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Full length article

Influence of endometrial nerve fibers and hormones on pain in women with endometriosis

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ABSTRACT

Objective: The mechanisms behind endometriosis-related pain are not yet fully understood. To determine if there is a difference in the density of endometrial nerve fibers between women with endometriosis and healthy controls, and to explore how the density of these nerve fibers and hormone levels correlate with the severity of symptoms experienced by the women.

Study Design: In this case-control study, 76 women with endometriosis and 24 healthy controls were included. The patient group was divided into two subgroups: those with and without hormonal treatments. Endometrial biopsies were taken and stained to detect PGP 9.5, a nerve fiber marker. Blood samples were collected for hormone analysis. Pain symptom severity was measured using VAS and EHP30.

Results: Women with endometriosis had a higher density of endometrial nerve fibers than healthy controls (median [range]: 2.0 [2.0–4.0] vs. 1.0 [0.0–1.0] fibers/mm^2, $P < 0.001$). This increased density was associated with more severe pain ($\beta = 0.130$ [95 % CI: 0.019, 0.240], $P = 0.02$). Women with endometriosis, regardless of hormone treatment, had a higher density of endometrial nerve fibers (3.0 [2.0–4.0] and 2.0 [1.0–4.0] fibers/mm^2, respectively) compared with healthy controls (1.0 [0.0-u1.0] fibers/mm^2, both $P < 0.001$). The density was not significantly different between those receiving and not receiving hormone treatment. The allopregnanolone/progesterone ratio was greater in women with endometriosis not receiving hormone treatment (0.002 [0.001–0.004]) than in healthy controls (0.001 [0.000–0.005]) and women receiving hormone treatment (0.001 [0.000–0.006], $P = 0.02$ and 0.001, respectively). A greater allopregnanolone/progesterone ratio was associated with more severe pain ($\beta = 20.662$ [95 % CI: 0.202, 41.121], $P = 0.048$), but hormone levels (estrogen, progesterone, and allopregnanolone) were not associated with endometrial nerve fiber density.

Conclusion: Women with endometriosis have a higher nerve fiber density in the endometrium, linked to more severe pain, regardless of hormone treatment. Increased progesterone metabolism to allopregnanolone may be a target for managing endometriosis pain.

Introduction

Endometriosis is a benign, chronic, estrogen-dependent condition. Women with endometriosis commonly report various pain symptoms, including dysmenorrhea, dyspareunia, and chronic pelvic pain. Some pathologic mechanisms for pain have been suggested, including inflammation and peripheral and central sensitization, although the mechanisms are not fully understood. Previous studies have found a high density of nerve fibers in endometriotic lesions, and that the density correlates with the pain symptoms reported by the patients [1,2]. Moreover it has been shown that there is an imbalance in the types of nerve fibers expressed within endometriotic lesions, where the ratio of sensory nerve fibers compared to adrenergic nerve fibers is drastically increased in endometriotic lesions compared to healthy peritoneum, promoting increased nociception by increased sensory nerve fibers, and inflammation by decreased adrenergic immunosuppression [3].

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Additionally, nerve fibers have been found in the eutopic endometrium of women with endometriosis [4]. Although initially believed to be specific to endometriosis, Zhang et al. [5] also found nerve fibers in the endometrium of women with uterine fibroids or adenomyosis. Interestingly, these nerve fibers were only found in women who also suffered from pelvic pain [5]. This suggests that nerve fibers, not only in endometriotic lesions but also in the eutopic endometrium, could be important in the pathogenesis of pelvic pain in endometriosis.

Neurogenesis is influenced by sex hormones, with estradiol and progesterone being crucial in modulating pain perception [6–8]. During the menstrual cycle, changes in estradiol levels can provoke the pain associated with endometriosis. Although the exact role of progesterone in pain modulation is not fully understood, progesterone may interact with neuroendocrine pathways, potentially increasing pain sensitivity during the luteal phase [6]. Additionally, allopregnanolone, a neurosteroid derived from progesterone, may be a key hormone in pain modulation. Allopregnanolone is secreted from the adrenal glands during stress and exhibits analgesic effects in models of nociceptive and neuropathic pain [9,10]. Higher levels of allopregnanolone are associated with less severe self-reported pain symptoms in certain populations, such as war veterans with chronic pain [11]. Therefore, understanding the interplay among these hormones, neurogenesis, and pain could reveal important pain management strategies for women with endometriosis.

