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Estrogen receptor α expression in tumor-infiltrating lymphocytes from patients with endometrial cancer

MARCIN A. JEDRYKA[1,2], ANNA SLAWEK[3], PAULINA KUBIK[3], DARIA LOREK[3], ANNA EWA KEDZIERSKA[3], ANDRZEJ CZEKANSKI[1,2], RAFAŁ MATKOWSKI[1,4] and ANNA CHELMONSKA-SOYTA[3]

[1]Department of Oncology, Wroclaw Medical University, 53-413 Wroclaw, Poland; [2]Department of Oncological Gynecology, Lower Silesian Oncology, Pulmonology and Hematology Center, 53-413 Wroclaw, Poland; [3]Laboratory of Reproductive Immunology, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 53-114 Wroclaw, Poland; [4]Breast Cancer Clinic, Lower Silesian Oncology, Pulmonology and Hematology Center, 53-413 Wroclaw, Poland

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Abstract. The complex crosstalk between the tumor milieu, including the hormonal environment and immune system interactions, is important to tumor growth in endometrial cancer (EC). Estradiol-mediated estrogen receptor α (ERα) signaling is critical for the function of regulatory tumor-infiltrating lymphocytes (TILs) in patients with cervical cancer. Therefore, the present study investigated the relative ERα level in infiltrating lymphocytes derived from EC tissues and whether its variable expression is associated with clinicopathological features, including the molecular classification. Endometrial tumor and normal endometrium samples were collected from 82 patients diagnosed with EC; however, only 54 samples were assessed as sufficient and qualified for further study. The frequency of T helper lymphocytes (Th cells), cytotoxic T lymphocytes (CTLs) and B lymphocytes (B cells) as well as the percentages of these cells expressing ERα were examined using flow cytometry. Furthermore, the expression of ERα in these TIL subpopulations was evaluated using median fluorescence intensity (MFI) to assess the absolute level of ERα in the studied lymphocytes. Associations of ERα levels in TILs with clinicopathological characteristics, including molecular subtypes, were measured. All the studied TIL subpopulations showed a significantly lower ERα level compared with normal endometrial tissue, which constituted the control group. However, the frequencies of Th cells and Th cells expressing ERα were significantly increased, while the frequencies of CTLs and CTLs expressing ERα were significantly decreased in EC compared with the control. The frequency of B cells expressing ERα was significantly increased in high grade EC tumors and tumors harboring mismatch repair deficiency. ERα expression (demonstrated with MFI) on examined TIL subsets was negatively correlated with body mass index in patients but did not demonstrate other correlations with the examined clinicopathological prognostic factors. The mechanism of ERα decrease in TILs from endometrial tumors as well as its prognostic significance and potential role in therapeutic targeting needs further investigation, including further examination of its molecular background and functional validation experiments.

Correspondence to: Dr Marcin A. Jedryka, Department of Oncology, Wroclaw Medical University, Plac Hirszfelda 12, 53-413 Wroclaw, Poland
E-mail: marcin.jedryka@umw.edu.pl

Abbreviations: EC, endometrial cancer; ER, estrogen receptor; TILs, tumor-infiltrating lymphocytes; TCGA, The Cancer Genome Atlas; POLE, polymerase ε; MSI, microsatellite instability; PI3K, phosphoinositide 3-kinase; OS, overall survival; FoxP3, forkhead box P3; PD-1, programmed cell death-1; PD-L1, programmed cell death ligand-1; ESGO, European Society of Gynecological Oncology; ESTRO, European Society for Radiotherapy and Oncology; ESP, European Society of Pathology; Th cells, T helper lymphocytes; B cells, B lymphocytes; CTLs, cytotoxic T lymphocytes; MFI, median fluorescence intensity; BMI, body mass index; NSMP, non-specific molecular profile; MMR-d, mismatch repairs deficient; TME, tumor microenvironment

Key words: endometrial cancer, estrogen receptor α, tumor-infiltrating lymphocytes, median fluorescence intensity, The Cancer Genome Atlas, molecular clusters

Introduction

Endometrial cancer (EC) is the most common gynecological malignancy in developed countries, with a rising incidence and associated mortality (1,2). For a number of years, EC has been classified dichotomously, including by examining clinical prognostic factors combined with histological characteristics and sex hormone receptor status (3). However, contemporary translational research efforts have further characterized endometrial tumors to identify risk groups and potential molecular sensitivities to targeted therapies, which could improve overall prognoses. For instance, The Cancer Genome Atlas (TCGA) Research Network consortium have proposed a novel EC classification based on genomic analysis that includes polymerase ε somatic (*POLE*) mutations, *TP53* mutations, copy number alterations and microsatellite instability (MSI) (4). Despite these breakthrough molecular

discoveries, the complex crosstalk between the tumor milieu, including the hormonal environment and immune system interactions, is considered to be an important pathway for EC growth (5). Identifying the subpopulations of patients with EC harboring unfavorable genomic and immune predictive features and introducing targeted treatment could improve the final therapeutic outcome (5).

Exposure to unopposed estrogen activity has been recognized as the main trigger for EC development (6). Estrogen binds to estrogen receptors (ERs) that exist in two main forms, ERα and ERβ, forming a complex which then acts on the estrogen response element in the cell nucleus, activating the transcription of target genes (7). ERα and ERβ are considered to have different expression patterns and biological functions in gynecological tumors (8-10). Specifically, ERα expression has been shown to be markedly lower in endometrial stroma than in the glands, indicating that stromal cells lose ERα expression when tumor cells proliferate as result of EC progression. The absence of ERα expression in EC tumor tissue has also been significantly associated with advanced clinical stage and grade, as well as being indicated as a strong predictor of lymph node involvement (11). At the beginning of EC development, significant IL-17A-driven ERα upregulation by tumor-infiltrating macrophages results in increased local estrogen sensitivity of the endometrial lesion (12). During EC progression to advanced stages, more ERα-negative cells appear due to changes in the ERα sensitivity to estrogen, leading to the significant decrease in the expression of this receptor (10). The lack of ERα expression in EC has also been considered a robust predictive marker of epithelial-mesenchymal transition (EMT) and associated with reduced patient survival (13). Additionally, it has been found that ERα-negative tumors are associated with the activation of the phosphoinositide 3-kinase (PI3K) pathways, which could implicate ERα expression as a potential predictive biomarker of the therapeutic response to PI3K pathway inhibitors (13). Another important molecular mechanism underlying the role of ERα in endometrial tumor development is the methylation of its genes. Methylation of the *ERα-C* isoform is frequently found in EC tissue compared with normal endometrium (14-16). In a study, this methylation was detected in 94% of EC samples in which the *ERα-C* gene was inactivated, suggesting a mechanism for tumor development (14).

The expression of tumor-infiltrating lymphocytes (TILs) in cancer tissue has been demonstrated to be a strong prognostic factor in patients with EC (5,17-21). In a study, the presence of a high number of $CD8^+$ T lymphocytes predicted an improved overall survival (OS) of the entire EC cohort, while a high number of forkhead box P3 ($FoxP3^+$) T cells was associated with a decrease in the OS of patients with the endometrioid EC subtype and memory T lymphocytes ($CD45R0^+$) were independent predictors of an increased OS in patients with non-endometrioid EC (18). Čermáková *et al* (19) demonstrated that $CD3^+$ TIL counts decrease with an advanced EC stage. Furthermore, a high level of TILs has been shown to be negatively correlated with histological grade, myometrial invasion and lymph node metastasis, while increased densities of $CD8^+$ and $CD45R0^+$ T cells in EC tumors are associated with favorable outcomes (21).

