant cervical pathology, who were qualified for total hysterectomy as a final treatment. The pathology of the uterine corpus was not observed in any of the cases and each patient contributed only one specimen. Patients who: (1) had a history of malignancy; (2) smoked; (3) suffered from polycystic ovarian syndrome; and (4) were prescribed hormonal treatment within the past 5 years were not eligible for this study. '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.

Immunohistochemistry and immunohistochemical scoring

From each case, a representative tissue block was selected, deparaffinized and rehydrated as described previously [3, 4, 8]. A standard immunohistochemical technique was performed using a rabbit polyclonal antibody to DFF45 (Abcam, Cambridge, UK) at a dilution of 1 : 100, a rabbit polyclonal antibody to DFF40 (Abcam, Cambridge, UK) at a dilution of 1 : 50, and a monoclonal mouse anti-human antibody to Bcl-2 (Leica Microsystems GmbH, Wetzlar, Germany) at a dilution of 1 : 200. Slides were incubated with 3,3'-diaminobenzidine for 5 min and counterstained with hematoxylin for 30 s; the enzymatic reactivity was visualized. 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.

Two board-certified histopathologists blindly evaluated the DFF45, DFF40, and Bcl-2 staining for each slide using 5 high-power fields (40×) 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 to 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.

Statistical analysis

The clinical features of the study groups were compared using one-way analysis of variance (ANOVA) or the Kruskal-Wallis test, depending on the homogeneity of variance, while differences in the immunohistochemical scoring were evaluated using the Kruskal-Wallis test. *Post hoc* tests were used where appropriate. The non-parametric Wilcoxon paired test was used and the Mann-Whitney *U* test was applied to compare scorings between endometrial gland stroma and myometrium. Clinical features were shown as the mean ± standard deviation (SD). Data from the immunohistochemistry results were presented as the median and interquartile range (IQR). A multiple 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 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 [20]. 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).

Results

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). Table I presents clinical characteristics of donors.

DFF40 and DFF45 expression

DFF40 and DFF45 nuclear expression was observed in endometrium and myometrium (Figure 1). Median DFF40 expression in proliferatory and secretory glandular endometrium was significantly higher compared to glandular atrophic endometrium, whereas no such changes were observed in stromal endometrium and myometrium (Figure 2, Table II). The significantly highest median DFF40 scoring was observed in glandular endometrium compared the endometrial stroma and myometrium in pre- and postmenopausal specimens (Table II).

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 when compared with postmenopausal samples (Table II, Figure 2). No differences in DFF45 scoring were observed in endometrial stroma or myometrium with respect to menstrual cycle phases or menopausal status (Table II, Figure 2). Significantly higher median DFF45 expression was proved in endometrial glands compared to the stroma and myometrium with respect to phases of the menstrual cycle and menopausal status (Table II).

Bcl-2 expression

Bcl-2 presented cytoplasmic expression with the highest median scoring observed with regard to the glandular layer of the proliferative endometrium, and it differed significantly from the median expression observed in the endometrial stroma and myometrium derived during the proliferative phase of the menstrual cycle (Figure 1, Table II). 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 II). 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 (Figure 2, Table II).

Association of clinical features and DFF40, DFF45, and Bcl-2 endometrial and myometrial expression

The expression of DFF40, DFF45 and Bcl-2 in endometrial glands and stroma shows no association with age, age at menarche, body mass index (BMI), length of menstrual cycle and menses, parity or gravidity, while the presence of the menopause was associated negatively with DFF40 and DFF45 expression in endometrial glands (p = 0.006; p = 0.005, respectively), and DFF45 glandular expression alone was dependent on the phase of the menstrual cycle (p = 0.002). Additionally, Bcl-2 glandular expression was dependent on the menstrual cycle phase (p < 0.001).

Myometrial DFF40, DFF45 and Bcl-2 expression was independent from all the above clinical features.