Our primary objective was to investigate whether the density of endometrial nerve fibers differs between women with endometriosis and healthy controls and whether the endometrial nerve fiber density is associated with the severity of pain symptoms. Our hypothesis was that women with endometriosis would have a greater density of nerve fibers within the functional layer of the endometrium than healthy controls, and that the increased nerve fiber density would be associated with more severe pain in women with endometriosis. Our secondary objectives were to study the difference in endometrial nerve fiber density between patients receiving and not receiving hormone treatment and to assess if the endometrial nerve fiber density is associated with serum levels of endogenous estradiol, progesterone and allopregnanolone.

Materials and Methods

Study participants

The case-control study was a collaboration between Umeå University (Sundsvall Research Unit, Sundsvall, Sweden) and Kanuni Sultan Suleyman Training and Research Hospital (Istanbul, Turkey). Women with endometriosis were recruited from the outpatient clinics at Kanuni Sultan Suleyman Training and Research Hospital and Sundsvall County Hospital. As a control group, healthy women were recruited from the outpatient clinic at Kanuni Sultan Suleyman Training and Research Hospital. Women aged 18–45 years with symptomatic endometriosis, visually confirmed by laparoscopy, were eligible regardless of whether they were receiving hormone treatment [12]. Healthy women who were confirmed to not have endometriosis during laparoscopic sterilization were eligible for the control group. Women were excluded if they were pregnant and/or were diagnosed with a concomitant condition associated with pelvic pain. The study was performed in accordance with the ethical principles of the Declaration of Helsinki and was approved by the Regional Ethical Review Board of Umeå (October 2021; Dnr 2021–05236) and the Provincial Health Directorate, Kanuni Sultan Suleyman Training and Research Hospital Ethics Committee (November 2021; Dnr. 2021.11.281).

Study design

Women were recruited after receiving oral and written information about the study and signing the consent form. To obtain sufficient endometrial tissue for nerve fiber examinations, all women, except those on hormone treatment, were examined in the secretory phase of the menstrual cycle; the menstrual phase was confirmed by histological examination of the endometrium [13]. The participants' medical history was reviewed and they underwent gynecological and ultrasound examinations. Biopsy of eutopic endometrium was performed using a single-use endometrial suction curette (Pipelle, CooperSurgical, Trumbull, CT, USA) for immunohistochemical assessment of endometrial nerve fibers. Among women recruited in Turkey, a blood sample was taken for hormone analyses and the severity of symptoms of women with endometriosis was assessed using a visual analog scale (VAS) and the Endometriosis Health Profile 30 (EHP30) questionnaire.

Assessment of symptoms

All women with endometriosis enrolled in Turkey completed the VAS and EHP30 at enrollment to assess symptom severity. Their current level of pain was measured using a VAS, which is often used to evaluate a person's ongoing pain. In this VAS, the women estimated their pain by placing a mark on a 100-mm line, where 0 indicated no pain and 10 indicated the worst imaginable pain.

The impact of symptoms, including pain, on health-related quality of life was assessed using the EHP30, a validated questionnaire that was specifically developed for women with endometriosis. The questionnaire comprises 30 questions divided into five dimensions: pain, control and powerlessness, emotional well-being, social support, and self-image. Each dimension is scored from 0 to 100, where 100 indicates the worst possible negative impact on health-related quality of life [14].

Immunohistochemical assessment of the endometrial nerve fiber density

The endometrial biopsies were fixed in formalin, embedded in paraffin, and stored at room temperature until immunohistochemistry was performed at the Department of Pathology, Istanbul Kanui Sultan Suleyman Training and Research Hospital, Istanbul, Turkey. Manual immunohistochemistry was performed to detect protein gene product (PGP) 9.5. PGP9.5 is a neuron-specific protein used as a marker of nerve fibers [15].

