Decreased expression of estrogen receptors alpha and beta in peripheral blood lymphocytes from the endometrial cancer patients and women with endometriosis

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Summary
Aim: Endometriosis and endometrial cancer are described as typical estrogen-dependent gynaecological diseases. The question arises if estrogens, working through their receptors, could influence the immunological activity of lymphocytes in these disorders. Here, we evaluated transcriptional expression of the estrogen receptors alpha and beta (ERα and ERβ) in T lymphocytes isolated from the peripheral blood in endometrial cancer and endometriosis patients.

Methods: Peripheral blood was collected from ten patients with endometrial cancer, nine with endometriosis, and ten disease-free controls. After isolation of the T lymphocytes, purity was confirmed by flow cytometry and the relative level of ERα and ERβ mRNA was determined using RT-qPCR analysis.

Results: Both ERα and ERβ were significantly decreased in T lymphocytes isolated from patients with either endometrial cancer or endometriosis when compared to the healthy controls. We measured no difference in the mRNA levels of ERα between endometrial cancer patients and endometriosis group, but ERβ expression in endometrial cancer women was twice as high than in the endometriosis group.

Conclusion: Decreased transcription of nuclear estrogen receptor isoforms characterizes T lymphocytes from women with endometrial cancer and endometriosis.

Key words: Endometriosis; Pelvic Pain; Endometrium Cancer; Steroid Hormones; Hormone Receptors; Molecular Medicine; Growth Factors.

Introduction
Estrogens influence the action of immune system cells principally via their intracellular estrogen receptors alpha (ERα) and beta (ERβ), and estrogen receptor expression in different types of lymphocytes is well established [1-4]. Moreover, the effects of estrogens and their receptor subtypes on T lymphocyte activity, under both physiological and pathological conditions, have been extensively studied. For example, estrogen dependent expansion of T regulatory lymphocytes (CD4+CD25+FOXP3+) is a crucial mechanism controlling pregnancy maintenance in mice [5]. Alternatively, in patients with systemic lupus erythematosus (SLE), both ER subtypes activated the disease markers calcineurin and CD154 in T cells when treated with specific ER agonists [6], and females with SLE exhibited low ERβ expression and high serum levels of anti-estrogen ERα antibodies that increased SLE activity [7]. Nevertheless, the expression of ERα and ERβ in lymphocytes of some gynaecological diseases traditionally considered estrogen-dependent, such as endometrioid type endometrial carcinoma and endometriosis, remains unknown.

Endometrial cancer is the most common malignancy of the female genital tract arising principally in postmenopausal women from wealthy societies. This malignancy is recognized as estrogen-dependent in almost 90% of cases [8] and expression of estrogen and progesterone receptors in malignant tissue is considered an important predictive factor in this disease [8, 9]. A recent study by Sliwinska et al. showed that polymorphisms within the ERα gene, as well as the CYP1B1 gene, were associated with the development of endometrial cancer [10]. On the other hand, infiltrating macrophages can induce ERα expression in endometrial cancer cells through an IL-17A-mediated epigenetic mechanism resulting in the sensitization of cancer cells to estrogen [11].

Endometriosis, a benign disease affecting at least 10% of reproductive age females, is also an estrogen-dependent disorder as the majority of endometriosis patients react well to estrogen-suppressive therapy [12, 13]. Estrogens may influence the immune system and this effect may be crucial in the development of endometriosis. Estrogens cause migration of Natural Killer (NK) cells to the uterus as well as promoting differentiation and propagation of T regulatory lymphocytes due to ERα and ERβ [5]. ERα and ERβ mRNA and protein were also detected in peripheral blood mononuclear cells in healthy humans, although their func-
containing 10% DMSO and frozen at -70°C. The lymphocytes were then put into medium and analyzed by flow cytometry. In each sample, we counted lymphocytes with CD3 surface antigen as well as a marker for T lymphocytes (CD4 and CD8). Data obtained were recorded in a disease-free control group.

**Material and Methods**

Our study population consisted of three groups: ten postmenopausal patients diagnosed with endometrial carcinoma (low risk patients: endometrioid tumor type, stage I and grade 1 or 2); nine premenopausal women with advanced pelvic endometriosis; ten women of reproductive age with simple functional ovarian cysts served as the control group. After receiving study approval from the Wroclaw Medical University Ethics Committee, an informed consent form was obtained from each patient. Endometrial cancer patients, patients with endometriosis, and control patients were surgically treated in the Department of Oncological Gynaecology, Lower Silesian Cancer Center, Wroclaw, Poland. During the operation, a sample of 8-10 mL of peripheral blood was collected from each patient and immediately sent to the Laboratory of Reproductive Immunology at the Ludwik Hirszfeld Institute of Immunology and Experimental Medicine, Polish Academy of Sciences in Wroclaw for testing. The diagnosis of endometrial cancer or endometriosis was confirmed by a pathologist experienced in gynaecology and gynaecological oncology.

