CORRELATIONS BETWEEN THE LYMPHOID INFILTRATE AND THE STEROID HORMONE RECEPTORS IN ENDOMETRIOSIS

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CORRELATIONS BETWEEN THE LYMPHOID INFILTRATE AND THE STEROID HORMONE RECEPTORS IN ENDOMETRIOSIS (Abstract): Aim: Endometriosis, a chronic estrogen-dependent disease, is associated with persistent pelvic pain and is classified as a neuro-inflammatory disorder. This study aimed to decipher the inflammatory milieu in endometriosis, focusing on the lymphoid infiltrate represented by the CD4+ cells, CD8+ cells, and CD68+ macrophages, and the possible correlation with steroid hormone receptor expressions.

Material and methods: Research conducted at the “Elena Doamna” Clinical Hospital and the “Grigore T. Popa” University of Medicine and Pharmacy in Iaşi, Romania, involved retrospective analysis of 53 patients with endometriosis from 2018 to 2023. Results: Through immunohistochemical assays, we observed correlations between CD4 and CD8 expressions, and between CD4, CD68, and the PR score. Also, a possible causality between the macrophage infiltrate and PR expression could be suggested. Conclusions: The inflammatory infiltrate’s analysis in endometriosis provides a deeper knowledge of its pathological development and the interplay of inflammatory cells. Understanding these receptors might guide future treatment strategies for better disease management. Keywords: ENDOMETRIOSIS, INFLAMMATION, LYMPHOID INFILTRATE, STEROID HORMONE RECEPTORS.

Endometriosis is a chronic, estrogen-dependent disease characterized by the ectopic growth of endometrial tissue (encompassing both glands and stroma) outside the uterine cavity. This pathology is frequently correlated with persistent pelvic pain and is contemporarily classified as a neuro-inflammatory disorder (1).

The etiopathogenesis of endometriosis has been extensively studied, yet definitive understanding remains evasive. We currently have data about the roles of genetic predisposition, estrogenic modulation, progesterone resistance, and inflammatory pathways, in conjunction with altered expression of hormonal receptors such as estrogen receptors (ER) and progesterone receptors (PR) (1, 2). Immunological components are hypothesized to be instrumental in both the inception and evolution of
endometriosis (3-5).

Within the inflammatory cellular milieu associated with endometriosis, CD4+ T cells and CD8+ T cells are discernible, albeit their precise pathological roles remain only partially delineated (6). Concurrently, macrophages are posited to be pivotal in the proliferation, angiogenesis, innervation, and nociception of endometriotic lesions (7-10).

The heterogeneity of the intralesional inflammatory infiltrate across various histological manifestations of endometriosis holds potential for profound insights into the sequential pathophysiological events and the multifaceted immune responses in endometriosis (11). Our study seeks to elucidate the composition of the inflammatory milieu through a semi-quantitative analysis of CD4+ cells, CD8+ cells, and CD68+ macrophages in ovarian and cutaneous endometriosis. Complementarily, we assessed the immunohistochemical expressions of estrogen and progesterone receptors, trying to find correlations and eventual causalities.

MATERIAL AND METHODS

The research was undertaken at both the “Elena Doamna” Clinical Hospital and the “Gr. T. Popa” University of Medicine and Pharmacy located in Iași, Romania. This study encompassed a retrospective review of clinical records combined with histopathological examinations of surgical samples (ranging from nodule removals to full hysterectomies with adnexectomy) from 53 patients diagnosed with endometriosis between the years 2018 and 2023.

Slides stained with Hematoxylin-Eosin (HE) were viewed by a pair of independent pathologists to verify the diagnosis. An immunohistochemical (IHC) test followed, utilizing specific antibodies (tab. I). Upon completion of the IHC procedure, a semi-quantitative evaluation of the immunoreactivity of the inflammatory infiltrate in the epithelium and stroma has been carried out. Also, the ERα and PR quantification used a score based on the positivity index added to the staining intensity. The analysis has been performed using their respective scores available in literature (12-16).