Formalin-fixed, paraffin-embedded tissues were cut into 3-μm-thick sections, which were transferred to a positively charged slide. Deparaffinized and dehydrated sections were placed in distilled water. For the pre-enzyme procedure, EnVision FLEX target retrieval solution, high pH (Agilent Technologies, Santa Clara, CA, USA) was added to the pretreatment device (Agilent Technologies). The preheating temperature was set to 65 °C and the epitope retrieval temperature was set to 97 °C. The tissues were kept in the device for 20 min, cooled to 65 °C, and then immediately transferred to Tris-buffered saline (TBS; ScyTek, West Logan, UT, USA) and washed with TBS twice. Hydrogen peroxide (3 %) solution was applied for 10 min at room temperature. Rabbit anti-human PGP9.5 antibody (GeneTex, Irvine, CA, USA) was applied at a dilution of 1:1000 and incubated for 30 min. The tissues were then incubated with anti-polyvalent biotinylated antibodies (ScyTek) for 20 min, followed by horseradish peroxidase (ScyTek) for 20 min. The samples were incubated with 3,3′-diaminobenzidine chromogen substrate solution for 10 min at room temperature and counterstained with hematoxylin.

Following immunostaining, the endometrial biopsy was scanned at low power (×100) using a microscope (BX51, Olympus, Tokyo, Japan) to identify hot spots representing the areas of highest innervation. Individual nerve fibers were then counted under a higher power (×400) to determine the number of nerves per unit area. The mean nerve count in five hot spots was calculated per sample[16]. The results were expressed as the mean number of nerve fibers per millimeter squared in each specimen from all endometrial samples. A single observer, blinded to the sample background, counted the number of PGP9.5-positive nerve fibers.

Hormone analyses

Blood samples (20 mL) were obtained by venipuncture in the clinic between 08:00 and 13:00. The samples were centrifuged, and sera were immediately transferred to a freezer for storage at −80 °C. The estradiol and progesterone assays were performed at the Department of Biochemistry, Istanbul Kanui Sultan Suleyman Training and Research Hospital using competitive electrochemiluminescence assays (Elecsys Estradiol Gen III and Elecsys Progesterone Gen III, Roche Diagnostics, Mannheim, Germany) on an automated analyzer (Cobas 601, Roche Diagnostics) according to the manufacturer's instructions. According to the manufacturer's datasheets, the assay ranges for estradiol and progesterone were 18.4–11,010 pmol/L (5.0–3000 pg/mL) and 0.159–191 nmol/L (0.050–60.0 ng/mL), respectively, and the intra-assay coefficients of variation were 1.7 %-2.6 % and 1.4 %-2.8 %, respectively.

Allopregnanolone serum levels were determined at Admescope Oy Laboratory (Oulu, Finland) using a previously described method [17]. Allopregnanolone was quantified by ultra-high-performance liquid chromatography (Waters Acquity, Waters Corp., Milford, MA, USA) using a C18 column on a triple quadrupole mass spectrometer (XEVO-TQ-S, Waters Corp.). The detection limit was 0.0318–318 nmol/L (0.01–100 ng/mL).

Allopregnanolone influences the excitability of neurons by interacting with gamma-aminobutyric acid (GABA)A receptors [18], potentially explaining its effects on stress (pain) responses. In women, allopregnanolone levels typically fluctuate in sync with its precursor progesterone throughout the menstrual cycle[19]. However, in certain conditions, such as premenstrual syndrome (PMS), the correlation between serum concentrations of allopregnanolone and progesterone may vanish [20]. Thus, the allopregnanolone/progesterone ratio can be instrumental in identifying hormonal imbalances that may contribute to pain symptoms. The allopregnanolone/progesterone ratio was calculated to provide insights into the regulation of allopregnanolone production and its fluctuation in each group.

Statistical analysis

All statistical analyses were performed using SPSS version 28 (IBM Corp., Armonk, NY, USA). Continuous variables are presented as the median (interquartile range). Categorical variables are presented as the frequency count and percentage. Pearson's χ^2 test was used to determine the significance of between-group differences in categorical variables. Continuous variables were compared using the Mann-Whitney *U* test for comparisons between two groups (i.e., healthy controls and women with endometriosis). The Kruskal-Wallis test was used for comparisons among three groups (healthy controls, women with endometriosis receiving hormone treatment, and women with endometriosis not receiving hormone treatment), and *P*-values were adjusted for multiple testing using Bonferroni correction. Linear regression was used to determine associations between explanatory variables and the outcome variables; each analysis was performed using separate models in which hormone treatment and age were included as potential confounding variables. The residual analysis, as a diagnostic test to evaluate the regression model, confirmed that the assumptions of linearity and residuals were satisfied. The linearity assumption was validated by the absence of patterns in the residuals versus predicted values plot. The results are presented as β coefficients with 95 % confidence intervals (CI). *P*-values of < 0.05 were considered significant.