Table I. Clinical characteristics of tissue specimen donors

Parameter	Endometrium			Myometrium			*P*-value
	Proliferatory phase (*n* = 25)	Secretory phase (*n* = 26)	Post-menopausal (*n* = 27)	Proliferatory phase (*n* = 24)	Secretory phase (*n* = 20)	Post-menopausal (*n* = 21)	
Age, mean ± SD, range [years]*	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$^{\$\#}$
BMI, mean ± SD, range [kg/m^2]*	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$^{\$}$
Age of first menstrual period, mean ± SD, range [years]*	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$^{\$}$
Age of menopause, mean ± SD [years]*	–	–	51.33 ±2.08 46.00–55.00	–	–	51.48 ±1.86 47.00–55.00	0.806$^{\$\$}$
Duration of menstrual cycle, mean ± SD, range [days]*$^{\&}$	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	23 (92.00%)	24 (92.31%)	20 (74.07%)	22 (91.67%)	18 (90.00%)	17 (80.95%)	0.273**
Irregular	2 (8.00%)	2 (7.69%)	7 (25.93%)	2 (8.33%)	2 (10.00%)	4 (14.83%)	
Menstrual cycles$^{\&}$:							
Painful	3 (12.00%)	4 (15.38%)	3 (11.12%)	2 (8.33%)	1 (5.00%)	2 (5.00%)	0.902**
Painless	22 (88.00%)	22 (84.62%)	24 (88.88%)	22 (91.67%)	19 (95.00%)	19 (95.00%)	
Duration of menstruation, mean ± SD, range [days]*	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$^{\$}$
Type of menstrual bleeding$^{\&}$:							
Scant	2 (8.00%)	2 (7.69%)	3 (11.11%)	2 (8.34%)	1 (5.00%)	1 (4.77%)	0.997**
Normal	22 (88.00%)	22 (84.62%)	22 (81.48%)	20 (83.32%)	17 (85.00%)	18 (85.71%)	
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, **χ^2 test, $^{\$}$ANOVA – one-way analysis of variance, $^{\$\$}$Student's t-test, $^{\#}$p-value statistically significant, $^{\&}$for the last 24 months of reproductive age.*