Considering the new molecular TCGA classification of EC tumors and the variation in TIL activities and immunosuppressive characteristics between the molecular EC subtypes, the assessment of such an immune marker may improve the predicted response to immunotherapy (22). MSI and high-grade *POLE* wildtype/microsatellite-stable EC clusters have been confirmed to be highly immunogenic subtypes with a significant high-density TIL infiltrate, especially within the invasive front of the tumor (23). *POLE*-mutated and MSI EC tumors possess a high neoantigen load and number of TILs, which is counterbalanced by the upregulation of programmed cell death-1 (PD-1) and programmed cell death ligand-1 (PD-L1), making these tumors excellent candidates for PD-1-targeted immunotherapies (5,24). Estradiol-mediated ERα signaling has been reported to be critical for the regulation of TIL function in patients with cervical cancer. Specifically, Adurthi *et al* (25) presented evidence that estradiol and ERα interactions with the *FOXP3* locus exerted potent effects on gene expression and could modulate the suppressive function of primary human tumor-infiltrating regulatory T cells in patients with cervical cancer. This proposed model may be universal as ERα has been shown to regulate genes in a similar manner in MCF-7 breast cancer cells (26); however, to the best of our knowledge, this model has not been studied in an EC population thus far.

In our previous research, decreased gene expression of both ER isoforms was observed in the peripheral blood lymphocytes isolated from patients with EC compared with individuals of reproductive age with simple functional ovarian cysts (27). However, the tumor environment crosstalk between TILs and cancer cells may be different due to distinct tissue conditions compared with the peripheral blood.

Based on these findings, we hypothesized that ERα expression in infiltrating lymphocytes from EC tissue may change during disease progression and may depend on the EC molecular subtype; therefore, the present study aimed to investigate ERα manifestation in endometrial tumor-derived TILs. The potential prognostic value of the ERα level in TILs from patients with EC may help to translate the findings of previous studies into improved diagnostic and therapeutic approaches.

Materials and methods

Patients. The study group was selected from a cohort of patients with EC who underwent surgery as their primary treatment at the Department of Oncological Gynecology within the Lower Silesian Oncology, Pulmonology and Hematology Center (Wroclaw, Poland) between September 2019 and June 2023. All patients underwent hysterectomy with bilateral salpingo-oophorectomy followed by pelvic and paraaortic lymph node dissection or a sentinel lymph node biopsy procedure, depending on the preoperative evaluation and assumed clinical stage. This study was approved by the Wroclaw Medical University Bioethics Committee (registration no. 166/2019; March 5, 2019). Written informed consent for study participation was obtained from each qualified patient prior to surgery, in accordance with the Declaration of Helsinki.

The inclusion criteria were as follows: i) The diagnosis of primary EC (both endometrioid and non-endometrioid) and a surgical intervention treatment plan; ii) endometrial

tumor size of at least 1 cm in the largest diameter to ensure sufficient tissue both for routine pathological assessment and for the present study; iii) no previous oncological treatment of any type and no previous immunotherapy of any type for any reason; and iv) patients aged >18 years old who provided written, informed consent.

The pathological assessments included a standard institutional protocol for EC, such as immunohistochemical staining tests for mismatch repair (MMR) status and p53 protein expression and Sanger sequencing of EC tissues to determine the somatic *POLE* mutation status (28), the results from which were obtained from the medical records. All cases were staged according to the 2018 International Federation of Gynecology and Obstetrics classification based on the final histopathological reports (29), and divided into molecular clusters based on TCGA classification (4). Additionally, the study population was defined and stratified to specific risk groups due to the presence of prognostic factors according to the European Society of Gynecological Society (ESGO)/European Society for Radiotherapy and Oncology (ESTRO)/European Society of Pathology (ESP) 2021 guidelines (30). The general study group characteristics are presented in Table I.

The primary aim of the present study was to examine ERα expression in all studied subgroups of TILs derived from endometrial tumors compared with healthy endometrial tissue. The secondary aims included examining the correlations of ERα expression in TILs with the relevant clinicopathological features (including molecular clusters) of the studied endometrial tumors.

Table I. General clinical characteristics of the study population (n=54).

Parameters	Value
Median age, years (range)	67 (29-82)
Median BMI, kg/m^2 (range)	32.5 (23-55)
Median tumor size, cm (range)	3.0 (1-11)
FIGO stage, n (%)	
Stage I	40 (74.1)
Stage >I	14 (25.9)
Myometrial infiltration, n (%)	
<50%	30 (55.6)
≥50%	24 (44.4)
Parametria infiltration, n (%)	
Negative	52 (96.3)
Positive	2 (3.7)
Histological grade[a], n (%)	
Low grade	39 (72.2)
High grade	15 (27.8)
Histological type[b], n (%)	
Type I	48 (88.9)
Type II	6 (11.1)
LVSI, n (%)	
Not identified	37 (68.5)
Present	17 (31.5)
Clinical risk group, n (%)	
Low	21 (38.9)
Intermediate	9 (16.6)
High-intermediate	13 (24.1)
High	11 (20.4)
MMR, n (%)	
MMR proficient	32 (59.2)
MMR deficient	22 (40.8)
TCGA, n (%)	
NSMP	26 (48.1)
MSI	22 (40.8)
p53 mutant	6 (11.1)

[a]Low grade: Grades 1 and 2, high grade: Grade 3. [b]Type I: endometrioid tumor, type II: Non-endometrioid tumor. BMI, body mass index; FIGO, International Federation of Gynecology and Obstetrics; LVSI, lymphovascular space involvement; MMR, mismatch repair; TCGA, The Cancer Genome Atlas; NSMP, non-specific molecular profile; MSI, microsatellite instability.

Sample collection. Immediately after the hysterectomy, the uterus was halved to expose the endometrial tumor and the macroscopically normal endometrium. Both EC tissue and control tissue (healthy endometrium adjacent to the tumor tissue) were sampled by an experienced gynecological oncologist and then immediately transported to the Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland for further processing. The postoperative samples were thoroughly examined by an experienced pathologist who also verified the sample collection sites by visual assessment to confirm that the macroscopic healthy endometrium was non-malignant.

A total of 82 samples were collected out of the 100 planned. However, lymphocyte isolation failed in some cases, especially when specimens were very scant. Furthermore, the timing of sample collection was related to the peak of the COVID-19 pandemic, which inferred logistical challenges. Therefore, only 54 cases (with sufficient tumor and control tissue samples) were ultimately enrolled and investigated, which was a limitation of the present study.

Enzymatic digestion. EC and control tissues (~300 mg) were roughly minced with scissors into small pieces in Hanks' Balanced Salt Solution with Ca^{2+}/Mg^{2+} (Biowest) supplemented with 30 µg/ml DNase I (Roche Diagnostics) and 1 mg/ml collagenase type IV (BioShop Canada, Inc.), then incubated in a water bath for 15 min at 37˚C. The tissue dispersing process was repeated, including a further incubation for 15 min at 37˚C. Then, the samples were briefly shaken and incubated for 15 min at 37˚C. This step was repeated three times. The digested tissues were passed through 100 and 40 µm cell strainers. The purified cells were centrifuged at 300 x g for 4 min at 4˚C, then the cell pellet was resuspended in sorting buffer [phosphate-buffered saline (PBS) supplemented with 2 mM EDTA and 2% fetal bovine serum (Biowest)] and incubated for 30 min at 37˚C. The samples were then centrifuged at 300 x g for 4 min at 4˚C and the cell pellet was resuspended in RBC Lysis Buffer (BioLegend, Inc.). After 15 min of incubation at room

temperature in the dark, according to the manufacturer's instructions, the cells were centrifuged at 300 x g for 5 min at 4˚C and finally washed with PBS buffer.