A power analysis was performed based on the assumption that PGP9.5-positive nerve fibers are present in the functional layer of the endometrium in women with endometriosis during the secretory phase but not in healthy women [4]. This assumption underlies the power analysis, which determined that, with a significance level (α) of 0.05 and a power of 90 %, a sample size of four women per group would be sufficient to detect a significant difference in the main outcome.

Results

Characteristics of women with endometriosis and healthy controls

In total, 98 women with endometriosis were recruited. Of these, 18 were excluded due to concomitant painful conditions (fibromyalgia: $n = 15$; irritable bowel syndrome [IBS]: $n = 2$; fibromyalgia and IBS: $n = 1$). Two patients were excluded due to insufficient material in the endometrial biopsy. Two patients were excluded because they were not in the secretory phase according to the histologic assessment. Therefore, 76 women with endometriosis were included in the study, of whom 18 % ($n = 14$) were recruited in Sundsvall, Sweden and 82 % ($n = 62$) were recruited in Istanbul, Turkey. However, a comparison of baseline characteristics revealed significant differences between the women recruited in Sweden and Turkey regarding their age (28.0 [23.5–30.25] vs. 36.0 [33.0–39.0] years, respectively, $P < 0.001$), the proportion of nulligravid women (6/14 vs. 3/62, respectively, $P < 0.001$), the proportion of nulliparous women (9/14 vs. 3/62, respectively, $P < 0.001$) and endometrial nerve fiber density (1.0 [1.0 – 2.0] vs 3.0 [2.0 – 4.0], respectively, P < 0.001). Baseline characteristics among the populations recruited in Turkey and Sweden are shown separately in supplemental table 1.

The control group comprised 45 women who underwent laparoscopic sterilization. After thorough investigation, 19 women were excluded due to a concomitant painful condition (fibromyalgia: $n = 13$; IBS: $n = 4$; fibromyalgia and IBS: $n = 2$) and two women were not in the secretory phase. Therefore, 24 healthy controls were included in the study, all of whom were recruited in Turkey (Fig. 1).

There were no significant differences in the age or body mass index between the women with endometriosis and healthy controls (Table 1), although the proportion of nulliparous women was greater among women with endometriosis. Among the women with endometriosis, 18 % ($n = 14$) were diagnosed with peritoneal endometriosis, 80 % ($n = 61$) were diagnosed with ovarian endometriomas, and 1 % ($n = 1$) had deep infiltrating endometriosis. At the time of recruitment, 43 % ($n = 33$) of the women with endometriosis were receiving hormone treatment and were amenorrheic; the hormone treatments included dienogest in 58 % ($n = 19$), combined oral contraceptives in 12 % ($n = 4$), and dydrogesterone in 30 % ($n = 10$). The median duration of hormone treatment was 10.0 (8.0–14.5) months.

Endometrial nerve fiber density and pain symptoms

Women with endometriosis had a greater density of PGP9.5-positive nerve fibers in the functional layer of the endometrium than healthy controls (median of 2.0 [2.0–4.0] vs. 1.0 [0.0–1.0] fibers/mm^2, respectively, $P < 0.001$; Fig. 2). Furthermore, among women with endometriosis, a greater endometrial nerve fiber density was associated with more severe pain in terms of an elevated VAS score ($\beta = 0.130$ [95 % CI: 0.021, 0.239], $P = 0.02$). This association remained after adjusting for age and hormone treatment as possible confounders ($\beta = 0.130$ [95 % CI: 0.019, 0.240], $P = 0.02$, Table 2).

Endometrial nerve fiber density and hormone treatment

The endometrial nerve fiber density was significantly greater among women with endometriosis who were and were not receiving hormone treatment (3.0 [2.0–4.0] and 2.0 [1.0–4.0] fibers/mm^2, respectively) than healthy controls (1.0 [0.0–1.0] fibers/mm^2, both $P < 0.001$). However, among women with endometriosis, the nerve fiber density was not significantly different between those who were and were not receiving hormone treatment ($P > 0.9$).