### TABLE I.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Clone</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>Mouse Monoclonal</td>
<td>4B12/Novocastra</td>
<td>1/100</td>
</tr>
<tr>
<td>CD8</td>
<td>Mouse Monoclonal</td>
<td>1A5/Novocastra</td>
<td>1/50</td>
</tr>
<tr>
<td>CD68</td>
<td>Mouse Monoclonal</td>
<td>KP1/Novocastra</td>
<td>1/400</td>
</tr>
<tr>
<td>ERα</td>
<td>Mouse Monoclonal</td>
<td>6711/Novocastra</td>
<td>RTU</td>
</tr>
<tr>
<td>PR</td>
<td>Mouse Monoclonal</td>
<td>312/Novocastra</td>
<td>RTU</td>
</tr>
</tbody>
</table>

In our statistical analysis, we employed the Hoeffding measure, a non-parametric statistical method tailored for assessing the correlation between two random variables. The Hoeffding correlation measure is adept at detecting both linear and non-linear associations, useful in situations where relationships between variables might not always adhere to a linear paradigm. The Hoeffding’s D measure offers a robust quantification of association without being confined to a specific form or pattern of
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relationship between the variables under investigation.

We also utilized the Pearson correlation coefficient, a widely recognized parametric statistical method that quantifies the linear relationship between two continuous variables. The Pearson test measures the strength and direction of the linear relationship, providing a coefficient value between -1 and 1. A value closer to 1 implies a strong positive linear relationship, a value closer to -1 indicates a strong negative linear relationship, and a value near 0 suggests no linear correlation. Given its sensitivity to linear associations, this test is pivotal when aiming to ascertain direct proportionality or inversely proportional relationships between variables.

Informed consent was secured from all the patients, adhering to guidelines that guarantee participants make well-informed decisions and that their rights are safeguarded (17). The study received approval from the Research Ethics Committee of the “Grigore T. Popa” University of Medicine and Pharmacy Iasi (Approval No. 285/26.03.2023), in alignment with the Declaration of Helsinki’s principles concerning medical research involving human participants.

RESULTS

The results were expressed as CD4 percent, CD8 percent, CD68 percent, ERα score and PR score. In order to investigate an eventual reciprocal influence of inflammation and hormonal imbalance in the endometriotic lesions, we performed a Hoeffding and also a Pearson test (figs. 1, 2). Both methods were used in order to compare their results, but also to capture both linear and potentially non-linear relationships.

<table>
<thead>
<tr>
<th></th>
<th>CD4 percent</th>
<th>CD8 percent</th>
<th>CD68 percent</th>
<th>ERα score</th>
<th>PR score</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 percent</td>
<td>1.000000</td>
<td>0.058491</td>
<td>0.014912</td>
<td>0.007265</td>
<td>-0.006719</td>
</tr>
<tr>
<td>CD8 percent</td>
<td>0.058491</td>
<td>1.000000</td>
<td>-0.009299</td>
<td>-0.007093</td>
<td>0.005025</td>
</tr>
<tr>
<td>CD68 percent</td>
<td>0.014912</td>
<td>-0.009299</td>
<td>1.000000</td>
<td>-0.003800</td>
<td>0.038728</td>
</tr>
<tr>
<td>ERα score</td>
<td>0.007265</td>
<td>-0.007093</td>
<td>-0.003800</td>
<td>1.000000</td>
<td>0.001113</td>
</tr>
<tr>
<td>PR score</td>
<td>-0.006719</td>
<td>0.005025</td>
<td>0.038728</td>
<td>0.001113</td>
<td>1.000000</td>
</tr>
</tbody>
</table>

Fig. 1. *Hoeffding correlation* coefficient regarding the association between lymphoid infiltrate and steroid hormones expressions