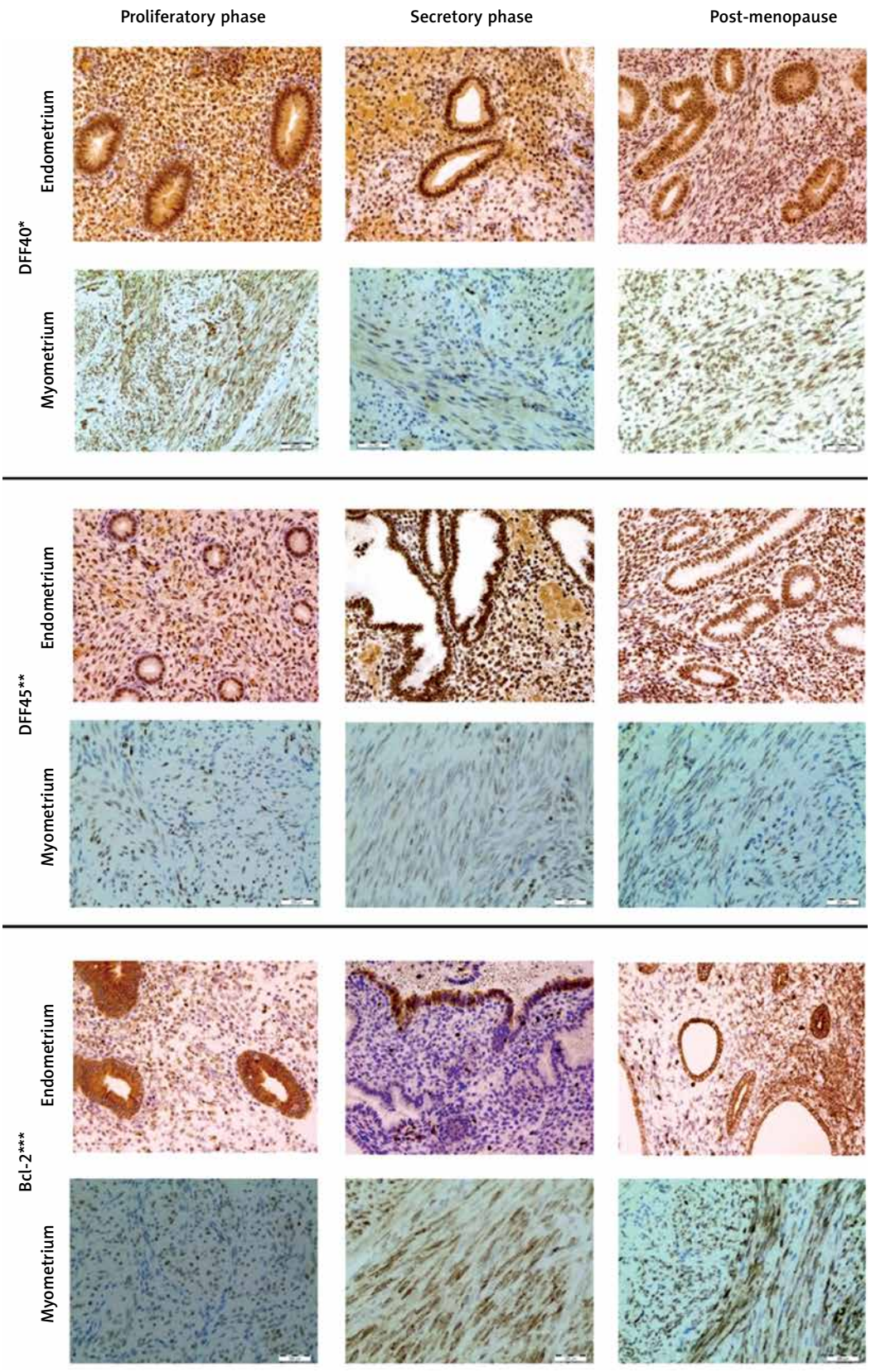

Figure 1. Expression of DFF40*, DFF45** and Bcl-2*** (200×) in normal human endometrium and myometrium
**DNA fragmentation factor 40, **DNA fragmentation factor 45, ***B-cell lymphoma 2.*

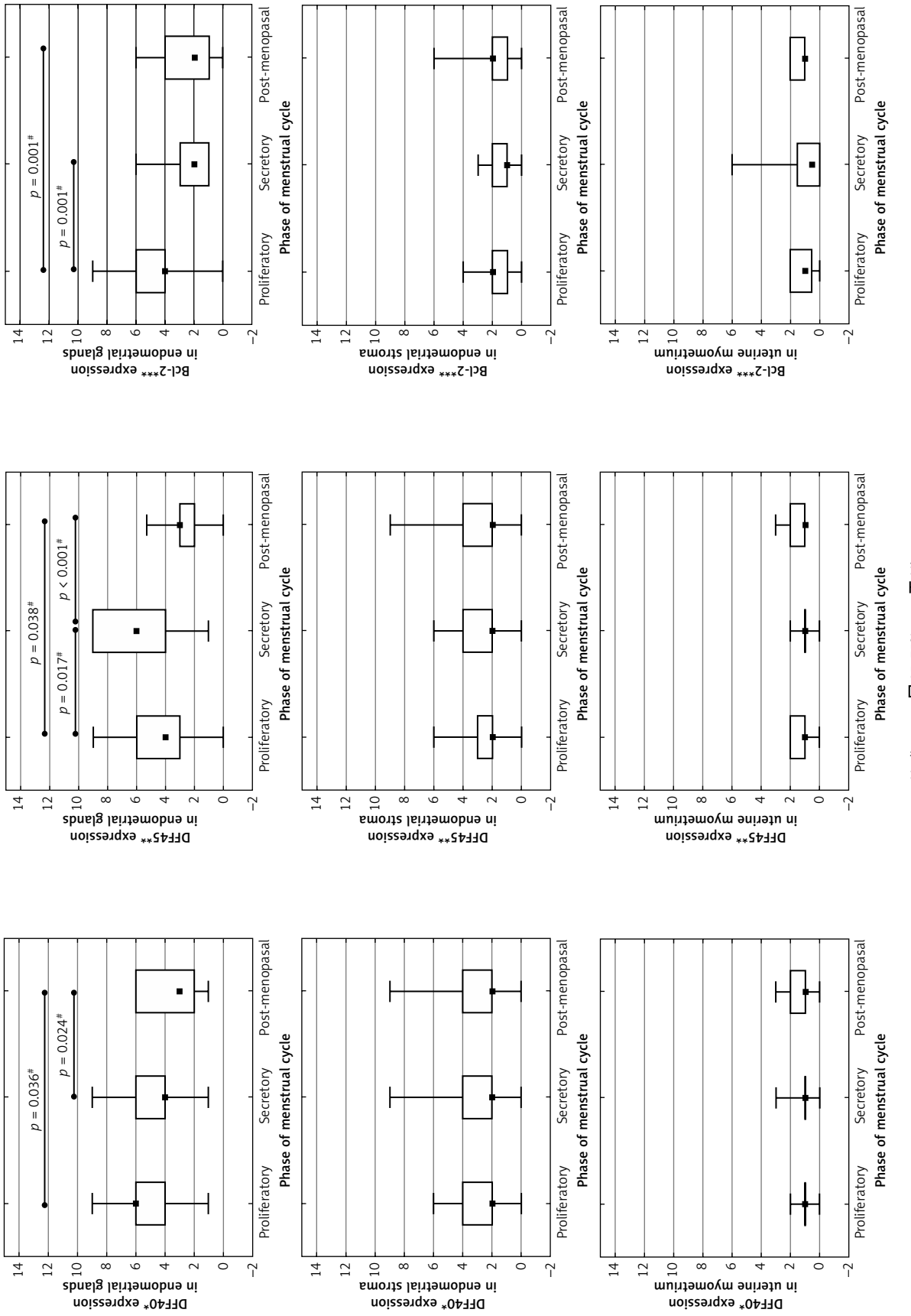

Figure 2. Median DFF40*, DFF45**, and Bcl-2*** expression in the normal endometrial glands, stroma, and uterine myometrium