Flow cytometry. Isolated cells (1x10^6) were stained with a cocktail of fluorescently labeled antibodies: PE/Cy7 Anti-CD3 (clone: UCHT1; cat. no. 30042), APC/Cy7 anti-CD4 (clone: RPA-T4; cat. no. 300518), BV421 anti-CD8a (clone: RPA-T8; cat. no. 301036), APC anti-CD19 (clone: HIB19; cat. no. 302212) and AF700 anti-CD45 (clone: HI30; cat. no. 304024) (all from BioLegend, Inc.) for 30 min at 4˚C in the dark. Then, the cells were washed twice, with staining buffer (0.5 mM EDTA, 0.002% sodium azide, 1% fetal bovine serum), and intracellular staining was performed according to the manufacturer's protocol (True-Nuclear Transcription Factor Buffer Set; BioLegend, Inc.). Briefly, cells were fixed for 14 h at 4˚C in the dark, washed twice with permeabilization buffer and stained for 1 h at 4˚C in the dark with a recombinant AF488 anti-ERα antibody (clone: E115; cat. no. AB194150; Abcam) or appropriate isotype control (AF488 rabbit IgG, clone: EPR25A; cat. no. AB199091; Abcam) at the same concentration as the specific antibody. The samples were washed twice with permeabilization buffer, fixed with buffered 1% paraformaldehyde at 4˚C and then the cells were analyzed using a BD LSR Fortessa™ flow cytometer (BD Biosciences). Only viable cells (stained with the Zombie Red™ Fixable Viability Kit at room temperature, in the dark, for 15 min, according to the manufacturer's instructions; BioLegend, Inc.) were analyzed. Before extracellular and intracellular staining, Fc receptor blocking with Human TruStainFcX (5 µl in 100 µl staining volume, 10 min at room temperature; BioLegend, Inc.) was performed according to the manufacturer's instructions. The relative level of ERα is shown as the specific median fluorescence intensity (MFI) based on the difference between the median fluorescence intensity of the specifically stained cells and the isotype-matched control cells gated for the populations of interest. All analyses were performed using FlowJo v10.7.2 (BD Biosciences). Representative dot plots showing the gating strategy of the cells of interest are shown in Fig. 1.

Statistical analysis. Continuous variables are expressed as mean ± standard deviation and median with interquartile range. STATISTICA version 13.3 (TIBCO Software, Inc.) and R statistical software version 4.1.0 (The R Foundation; http://www.R-project.org) were used to perform the statistical procedures. Differences in categorical factors were determined with Fisher's exact test. Normality was assessed using Shapiro-Wilk tests. Differences in continuous values between two groups were assessed with non-parametric Mann-Whitney U tests for non-normally distributed variables. Differences in continuous variables among three or more groups were assessed with the Kruskal-Wallis test. The Dunn test was used for adjustment for multiple testing. To assess the correlation between risk factors and the subsets of tested lymphocytes in the tumor tissue, Spearman's rank correlation coefficient test (ρ) was performed. The Wilcoxon signed-rank test was used to compare the level of the two dependent parameters. For multiple comparisons, the Bonferroni correction was used. All tests were two-sided. P<0.05 was considered to indicate a statistically significant difference.

Results

Frequencies of TILs in EC tissues vary depending on the lymphocyte type. In the present study, three subpopulations of lymphocytes were investigated: T helper lymphocytes (Th cells; $CD45^+CD3^+CD4^+$), cytotoxic T lymphocytes (CTLs; $CD45^+CD3^+CD8^+$) and B lymphocytes subset (B cells; $CD45^+CD3^-CD19^+$). The frequency of Th cells increased significantly in EC tissues compared with the control tissues (41.4 vs. 36.1%; P<0.001). By contrast, the frequency of CTLs was significantly lower in EC tissues (37.0 vs. 42.5%; P=0.002), while there were no differences in the B cells frequencies between EC and control tissue (5.3 vs. 4.8%; P=0.928) (Fig. 2).

Similarly, the prevalence of ERα-expressing TILs was dependent on the subpopulation. The number of $ERα^+$ Th cells was significantly higher in EC tissues compared with the control tissue (40.9 vs. 33.7%; P<0.001), whereas the frequency of $ERα^+$ CTL cells was significantly decreased in EC tissues (35.9 vs. 40.3; P<0.006). However, significant differences in the frequency of $ERα^+$ B cells were not observed between the EC and control tissues (Fig. 3).

ERα expression is significantly decreased in TILs from EC tissues. The main objective of the present study was to quantify ERα expression in TILs based on MFI, which was its absolute level, while the frequency of ERα-positive TILs only reflects their presence in tumor tissue. The expression of ERα (presented as MFI) of all tested endometrial tumor-derived TIL subpopulations was significantly decreased compared with control tissues of normal adjacent endometrium (ERα-MFI in Th cells was 1,721 vs. 2,036 in the control; P<0.001; ERα-MFI in CTLs was 1,871 vs. 2,006; P=0.001 and ERα-MFI in B cells was 1,546 vs. 1,898; P<0.001; Fig. 4).

Association of the TIL and $ERα^+$ TIL frequencies with the clinicopathological characteristics of the patients. The associations between the measured TIL subpopulation frequencies and important prognostic factors, such as the clinical risk group according to ESGO/ESTRO/ESP recommendations (27), myometrial infiltration (≤50 vs. >50%), parametrial involvement (present vs. absent), lymphovascular space involvement (present vs. absent), histological type (endometrioid vs. non-endometrioid), histological grading (low vs. high Grade), stage (I vs. II and III), body mass index (BMI; <30 vs. 30-35 vs. >35), tumor size (<2 cm vs. 2-4 cm vs. >4 cm), p53 mutation (present vs. absent), MMR protein expression (proficient vs. deficient) and type of TCGA molecular clusters [*POLE* mutated tumors vs. MMR deficient tumors (MMR-d) or with microsatellite instability (MSI) vs. non-specific molecular profile tumors (NSMP) and p53 mutated tumors], were estimated for every patient. However, no cases with *POLE* mutations were found in the study cohort; therefore, analysis was only possible in the three remaining cluster subpopulations. All the data are presented in Tables II and III. The B cell frequency was significantly increased in high-grade EC tumors compared with low grade (6.7 vs. 3.9; P<0.024), but no significant difference in grade was observed in the Th and CTLs subsets. MMR-d tumors showed an increased B cell frequency compared with MMR proficient EC tissues (5.9 vs. 4.7; P<0.028). However,

Figure 1. Representative dot plots for the gating of T helper lymphocytes (CD45⁺CD3⁺CD4⁺), cytotoxic T lymphocytes (CD45⁺CD3⁺CD8a⁺) and B lymphocytes (CD45⁺CD3⁻CD19⁺) and representative histograms of the ERα expression (red histograms) overlayed with the respective isotype-matched controls (blue histograms) in these cells. ERα, estrogen receptor α.