<table>
<thead>
<tr>
<th></th>
<th>CD4 percent</th>
<th>CD8 percent</th>
<th>CD68 percent</th>
<th>ERα score</th>
<th>PR score</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 percent</td>
<td>1.000000</td>
<td>0.533522</td>
<td>0.338297</td>
<td>0.108531</td>
<td>0.158922</td>
</tr>
<tr>
<td>CD8 percent</td>
<td>0.533522</td>
<td>1.000000</td>
<td>0.247454</td>
<td>0.143872</td>
<td>-0.190404</td>
</tr>
<tr>
<td>CD68 percent</td>
<td>0.338297</td>
<td>0.247454</td>
<td>1.000000</td>
<td>0.106933</td>
<td>0.300278</td>
</tr>
<tr>
<td>ERα score</td>
<td>0.108531</td>
<td>0.143872</td>
<td>0.106933</td>
<td>1.000000</td>
<td>0.220835</td>
</tr>
<tr>
<td>PR score</td>
<td>0.158922</td>
<td>-0.190404</td>
<td>0.300278</td>
<td>0.220835</td>
<td>1.000000</td>
</tr>
</tbody>
</table>

Fig. 2. *Pearson correlation* coefficient regarding the correlations between lymphoid infiltrate and steroid hormones expression
The non-linear correlation coefficient suggested a potential relationship between the expressions of CD4 and CD8, as well as the expressions of CD4 and CD68 and between CD68 and PR score. No correlation was found between CD68 and ERα expression, or between CD4, CD8, and hormonal receptors in the analyzed samples.

While correlation between random variables does not imply nor reveal causal directionality, this can be assessed with the help of an Additive Noise Model (ANM) (18, 19). We made use of the popular WhyPy Python packages to estimate through both Maximum Likelihood and Independence of Residuals the potential causal directionality for pairs of correlated variables in our data.

The ANM study results of CD4 (X0) and CD8 (X1) data are shown in figures 3 and 4. These results indicated that the box plots describing noise distribution in the modelling of dependencies X0~f(X1) and X1~f(X0), respectively, mostly overlap in both Maximum Likelihood (fig. 3) and Independence of Residual (fig. 4). These results did not support any clear causal directionality hypothesis.

Similarly, the ANM study of CD4 (X0) and CD68 (X1) revealed overlapping noise distributions as shown in figures 5 and 6, which is not indicative of any potential causal directionality.

Finally, the results of the ANM study of CD68(X0) and PR Score (X1) are shown in figs. 7 and 8. The Maximum Likelihood test shown in figure 7 supported a hypothesis for causal direction in which CD68 causes PR Score, while the Independence of Residuals test (fig. 8) does not result in clear support for either direction. Should a causal link exist between CD68 and PR Score, indicates that it is more likely that the first causes the second (fig. 7).
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**Fig. 6.** ANM Independence of Residuals for CD4 (X0) and CD68 (X1)

**Fig. 7.** ANM Maximum Likelihood for CD68 (X0) and PR (X1)

**Fig. 8.** ANM Independence of Residuals for CD68 (X0) and PR (X1)

**DISCUSSION**

A key role in the pathogenesis of endometriosis is attributed to the menstrual reflux of endometrial tissue (including stem cells) into the peritoneal cavity. Nevertheless, this phenomenon is not sufficient for the development of the disease, and the microenvironment factors that stimulate the functions of stem cells and allow the development of endometriotic implants are very important adjuncts to the mechanism of retrograde menstruation (20). Also, considering the widespread distribution of endometriosis localization in the human body, modern theories attempt to combine the effect of several factors that contribute to its development, as multifactorial, multicompartmental pathogenic phenomena such as estrogen dependence, progesterone resistance, genetic susceptibility, all under the influence of environmental factors (11). Furthermore, the pathogenic mechanisms are added to the immune system’s inability to neutralize ectopic endometrial cells. Additionally, the interplay between hormonal levels, inflammation, and the progression of the disease has been extensively researched.

Since 1987, Halme et al. proposed an immunological aspect to endometriosis (21). They observed elevated levels of activated macrophages, belonging to the cellular component of the innate immune system, in both the peritoneal fluid and the intact endometrial tissues (referred to as eutopic endometrium) of endometriosis patients compared to women without the condition (21). Given that macrophages, which have widespread tissue presence, play a pivotal role in many inflammatory and cancer-related diseases (22), they are believed to be crucial in influencing various aspects of endometriosis. This includes the growth, development, vascularization, and nerve supply of endometriotic lesions, and even in the creation and sensation of pain (7-9).