Heparinized blood samples were overlaid with Histopaque-1077 using a 3 to 1 ratio, respectively, and then centrifuged for 30 minutes at 400 xg at room temperature. Following this, peripheral blood mononuclear cells (PBMC) were removed, rinsed twice with phosphate-buffered saline (PBS), stained with anti-CD3 antibody and analyzed by flow cytometry. In each sample, we counted lymphocytes with CD3 surface antigen as well as a marker for T lymphocytes (CD4 and CD8). Data obtained showed that 70-80% of the PBMC fraction consisted of T lymphocytes. The lymphocytes were then put into medium containing 10% DMSO and frozen at -70°C.

The isolation of total RNA from isolated lymphocytes was performed on two independent samples from each patient to assure reproducibility. A protocol using TRIzol/chloroform, isopropanol, and ethanol was used as outlined in the manufacturer’s instructions. RNA quality was assessed by agarose gel electrophoresis and RNA concentrations measured with a NanoDrop 1000 instrument. 2 μg of RNA was reverse transcribed in a 20 μl reaction volume using a High-Capacity cDNA Reverse Transcription Kit. cDNA synthesis reactions also contained RNase Inhibitor and random hexamers as primers and thermal cycling conditions used were: 25°C 10 min, 37°C 120 min, 85°C 5 min. Quantitative PCR (qPCR) was performed using a StepOnePlus Real Time PCR System. Reaction mixtures (20 μl total volume) contained 10 μl Real-Time 2x HS-PCR Master Mix containing SYBR green (manufacturer), 4 μl of 10X diluted cDNA, 2 pmol (0.2 μl) of each primer, and 5.6 μl of nuclease free H$_2$O.

qPCR analyses were designed to compare relative quantities of alpha- and beta-estradiol receptor (ERα and ERβ) mRNA in T lymphocytes using the ∆∆Ct model with the GAPDH transcript as the internal reference. Primers chosen for analysis were: GAPDH Forward 5’-CATGAGAAGTATGACACAGCCT-3’ and GAPDH Reverse 5’-AGTTCTTCCACGATACCAAGGT (PrimerBank ID 7669492a3); ERα Forward 5’-AATGTTGCTGGCTAGATGCCT-3’ and ERα Reverse 5’-CTGGTCCAAGAGCAATGGAG-3’; ERβ Forward 5’-GATCGCTAGACACACCTTACCTGT-3’ and ERα Reverse 5’-GGGCAACCGGTACCCTA-3’. qPCR reactions were denatured at 95°C for 10 min, followed by amplification for 40 thermocycles (95°C 30 sec denaturation, 54°C or 59°C 30 sec annealing of primers for: GAPDH an ERα and ERβ, respectively, and extension at 72°C 30 sec). Melting curve analysis was conducted in the temperature range between 60°C and 95°C and every sample was assayed in triplicate.

The distribution of the results gathered in these qPCR experiments was estimated using the Shapiro and Wilk test but did not fulfill the criteria for normality. qPCR results are presented as mean ± standard deviation, as well as median & interquartile range. To study the ERα and ERβ expression in T lymphocytes we used the Wilcoxon-Mann-Whitney test, suitable for unpaired results from two independent samples. A two-tailed $p \leq 0.05$ was considered statistically significant when comparing different sample medians.

<table>
<thead>
<tr>
<th></th>
<th>ER alpha</th>
<th>ER beta</th>
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<tbody>
<tr>
<td>Healthy</td>
<td>4.67</td>
<td>1.8</td>
</tr>
<tr>
<td>Endometriosis</td>
<td>1.96</td>
<td>16.78</td>
</tr>
<tr>
<td>Cancer</td>
<td>1.84</td>
<td>4.12</td>
</tr>
<tr>
<td>Healthy</td>
<td>4.16</td>
<td>1.27</td>
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<tr>
<td>Endometriosis</td>
<td>1.95</td>
<td>10.67</td>
</tr>
<tr>
<td>Cancer</td>
<td>1.3</td>
<td>2.93</td>
</tr>
</tbody>
</table>

*Table 1. — mRNA expression of ERα and ERβ from the isolated lymphocytes presented as mean ± standard deviation and median & interquartile range in three studied groups: healthy, endometriosis and endometrial cancer.*

<table>
<thead>
<tr>
<th></th>
<th>Healthy</th>
<th>Endometriosis</th>
<th>Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>4.67</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td>± 2.70</td>
<td>± 1.41</td>
<td>± 0.53</td>
</tr>
<tr>
<td>Median</td>
<td>4.16</td>
<td>1.27</td>
<td>1.95</td>
</tr>
<tr>
<td>Interquartile range</td>
<td>2.83</td>
<td>0.8</td>
<td>0.17</td>
</tr>
</tbody>
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Results