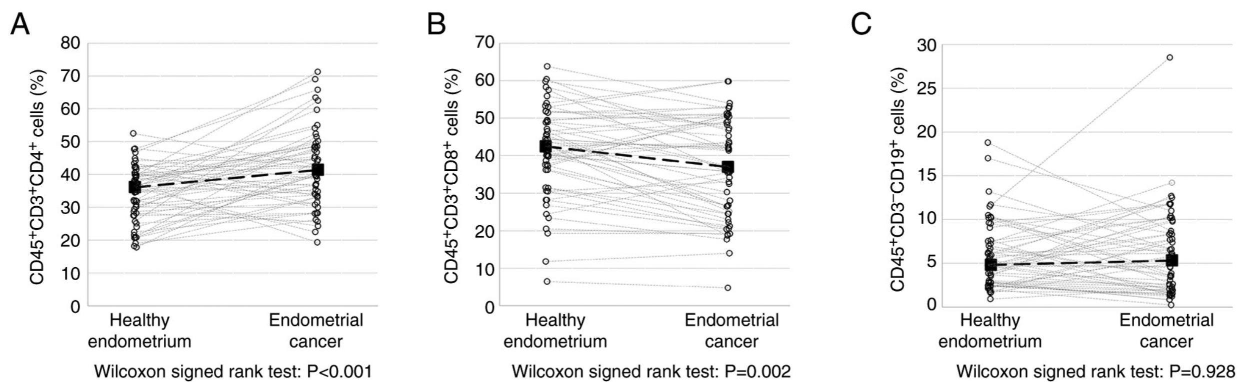

Figure 2. Frequency of the three studied subpopulations of tumor-infiltrating lymphocytes, (A) T helper lymphocytes (CD45⁺CD3⁺CD4⁺), (B) cytotoxic T lymphocytes (CD45⁺CD3⁺CD8⁺) and (C) B lymphocytes (CD45⁺CD3⁻CD19⁺), in the collected cancer and healthy endometrium samples. The results were compared using Wilcoxon signed rank test.

the frequency of the examined Th cells was significantly decreased in EC tissues with MSI (30.8 vs. 37.3; P<0.037). Furthermore, the frequency of Th cells significantly decreased in obese patients (BMI >35) compared with normal weight patients (BMI <30) (29.8 vs. 37.9; P<0.048).

Notably, the frequency of the examined B cell subset expressing ERα demonstrated a significant increase in high-grade EC tumors compared with low-grade tumors (5.4 vs. 3.8; P<0.016, MMR-d tumors compared with MMR-p tumors (5.6 vs. 3.9; P<0.018) and the TCGA MSI molecular cluster compared with the NSMP and p53 mutant clusters (5.6 vs. 3.6 vs. 2.4; P<0.033) (Table III and Fig. 5).

ERα expression in TILs from EC tissue was not significantly associated with relevant prognostic factors, except BMI. ERα expression (when measured by MFI) in all the studied

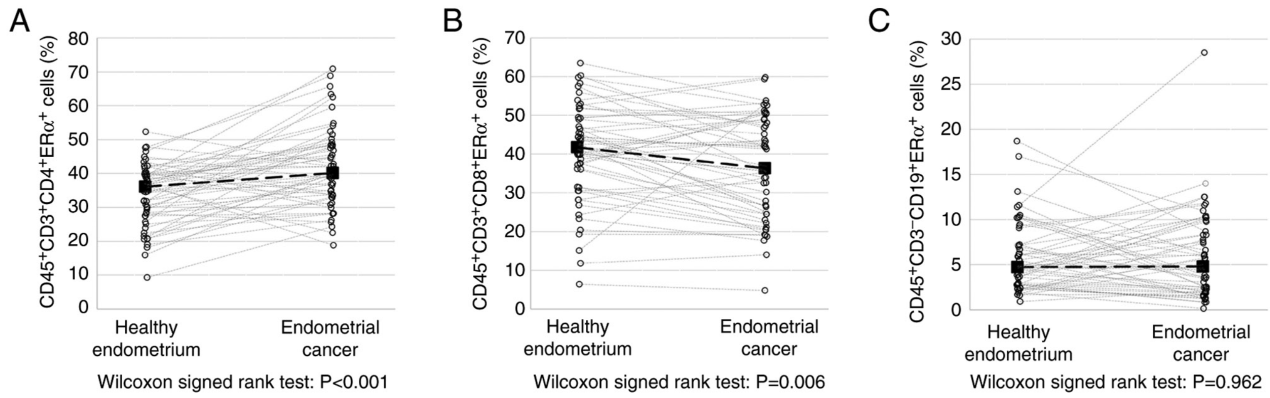

Figure 3. Frequency of the three studied subpopulations of tumor-infiltrating lymphocytes expressing ERα, (A) T helper lymphocytes expressing ERα ($CD45^+CD3^+CD4^+ERα^+$), (B) cytotoxic T lymphocytes expressing ERα ($CD45^+CD3^+CD8^+ERα^+$) and (C) B lymphocytes expressing ERα ($CD45^+CD3^-CD19^+ERα^+$), in the collected endometrial cancer and healthy endometrium samples. The results were compared using Wilcoxon signed rank test. ERα, estrogen receptor α.

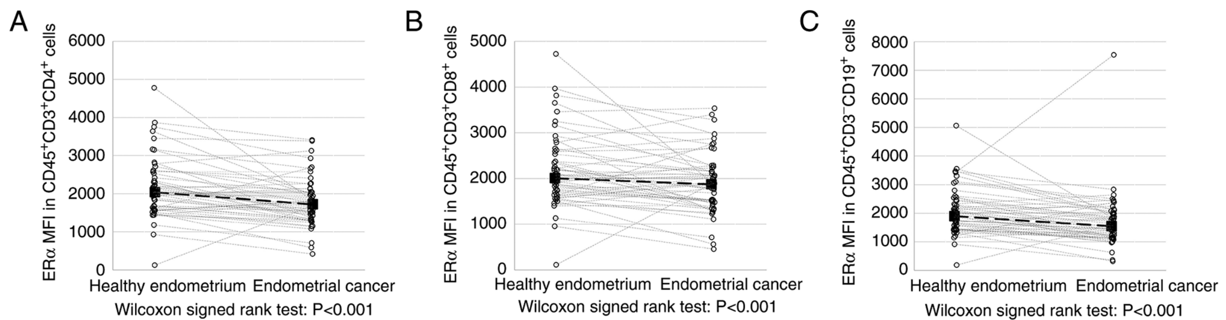

Figure 4. Expression of ERα (measured as MFI) in the three studied tumor infiltrating lymphocyte subpopulations, (A) T helper lymphocytes ($CD45^+CD3^+CD4^+$), (B) cytotoxic T lymphocytes ($CD45^+CD3^+CD8^+$) and (C) B lymphocytes ($CD45^+CD3^-CD19^+$), in the collected endometrial cancer and healthy endometrium samples. The results were compared using Wilcoxon signed rank test. ERα, estrogen receptor α; MFI, median fluorescence intensity.

TIL subgroups was not significantly associated with the relevant clinicopathological prognostic factors, except for BMI (Table IV). Furthermore, decreased ERα expression in each TIL subset was inversely correlated with the BMI of the studied patients (Fig. 6).

Discussion

In the present study, three main subsets of TILs demonstrated a different frequency pattern in EC samples compared with healthy endometrial tissues from the same patient: Th cells were increased, CTLs were decreased and no significant change in B cells was observed. The same results were observed for these TIL subsets with ERα positivity. However, when the ERα level was presented as MFI, all TIL subsets with ERα positivity were significantly decreased in EC tissues compared with healthy endometrial tissue. ERα expression (presented as MFI) in all studied TILs was not associated with any clinically relevant prognostic factors, except for BMI. Furthermore, an increase in BMI was inversely correlated with a decrease in ERα levels. Moreover, an increased frequency of ERα-positive B lymphocytes in patients with high-grade endometrial tumors classified as the MSI molecular group was observed. To the best of our knowledge, the present preliminary study was the first to investigate ERα expression in TILs derived from endometrial tumors.