**DNA fragmentation factor 40, **DNA fragmentation factor 45, ***B-cell lymphoma 2, #p-value statistically significant.*

Table II. Immunoexpression of DFF40; DFF45 and Bcl-2 in normal human endometrium and normal human uterine myometrium in different phases of menstrual cycle and postmenopausally

Variable	Menstrual cycle phase				Post-menopausal		
	Proliferatory			Secretory			
Median DFF40* expression, (IQR) and min.–max.:							
Endometrial glands	$p < 0.001^{\#}$	$p < 0.001^{\$}$	6.00 (2.00) 2.00–9.00	$p < 0.001^{\#}$	$p = 0.014^{\$}$	6.00 (3.00) 1.00–9.00	$p < 0.001^{\#}$	4.00 (2.00) 1.00–6.00
Endometrial stroma		$p = 0.001^{\#}$	3.00 (2.00) 0.00–9.00		$p = 0.001^{\$}$	3.00 (2.00) 0.00–9.00		$p = 0.001^{\#}$ 3.00 (2.00) 1.00–9.00
Uterine myometrium			1.00 (1.00) 0.00–4.00			1.00 (0.50) 0.00–3.00		2.00 (1.00) 0.00–3.00
Median DFF45** expression (IQR) and min.–max.:								
Endometrial glands	$p < 0.001^{\#}$	$p = 0.018^{\$}$	3.00 (2.00) 0.00–6.00	$p < 0.001^{\#}$	$p < 0.001^{\$}$	4.00 (2.00) 2.00–9.00	$p = 0.021^{\#}$	$p < 0.004^{\$}$ 2.00 (2.00) 0.00–6.00
Endometrial stroma			2.00 (2.00) 0.00–4.00		$p = 0.006^{\#}$	2.00 (2.00) 0.00–6.00		1.00 (1.00) 0.00–2.00
Uterine myometrium			1.00 (1.00) 0.00–3.00			1.00 (0.00) 0.00–2.00		1.00 (0.00) 0.00–2.00
Median Bcl-2*** expression, (IQR) and min.–max.:								
Endometrial glands	$p < 0.001^{\#}$	$p < 0.001^{\$}$	4.00 (0.00) 2.00–9.00			1.00 (2.00) 0.00–6.00		2.00 (3.00) 0.00–6.00
Endometrial stroma		$p < 0.001^{\#}$	2.00 (1.00) 0.00–4.00			1.00 (1.00) 0.00–2.00		2.00 (1.00) 0.00–6.00
Uterine myometrium			1.00 (1.50) 0.00–4.00			0.50 (1.50) 0.00–6.00		1.00 (1.00) 1.00–4.00

**DNA fragmentation factor 40, **DNA fragmentation factor 45, ***B-cell lymphoma 2, #Mann-Whitney U-test, $paired Wilcoxon test.*

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: $\kappa = 0.96$ ($p < 0.001$).

Discussion

To the best of our knowledge, this study is 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. 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 [3, 4, 8].

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 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. These findings are consistent with those of Matsumoto *et al.*, who observed that apoptosis most frequently appeared in the epithelial endometrium [21]. However, it must be noted that DFF40 alone is not sufficient to execute DNA fragmentation, because when DFF40 is in the nucleus, it remains bound to DFF45, which inhibits the activity of DFF40. 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 [22, 23]. 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 makes it potentially susceptible to apoptosis. Regression analysis proved that DFF40 and DFF45 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 the endometrial glands. Similarly, DFF45 endometrial glandular expression was dependent only on the menstrual cycle phase and menopausal status. 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. These results are consistent with those of Otsuki *et al.*, 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) [24]. Furthermore, these authors found that the disappearance of Bcl-2 expression was correlated with the appearance of apoptosis. In addition, Li *et al.* [25] 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 and increased DFF45 expression that occurs during the secretory phase of the menstrual cycle may enhance apoptosis.

Although our outcomes are consistent with the data obtained by other researchers, 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. However, the roles that the DFF40/DFF45 complex and Bcl-2 play in apoptosis have already been explained, and this goal was not the aim of our study. In addition, we realize that an immunostaining analysis is subjective. Therefore, two experienced pathologists 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 [3, 4, 8–12, 16]. 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. Suitable selection of cases, based on their medical history, hysteroscopic and histopathology results, allowed us to avoid bias related to underlying and unidentified endometrial and myometrial pathology.

In conclusion, 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 that their levels are stable in the normal uterine myometrium. This comprehensive evaluation provides a better understanding of other findings concerning DFF45 and Bcl-2 expression in female genital tract pathologies, including malignancies.

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Conflict of interest

The authors declare no conflict of interest.

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