The relative abundance of ERα and ERβ in T lymphocytes was compared to the abundance of the reference transcript GAPDH in groups of ten women with endometrial cancer, nine with endometriosis, and ten healthy controls. We found a significant decrease in the mRNA levels of both ERα and ERβ in peripheral blood T lymphocytes from patients with endometrial carcinoma and endometriosis when compared to healthy control women. When we compared relative ERα expression in lymphocytes derived from women with endometrial malignancy to ERβ expression measured in endometriosis patients, we did not observe any significant difference in ERα mRNA levels between those two groups. Interestingly, the ERβ/ERα expression ratio in peripheral blood lymphocytes was twice as high in endometrial cancer patients compared to women with endometriosis (Table 1 and Figure 1).

Discussion

In the estrogen-dependent disorders endometriosis and endometrial cancer the increase of local estrogenic milieu leads to disease development. Estradiol levels in endometrial cancer tissues correlated with both tumor grade and tumor invasion in pre- and postmenopausal women [15]. In endometriosis, cell survival in ectopic localizations and during inflammation, both of which are enhanced by locally high levels of estrogens, are responsible for chronic pelvic pain and infertility, the primary symptoms of this disease [16].

Estrogen action is classically mediated by its receptors alpha (ERα) and beta (ERβ) that function as ligand-activated transcription factors [17]. Studies with targeted deletion of the ERα gene showed that this ER subtype is essential in uterine cell proliferation and expression of the progesterone gene [18]. ERβ may act as an inhibitory modulator of ERα-stimulated gene transcription [19]. When ERβ mRNA decreases in the postmenopausal endometrium, it renders this tissue more sensitive to proliferation effect of estrogen due to unopposed ERα action [17, 20]. Thus, an imbalance of ER isoform expression could lead to endometrial hyperplasia and, consequently, to endometrial malignancy especially when correlated with inovulatory cycles following progesterone decrease during the menopausal transition [21].

In cases of endometriosis, estrogen receptor expression is dependent on localization. ERβ levels in endometriotic tissue are 142-fold higher than in endometrial stroma, whereas ERα levels are nine-fold lower in endometriotic stromal cells compared to endometrium stromal cells [22]. ERβ expression in endometriotic stromal cells downregulates the activity of the ERα promoter thus suppressing expression of ERα [22]. In turn, the increase of ERβ binding to the progesterone-receptor promoter in endometriotic stromal cells leads to a decrease in progesterone receptor [16]. As a consequence, endometriotic foci can develop in a high-estrogenic milieu unopposed by progesterone.

In our study, we examined the levels of ERα and ERβ in patients suffering from endometriosis and endometrial cancer. However, unlike previous studies, ER expression was tested not in uterine tissue (endometriotic or malignant) but in lymphocytes derived from peripheral blood (PBL). When we consider endometriosis and endometrial cancer as dist-
eases of the whole individual, not only its local organs, the question arises concerning alterations in distant tissues such as peripheral blood cells.

The present study determined that a significant decrease in the transcript levels of both ERα and ERβ occur in PBL in both endometriosis and endometrial cancer when compared to the healthy control donors. However, Jones et al. did not observe ER expression in leukocytes from eutopic and ectopic endometrial tissue in their immunohistochemical studies [23].

This discrepancy may be due to the fact that we studied ER expression at the mRNA level in peripheral blood lymphocytes. Nevertheless, another study confirmed ERs mRNA expression in peripheral blood T cells (CD4+) of healthy women, while ERβ mRNA was predominantly observed in B lymphocytes [2]. It is interesting that we also observed a strong imbalance between the levels of ERα and ERβ in the endometrial cancer patients and controls, but not in women with endometriosis. However, ERβ in endometrial cells may play a protective role against development of endometriosis as ectopic endometrial foci regress due to ERβ agonist administration. While this has been shown in an experimental animal model, the role of ERβ in lymphocytes in endometriosis requires further elucidation [24].

In conclusion, in this study we report that the expression of both ERα and ERβ in peripheral blood lymphocytes is significantly decreased in the estrogen-dependent diseases as endometriosis and endometrial cancer. Whether this phenomenon plays a role in the development of these diseases remains unclear, but may be worthy of further research.

Author contributions

Study idea and protocol (MJ, AC, ACS, RM), samples and data collecting (MJ), lab work (AC, DF), manuscript writing (MJ, AC), manuscript discussion and corrections (MJ, AC, ACS, RM), study funding obtaining (MJ, RM).

Ethics approval and consent to participate

This study was approved by Wroclaw Medical University Bioethics Committee (KB nr 247/2008). An informed consent form was obtained from each patient included to the study.

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Conflicts of interest

The authors of this manuscript declare having no competing financial interests in relation to the presented work.

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