The TIL frequency results from the present study are in concordance with another study that used flow cytometry to determine the Th cell and CTL distributions and levels in EC vs. non-neoplastic endometrium (31). A study by Jung *et al* (20), observed a negative correlation between the $CD8^+$ and $CD4^+$ infiltration rate (based on histopathological examination) and histological grade, myometrial invasion and lymph node metastasis (only $CD8^+$ rate), suggesting their role as important predictive factors. A number of studies have described the prognostic role of TIL involvement in EC tissue, highlighting the dominant positive correlation with high TIL density (especially $CD8^+$) in early stages and an overall good clinical outcome (18,19,21). Such prognostic correlations were not demonstrated in the present study. However, flow cytometry was used as the main tool to identify the prevalence of various TIL subsets in the present study, which was different from the typical methods of immunohistochemical staining and viewing whole microscopic slides in multiple fields to estimate the average percentage of TILs used by other investigators in their studies This methodological difference in TIL rate assessment could also explain the ambiguous differences observed in the lymphocyte groups examined in the present study in terms of their frequency in MMR-d endometrial tumors: Significant increase in B cells, significant decrease in Th cells and a negligible decrease in CTLs. This observation is contrary (except B cell frequency) to other studies

Table II. Frequencies of the three studied tumor infiltrating lymphocyte subsets, Th cells ($CD45^+CD3^+CD4^+$), CTLs ($CD45^+CD3^+CD8a^+$) and B cells ($CD45^+CD3^-CD19^+$), derived from the tumor according to certain endometrial cancer prognostic factors.

	Th cells (%)			CTLs (%)			B cells (%)		
Prognostic factor	Me	IQR	P-value	Me	IQR	P-value	Me	IQR	P-value
Clinical risk group			0.937			0.755			0.911
Low (n=21)	34.7	11.6		41.2	17.8		4.7	7.1	
Intermediate (n=9)	36.0	8.0		40.1	8.3		3.7	3.0	
High-intermediate (n=13)	36.8	11.3		42.5	8.4		5.9	3.7	
High (n=11)	31.8	17.1		45.6	16.8		4.9	3.8	
Myometrial infiltration			0.774			0.589			0.676
<50% (n=30)	35.6	12.4		40.8	12.8		4.7	7.1	
≥50% (n=24)	36.1	10.5		44.2	16.2		4.9	4.3	
Parametria involvement			0.647			0.714			0.697
Negative (n= 52)	36.1	12.0		42.5	13.2		4.8	4.8	
Positive (n=2)	36.8	13.3		38.5	14.2		5.5	1.4	
LVSI status			0.230			0.608			0.288
Not identified (n=37)	34.7	12.3		42.8	13.3		4.7	6.6	
Present (n=17)	37.3	11.6		41.2	17.5		5.9	2.5	
Histological type			0.611			0.752			0.474
Type I (n=48)	36.1	12.0		42.5	12.9		4.7	4.7	
Type II (n=6)	35.6	13.3		42.9	20.4		5.5	4.3	
FIGO stage			0.906			0.459			0.401
I (n=40)	36.2	12.0		41.9	15.3		5.2	6.5	
>I (n=14)	34.0	12.1		44.0	16.8		4.3	3.5	
Histological grade			0.870			0.446			0.024
Low grade (n=39)	36.0	11.7		41.2	17.8		3.9	4.5	
High grade (n=15)	36.2	17.1		45.6	14.6		6.7	5.4	
BMI, kg/m^2			0.048			0.309			0.367
<30 (n=14)	37.9	9.5		39.4	18.2		4.7	4.3	
30-35 (n=24)	34.0	11.9		42.6	8.2		4.7	4.0	
>35 (n=16)	29.8	16.0		46.5	14.6		6.7	5.9	
TU, cm			0.097			0.146			0.241
<2 (n=11)	36.1	12.7		39.2	24.4		4.8	5.6	
2-4 (n=27)	32.0	16.8		44.4	12.1		5.9	4.9	
>4 (n=16)	39.2	7.7		39.8	13.0		2.9	3.1	
p53			0.277			0.912			0.527
Normal (n=48)	35.4	12.3		42.5	13.2		4.7	4.8	
Mutant (n=6)	38.1	11.6		42.9	15.1		5.5	2.5	
MMR			0.037			0.238			0.028
MMR proficient (n=32)	37.3	10.4		39.8	17.9		4.7	4.0	
MMR deficient (n=22)	30.8	16.7		44.0	11.5		5.9	5.9	
TCGA			0.086			0.536			0.062
NSMP (n=26)	37.0	8.7		39.3	17.8		3.8	4.0	
MSI (n=22)	30.8	16.7		44.0	11.5		5.9	5.9	
p53 (n=6)	38.1	11.6		42.9	15.1		5.5	2.5	

Me, median; IQR, interquartile range; Th cells, T helper lymphocytes; CTLs, cytotoxic T lymphocytes; B cells, B lymphocytes; BMI, body mass index; FIGO, International Federation of Gynecology and Obstetrics; LVSI, lymphovascular space involvement; MMR, mismatch repair; TCGA, The Cancer Genome Atlas; NSMP, non-specific molecular profile; MSI, microsatellite instability; TU, tumor size.

Table III. Frequencies of the three studied tumor infiltrating lymphocyte subsets, Th cells ($CD45^+CD3^+CD4^+$), CTLs ($CD45^+CD3^+CD8a^+$) and B cells ($CD45^+CD3^-CD19^+$), expressing ERα derived from the tumor according to certain endometrial cancer prognostic factors.

	Th cells (%)			CTLs (%)			B cells (%)		
Prognostic factor	Me	IQR	P-value	Me	IQR	P-value	Me	IQR	P-value
Clinical risk group			0.916			0.744			0.792
Low (n=21)	34.7	12.3		40.0	16.0		4.5	7.1	
Intermediate (n=9)	36.0	7.7		40.1	8.0		3.6	2.0	
High-intermediate (n=13)	36.8	11.3		42.5	8.4		5.9	3.6	
High (n=11)	31.8	17.1		44.2	16.4		4.8	3.4	
Myometrial infiltration			0.663			0.433			0.486
<50% (n=30)	35.6	12.4		40.1	15.6		4.6	7.1	
≥50% (n=24)	36.1	10.4		44.1	16.1		4.8	4.2	
Parametria involvement			0.614			0.819			0.630
Negative (n=52)	36.1	12.3		41.8	15.4		4.7	4.4	
Positive (n=2)	36.7	13.4		38.5	14.1		5.5	1.5	
LVSI status			0.196			0.716			0.174
Not identified (n=37)	34.7	13.8		42.3	13.2		3.9	6.6	
Present (n=17)	37.0	11.4		41.1	15.1		5.9	2.5	
Histological type			0.554			0.620			0.401
Type I (n=48)	36.1	12.3		41.8	14.3		4.6	4.4	
Type II (n=6)	35.6	13.4		42.8	20.3		5.5	4.3	
FIGO stage			0.984			0.407			0.508
I (n=40)	36.3	13.2		40.7	16.8		4.7	6.4	
>I (n=14)	34.0	12.1		43.3	16.4		4.3	3.5	
Histological grade			0.985			0.374			0.016
Low grade (n=39)	36.0	12.4		41.1	16.6		3.9	3.8	
High grade (n=15)	36.2	17.1		44.4	14.5		6.7	5.4	
BMI, kg/m^2			0.087			0.177			0.270
<30 (n=14)	37.8	10.3		38.2	17.9		4.1	3.7	
30-35 (n=24)	34.0	11.9		42.6	6.0		4.6	3.8	
>35 (n=16)	29.8	16.0		46.5	14.5		6.6	5.6	
TU, cm			0.076			0.287			0.277
<2 (n=11)	36.1	12.8		39.2	24.4		4.8	5.5	
2-4 (n=27)	30.8	17.8		43.9	13.2		4.9	4.5	
>4 (n=16)	39.1	7.7		39.8	13.0		2.9	3.1	
p53			0.253			0.731			0.441
Normal (n=48)	35.4	13.2		41.8	15.4		4.6	4.4	
Mutant (n=6)	38.1	11.6		42.8	15.1		5.5	2.4	
MMR			0.060			0.170			0.018
MMR proficient (n=32)	37.0	12.0		38.7	16.6		3.9	3.9	
MMR deficient (n=22)	30.8	16.6		43.9	11.6		5.9	5.6	
TCGA			0.124			0.380			0.033
NSMP (n=26)	36.8	10.1		38.2	16.6		3.2	3.6	
MSI (n=22)	29.7	16.6		43.7	11.6		5.7	5.6	
p53 (n=6)	38.1	11.6		42.8	15.1		5.5	2.4	