Studies on the inflammatory cell types found in the female reproductive tract have produced varied and sometimes incon-
sistent findings (3, 5, 23). These discrepancies might be due to the changing nature of the menstrual cycle, as well as the ongoing morphological shifts in both the epithelial layer and stromal regions during different menstrual phases. However, the most commonly identified immune cells in this region include T cells, macrophages, dendritic cells, NK cells, neutrophils, and mast cells (24). Accordingly, it is considered that immune cells, such as NK cells, macrophages, dendritic cells, and neutrophils, may be involved in growth, angiogenesis, and invasion processes of endometriotic cells (24). Interestingly, B cells appear to be relatively rare in the female reproductive tract (25).

A key aspect of endometriosis pathogenesis is now considered to be linked to pelvic inflammation, but the exact pathways are yet to be determined. Several research studies have shown that cells like macrophages, lymphocytes, endometrial, and mesothelial cells can produce cytokines and inflammatory agents that further attract a variety of cells, including macrophages, lymphocytes, eosinophils, mast cells, and endometrial cells to the peritoneal cavity (25). This recruitment initiates a series of reactions that stimulate the proliferation of endometriotic cells. It appears to also promote their attachment to abnormal sites, blood vessel formation, and triggers the release of additional cytokines and chemokines, intensifying their overall impact.

Moreover, growing evidence admits that endometriosis is linked with an autoimmune status (24, 26), explained by the endometriosis-induced inflammation, leading to a deregulated immune response which may induce autoimmunity (26).

The endometriosis environment is also influenced by cytokines and defensins secreted by immune cells. In this regard, the levels of immune checkpoint inhibitors, which control the immune response, are different in endometriosis than those observed in healthy women (24).

Lymphocytes play essential roles in the process of survival, implantation, and proliferation of endometrial and endometriotic cells and numerous studies have shown aberrant functions of these cells in women with endometriosis. In this regard, recent research indicates a particular association of CD4+ T cells and CD8+ T cells with endometriosis (6). There is ongoing debate on whether changes in these cells are cause or a result of endometriosis.

CD4+ T lymphocytes are often referred as T helper cells because they aid to orchestrate and amplify the immune responses by interacting with other immune cells and releasing cytokines. CD4+ T cells can differentiate into various subsets (including Th1, Th2, Th17, and T regulatory (Treg) cells), depending on the cytokine milieu, each subset playing a unique role in immune responses.

CD8+ T lymphocytes (cytotoxic T cells) have the ability to recognize antigens presented by MHC class I molecules, which are present on almost all nucleated cells. CD8+ T lymphocytes provide immunosurveillance by constantly screening host cells for intracellular pathogens or malignancies.

Our study indicates that there is a weak correlation between the number of CD4+ T cells and CD8+ T cells in the endometriotic tissue. In contrast, other recent studies have found that the CD4/CD8 ratio in the peritoneal fluid leans towards CD8+ T cells, the preponderance of CD8+ T cells being comparable to the eutopic endometrium (28). Also, CD8+ T cells in the peritoneal fluid showed higher relative frequencies when contrasted with the peripheral blood (28).
It is considered that the endometriotic altered immune response may be correlated to damaged apoptotic processes. One study revealed in healthy women increased serum concentrations of HLA-DR- activated T cells and of CD8+ T lymphocytes throughout the luteal phase in comparison with the follicular phase (29). Contrary, in endometriosis there are no fluctuations in the peripheral blood concentrations of activated and cytotoxic lymphocytes during the menstrual cycle (26, 29).

Despite the identified correlation between the two cell populations, it is difficult to propose a hypothesis for causal direction in the endometriosis scenario. Our data suggests that the relationship between CD4+ and CD8+ T cells is multifaceted. Rather than a simple cause-and-effect manner of function, they are part of a complex immune network. Their collaboration is vital for a coordinated immune response.

Thus, T cells do not operate in isolation from the environment, their changes being able to play a role in the etiopathogenesis of endometriosis. Moreover, the immune status in endometriosis is largely regulated by epigenetic factors (26, 30).