ERα, estrogen receptor α; Me, median; IQR, interquartile range; Th cells, T helper lymphocytes; CTLs, cytotoxic T lymphocytes; B cells, B lymphocytes; BMI, body mass index; FIGO, International Federation of Gynecology and Obstetrics; LVSI, lymphovascular space involvement; MMR, mismatch repair; TCGA, The Cancer Genome Atlas; NSMP, non-specific molecular profile; MSI, microsatellite instability; TU, tumor size.

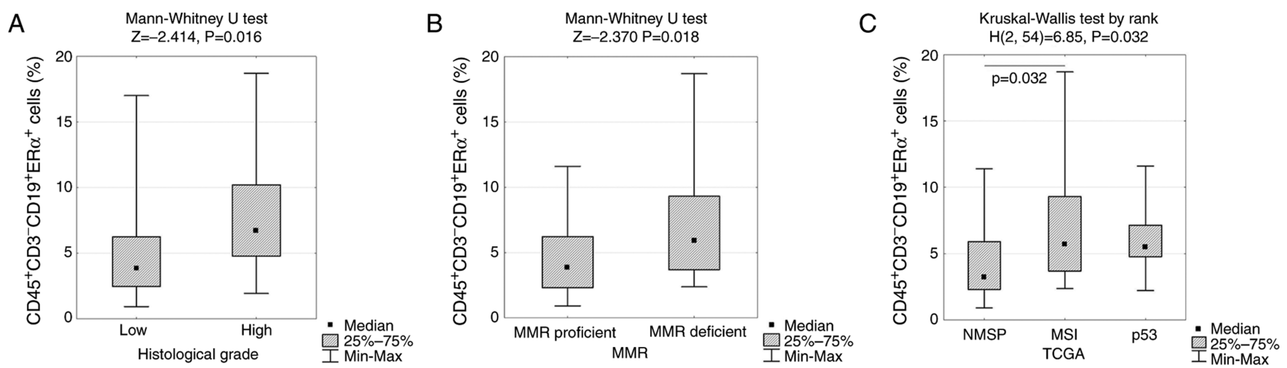

Figure 5. Relationship between the frequency of the B lymphocyte subset ($CD45^+CD3^-CD19^+$) expressing ERα derived from endometrial cancer tissue and certain prognostic features: (A) Histological grade, (B) MMR status and (C) TCGA cluster. The results were compared using Mann-Whitney U test or Kruskal-Wallis test followed by Dunn test. ERα, estrogen receptor α; MMR, mismatch repair; MSI, microsatellite instability; NSMP, non-specific molecular profile; p53, p53 mutated tumors; TCGA, The Cancer Genome Atlas.

that observed generally high TIL levels within the endometrial tumors of patients with *POLE* mutated and MMR-d EC (22,23,32). Notably, $CD3^+$ $PD-L^+$ and $CD3^+$ $PD-L1^+$ T-cell subpopulations showed significantly higher frequencies in EC tumors with MMR-d than with NSMP or p53 mutation-positive clusters (33). Specifically, MMR-d patients (with genetic mutation) demonstrated a significant increase in TILs compared with epigenetic MMR-d (MLH1 promoter hypermethylation) in EC tissues; however, no difference was demonstrated for PD-L1 expression (17). The clinical importance of higher TILs in mutated MMR-d EC tumors than in epigenetic MMR-d EC tumors is unknown, but it may associated with an improved prognosis of mutated MMR-d EC tumors and may be a favorable predictive marker for an improved response to PD-L1 targeted immunotherapy (17).

In contrast to T cells, B cells are considered not common in EC tumor epithelium, regardless of its genomic cluster but generally, elevated densities of epithelial and stromal T cells are accompanied by higher levels of B cells in the stromal compartment, with the highest expression levels demonstrated in MMR-d EC tumors (22). Moreover, tumor-associated B cells and their antibodies have recently been reported to be important factors of modulated immunity in the EC tumor microenvironment (TME) (34). Based on multiplex immunofluorescence and immunohistochemical staining of EC tissue microarrays, the immune response in the EC TME was found to be mediated by the binding of high levels of dimeric IgA (produced by tumor-associated B cells and plasma cells) to the polymeric immunoglobulin receptor, resulting in cell-intrinsic inflammatory pathways that promote T-cell-mediated immunity and the downregulation of DNA repair mechanisms, leading to an improved clinical outcome (35). Regardless of antigen recognition, irrelevant B-cell-derived IgA was shown to induce a series of inflammatory changes, ER stress and proapoptotic transcriptional pathways in EC cancer cells (35). The precise mechanism of B cell activation in the TME and the tumor-associated antigens specific for IgA remains unknown; however, we hypothesize that an increase in ERα-expressing B cells in the tumor milieu may play a role in this phenomenon, which should be clarified in further studies.

ERα plays a crucial role in endometrial malignant transformation as follows: i) Upstream regulators influence ERα transcription activity and thus EC cell proliferation; ii) ERα promotes EC occurrence with other co-regulators; and iii) ERα influences and mediates tumor proliferation, metastasis and apoptosis via downstream proteins or target genes (36). ERα has been shown to be a strong prognostic marker for EMT in EC and potentially predictive of the response to PI3K/mTOR pathway inhibitors (13). Kreizman-Shefer *et al* (37) demonstrated that ERα expression was significantly lower in EC compared with non-malignant tissue. Particularly, this decreased expression was observed in the stromal tissue of the EC tumor compared with the epithelial tissue, which may indicate an invasive tumor characteristic. In the present study, significantly decreased ERα expression (measured by MFI) was observed in the studied lymphocyte subpopulations derived from endometrial tumors compared with normal endometrium. We hypothesize that, with progressive endometrial tumor growth, TILs may exhibit decreased ERα levels compared with normal endometrial tissue due to a paracrine mechanism triggered by the development of tumor growth, with an increasing number of tumor progression mechanisms; however, this possibility was not investigated in the present preliminary study and the relationship between TIL frequency and ERα expression remains unknown. In contrast to the observation of decreased levels of ERα in TILs derived from EC tumors, ERα expression was shown to be increased in the tumor tissue itself. A study by Ning *et al* (12) observed that $CD68^+CD163^+$ macrophages infiltrating EC secrete a cytokine, IL17A, and thus enhance estrogen-dependent neoplastic cell proliferation by upregulating the ERα level in the tumor through ten-eleven translocation 1 mediated epigenetic gene modulation. This observation of the ERα promoting role in EC progression was supported by Hu *et al* (10), in which higher expression of ERα was noted in well differentiated and early tumors compared with poorly differentiated advanced EC tumors. The authors speculated that, during the process of gradual tumor progression, more ERα negative EC cells appear leading to a final low level of ERα, which is due to tumor heterogeneity and changes in ERα sensitivity to estrogen.

In a previous study, it was demonstrated that the estradiol-mediated ERα signaling pathway, under the transcriptional regulation of FOXP3, controls regulatory T-cell (T_{reg} cell) functions in patients with cervical cancer (25). The

Table IV. ERα expression (presented as MFI) in the three studied tumor infiltrating lymphocyte subpopulations, Th cells ($CD45^+CD3^+CD4^+$), CTLs ($CD45^+CD3^+CD8a^+$) and B cells ($CD45^+CD3^-CD19^+$), derived from the tumor according to certain endometrial cancer prognostic factors.

	ERα MFI in Th cells			ERα MFI in CTLs			ERα MFI in B cells		
Prognostic factor	Me	IQR	P-value	Me	IQR	P-value	Me	IQR	P-value
Clinical risk group			0.853			0.736			0.646
Low (n=21)	1,648	795		1,785	779		1,615	867	
Intermediate (n=9)	1,989	846		2,144	913		1,750	597	
High-intermediate (n=13)	1,788	546		1,861	546		1,736	723	
High (n=11)	1,804	721		1,890	728		1,409	1,113	
Myometrial infiltration			0.186			0.213			0.444
<50% (n=30)	1,744	795		1,901	772		1,630	777	
≥50% (n=24)	1,690	747		1,824	746		1,447	889	
Parametria involvement			0.099			0.131			0.060
Negative (n=52)	1,682	690		1,824	695		1,507	830	
Positive (n=2)	2,484	893		2,518	905		2,377	531	
LVSI status			0.440			0.284			0.440
Not identified (n=37)	1,687	700		1,913	748		1,564	780	
Present (n=17)	1,788	531		1,861	658		1,485	862	
Histological type			0.890			0.720			0.762
Type I (n=48)	1,721	688		1,871	713		1,507	822	
Type II (n=6)	1,679	719		1,761	728		1,838	1,144	
FIGO stage			0.890			0.767			0.407
I (n=40)	1,662	737		1,787	790		1,590	828	
>I (n=14)	1,838	721		1,942	657		1,443	766	
Histological grade			0.609			0.735			0.434
Low grade (n=39)	1,648	730		1,786	711		1,476	817	
High grade (n=15)	1,847	695		1,926	746		1,830	1,005	
BMI, kg/m^2			0.007			0.009			0.04
<30 (n=14)	1,979	902		2,194	1,127		1,806	1,068	
30-35 (n=24)	1,637	771		1,824	779		1,427	936	
>35 (n=16)	1,594	559		1,663	633		1,360	785	
TU, cm			0.872			0.870			0.881
<2 (n=11)	1,676	1,357		1,861	1,300		1,485	1,236	
2-4 (n=27)	1,687	685		1,787	750		1,528	894	
>4 (n=16)	1,838	601		1,885	611		1,697	764	
p53			0.912			0.847			0.409
Normal (n=48)	1,682	688		1,871	713		1,507	767	
Mutant (n=6)	1,818	719		1,891	728		2,127	1,355	
MMR			0.694			0.902			0.656
MMR proficient (n=31)	1,687	759		1,913	782		1,446	944	
MMR deficient (n=23)	1,788	696		1,861	680		1,564	750	
TCGA			0.914			0.969			0.666
NSMP (n=26)	1,682	662		1,849	720		1,428	845	
MSI (n=22)	1,727	696		1,871	680		1,546	636	
p53 (n=6)	1,818	719		1,891	728		2,127	1,355	

ERα, estrogen receptor α; MFI, median fluorescence intensity; Me, median; IQR, interquartile range; Th cells, T helper lymphocytes; CTLs, cytotoxic T lymphocytes; B cells, B lymphocytes; BMI, body mass index; FIGO, International Federation of Gynecology and Obstetrics; LVSI, lymphovascular space involvement; MMR, mismatch repair; TCGA, The Cancer Genome Atlas; NSMP, non-specific molecular profile; MSI, microsatellite instability; TU, tumor size.