Regarding the macrophage population, outlined in our study by the CD68 expressing cells, it is well-known that in women with endometriosis they consistently represent the majority of peritoneal immune cells throughout the menstrual cycle, accounting for 55%, with T cells following at 20% (28). The elevated counts of activated macrophages and adaptive immune cells, including T and B lymphocytes, coupled with the rise in inflammatory cytokines observed in patients with endometriosis, suggest the presence of an underlying immunological/inflammatory imbalance (31). Our results suggest that there is also weak correlation between the number of CD4+ T cells and CD68 cells in the endometriotic tissue, confirming the complex and intricate roles of the two types of cell populations and giving rise to hypothesis about their reciprocal influence. On the other hand, the ANM study of CD4 and CD68 revealed overlapping noise distributions, making it impossible to indicate any potential causal directionality at the moment.

Regarding the steroidal hormones in relation to the inflammatory microenvironment, reciprocal influences could be considered to form vicious cycle, as the presence of endometriotic implants boost the expression of ER-β while decreasing PR (32-35), triggering the production of genes responsible for various inflammatory mediators like IL-1 β, IL-6, IL-8, IL-17, TNF-α, and COX-2 (10, 11). On the other hand, the chronic inflammation accelerates the aromatase activity, causing an upsurge in estradiol levels (10).

However, hormonal imbalances play a crucial role in the endometriosis etiopathogenesis, the disease being considered as a progesterone (P4)-resistant and estrogen dependent process (26, 33).

A recent study by Hogg and associates emphasizes the relationship and the reciprocal influences between the macrophages and the steroidal hormones, describing that in the presence of estrogen, macrophages enhance their proliferative ability and become activated, transitioning to a phenotype more akin to a “wound healing” population (27, 28). Also, while endometrial macrophages don’t display the progesterone receptor (36), their gene expression markedly changes in reaction to progesterone receptor (37), indicating an indirect mode of regulation.

In the present study, no correlation was found between CD68 and ERα expression, possibly to the limited sample size or possibly due to the polymorphism of the estr
gen receptors. Nevertheless, our analysis of the CD68 and PR score quantifications suggest a correlation between the two. Moreover, the Maximum Likelihood test shown supported a hypothesis for causal direction in which the presence of macrophages (reflected by the CD68 percent) causes a rise in the PR score.

On the other hand, current research indicates that endometriosis commonly exhibits progesterone resistance due to diminished PR expression and functionality and there is notable dysregulation of PR expression and activity in target cells, both in eutopic and ectopic tissues, when compared to standard endometrium (38). These differences might arise from the polymorphism of PR, as PGR-B is generally considered to be the positive regulator of the effects of progesterone, and PGR-A has an inhibitory effect on PGR-B (38).

Also, some differences of opinion could be generated by the main limitation of our study, its reliance on observational analysis with a limited sample size (53 cases only). In this context, the authors were found to be in the impossibility to address potential confounders, leaving room for potential bias.

CONCLUSIONS

The analysis of the chronic inflammatory infiltrate in endometriosis offers a deeper knowledge of its pathological evolution and inflammatory cell interactions. As understanding these receptors is pivotal in ongoing research, future endometriosis treatments may focus on them for an improved disease management.

It is difficult to propose a hypothesis for causal direction regarding the CD4+ and CD8+ T cells, in endometriosis. The relationship between the two types of lymphocyte is multifaceted and cofactors that contribute to their tissular and humoral activity need to be further studied and taken into account. The present data shows that while both cell types communicate and influence each other, it is not a linear, one-to-one causation.

Our findings also indicate a weak correlation between the counts of CD4+ T cells and CD68 cells in endometriotic tissue. This underscores the intricate and multifaceted functions of these two cell types and prompts speculation regarding their mutual impact.

Also, our evaluation of the CD68 and PR score quantifications indicates a relationship between them, our evidence favoring a hypothesis where an increase in macrophages (as indicated by the CD68 percentage) leads to a heightened PR score.

Our data could lay the foundation for new treatment approaches, either in the development of macrophage-targeting medications or by developing methods to overcome resistance to progestin treatment.

There is still limited data regarding the immune landscape of endometriosis. To better understand the pathogenic mechanisms of endometriosis, a more comprehensive study is needed, with promising impact upon the treatment and randomized controlled trials might measure the effectiveness of new tailored interventions, in order to alleviate the burden of endometriosis.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.
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REFERENCES