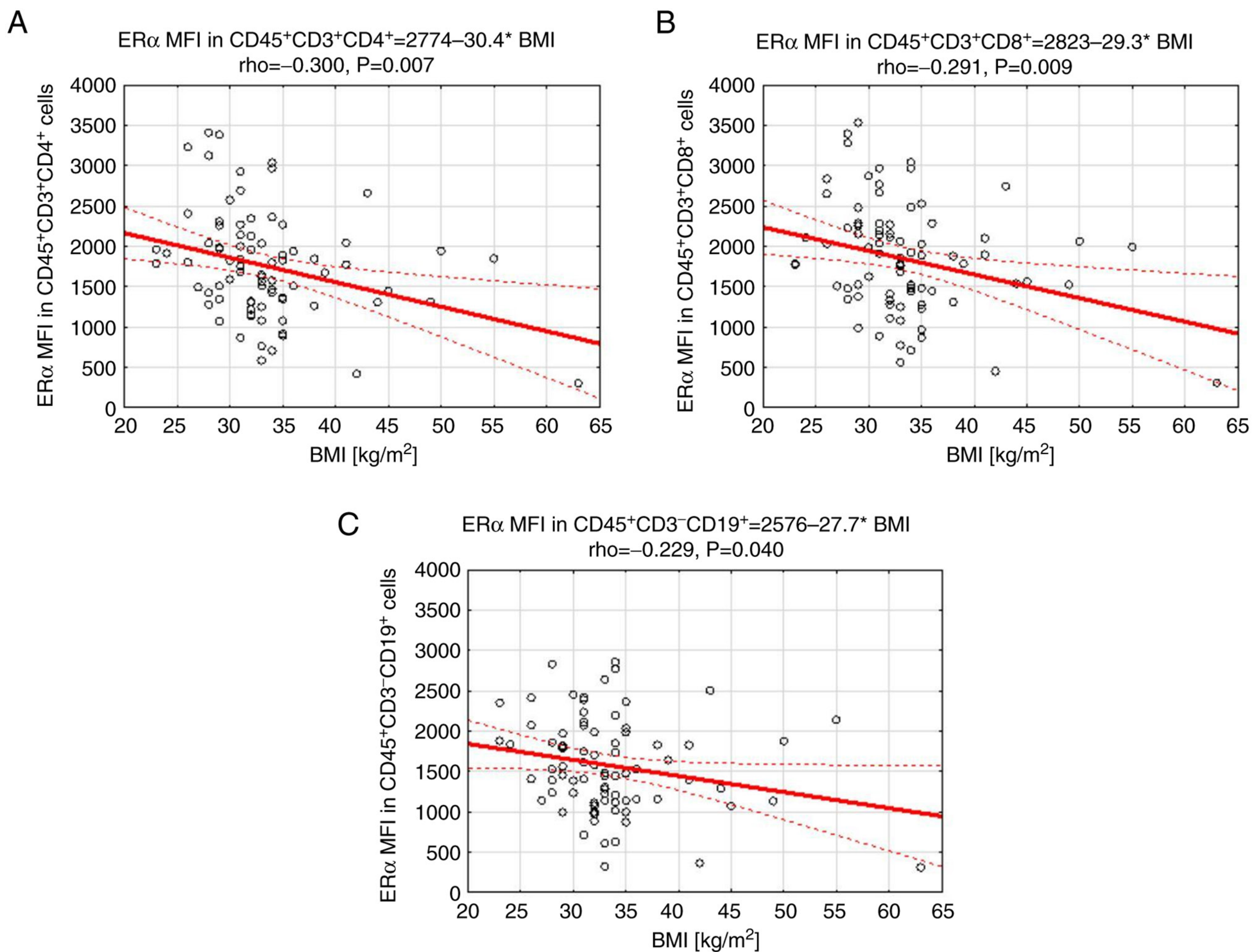

Figure 6. Spearman's ϱ coefficient correlation plot of BMI vs. ERα MFI in the three studied subpopulations of tumor infiltrating lymphocytes: (A) T helper lymphocytes ($CD45^+CD3^+CD4^+$), (B) cytotoxic T lymphocytes ($CD45^+CD3^+CD8^+$) and (C) B lymphocytes ($CD45^+CD3^-CD19^+$). BMI, body mass index; ERα, estrogen receptor α; MFI, median fluorescence intensity.

authors found high levels of both estradiol and ERα in T_{reg} cells ($CD4^+CD25^{high}CD127^{low}$ cells) isolated from cervical cancer tissues, suggesting an important role of ERα in the biology of T_{reg} cell subsets, whose suppressive activity can be enhanced by increasing FOXP3 expression. Marked FOXP3 expression was observed in T_{reg} cells derived from the peripheral blood and tumor tissue in patients with cervical cancer; however, minimal expression was noted in effector T cells and a total lack of expression was noted in naïve T cell populations from cervical cancer. To support this finding, a specific ERα antagonist, fulvestrant, was used, which significantly reduced the expression levels of both ERα and FOXP3 in cervical tumor T_{reg} cells. The T-cell response to estradiol is mainly mediated by the intracellular expression of ERα and ERβ, but their levels may differ according to cell distribution, including in $FOXP3^+$ subsets, within the tumor mass (25,38). In the present study, TIL subsets in endometrial tissue showed decreased ERα levels, which is partially consistent with the results of other immunofluorescence assays where a lack of ERα expression in a $CD45^+$ hematopoietic-lineage subset and in $CD68^+$ macrophages derived from cervical tumors was observed (39). ERα expression in the tumor cell environment is mainly restricted to cells negative for FSP-1 and CD34 (fibrocytes) and positive for α-smooth muscle actin, demonstrating that ERα may act primarily through paracrine stromal-to-tumor signaling (39).

ERα signaling may remodel the TME by exerting a direct effect on proliferation and suppressing the activity of both $CD4^+$ and $CD8^+$ T cells (39). The inhibition of ERα signaling could enhance the response to immune check-point blockade (ICB) therapy, which has been observed in a murine melanoma model (40). In this model, a tumor cell extrinsic activity of ERα increased the accumulation of macrophages and activated tumor-associated macrophages in the TME, suppressing adaptive immunity and promoting tumor growth. This activity was not observed in macrophages lacking ERα expression. Inhibition of ERα expression with fulvestrant decreased myeloid cell growth and increased the antitumor efficacy of ICB.

In the present study, a significant decrease in ERα expression (presented as MFI) in all studied TIL populations derived from endometrial tumors compared with normal endometrial tissue was observed. However, no significant correlations were observed between ERα expression and relevant clinicopathological features, with the exception of $ERα^+$ B cells, the frequency of which was significantly higher in MMR-d tumors. The functions of B cells in the TME of patients with EC remain essentially unknown as there have been few

relevant studies; however, Guo *et al* (41) observed a similar trend of increased B cell levels in MMR-d tumors and noted that an improved prognosis was associated with higher levels of $CD20^+$ B cells. Nevertheless, the increased frequency of ERα-positive B cells in the TME of patients with EC classified as MSI cluster demonstrated in the present study, as well as in high-grade endometrial tumors, requires further investigation.

Obesity is well established as the leading modifiable risk factor for the development of EC (42). After menopause (90% of the population in the present study), ovarian estradiol synthesis stops and adrenal androstenedione is converted, mostly in the fat tissue, by aromatase into estrone (43). Consequently, women with obesity present with 2 to 4-fold higher estrone levels, which correlate with a greater risk of ER-positive postmenopausal breast and endometrial cancer (44,45). Previous studies have shown that an increasing BMI (>30) is inversely correlated with $CD8^+$ tumor infiltration (but not with other immune cell subsets), and therefore obesity may reduce cytotoxic T cell immune surveillance of the endometrium; however, this mechanism can be reversed with weight loss (46,47). This observation could not be confirmed in the present study as the $CD8^+$ subset was not decreased; however, the density of Th cells ($CD4^+$) was significantly decreased in obese patients (BMI >35), suggesting a possible role for these cells in cancer growth in this specific population prone to developing EC. Notably, in the present study, a decrease in ERα expression in TILs was observed, which was correlated with a higher BMI. The significance of this phenomenon remains unknown, but the results suggest that maintaining a lower BMI may reduce estrogen-induced stimulation and modulate ERα expression in the TILs of the TME, potentially limiting the risk of ERα-positive EC in postmenopausal women.

The limitations of the present study include only focusing on ERα expression, resulting in an incomplete picture of the role of the ER family in TILs from endometrial tumors. ERβ may also be expressed in TILs and thus requires further investigation. The present study lacked functional validation experiments to verify the effect of ERα downregulation on the antitumor functions of TILs; however, the present study was designed as a pilot study and its small sample size (only 54 cases included) prevented further validation.

In conclusion, the results of the present study emphasized that TILs derived from EC tissue exhibit decreased ERα expression compared with healthy endometrial tissue. We hypothesize that estrogenic stimulation may regulate the TME in patients with EC, primarily through the ERα signaling pathway. This pathway influences the immune system (namely TILs), which in turn may modulate ERα expression through unknown paracrine effects. Future validation studies on TILs showing decreased ERα expression in terms of functions such as cytotoxicity and cytokine secretion, may help to understand this phenomenon. Subsequent investigation of the molecular mechanisms underlying ERα downregulation in TILs, such as ERα gene methylation and transcription factors, may prove crucial in translating this finding into clinical practice in the diagnosis and treatment of endometrial tumors.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MJ, AS, PK, DL, AK, AC, RM and ACS confirm the authenticity of all the raw data. MJ, AS, PK, DL, AK, AC, RM and ACS contributed to the design of the study. MJ, AS, PK, DL, AK and AC made substantial contributions to the acquisition of data. MJ, AS, PK, DL, AK, AC, RM and ACS made substantial contributions to the analysis and interpretation of data. MJ, AS and AK contributed to data curation by carefully and thoroughly collecting and reviewing key data. All authors read and approved to the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Wroclaw Medical University Bioethics Committee (Wroclaw, Poland; registration no. 166/2019, dated March 5, 2019), which reviews studies at cooperating hospitals in the region. Written informed consent for the participation in this study was obtained from each qualified patient prior to surgery, in accordance with the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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