Hormone levels

The analysis of serum hormone levels (estradiol, progesterone,

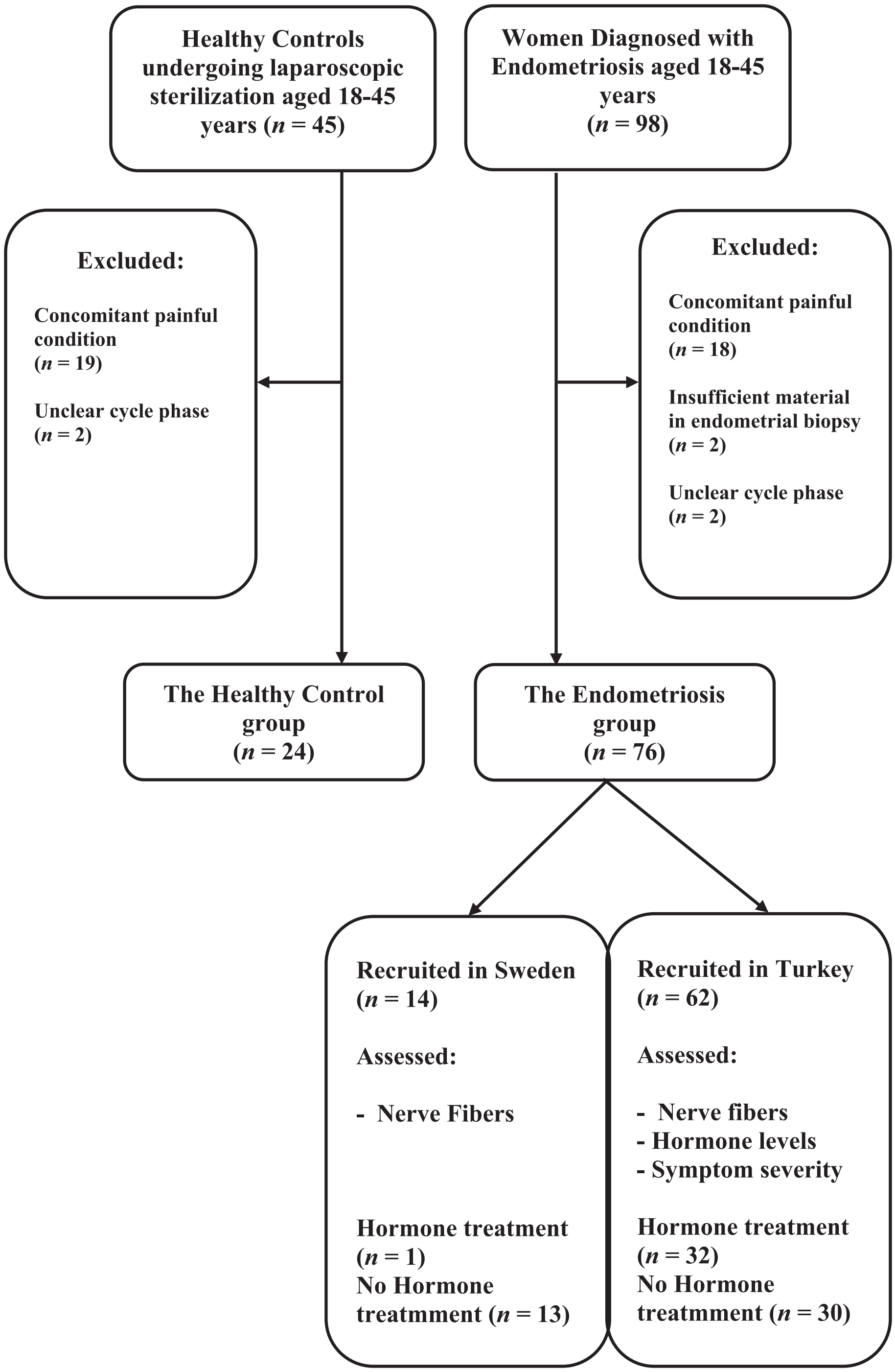

Fig. 1. Recruitment of study participants.

allopregnanolone, and allopregnanolone/progesterone ratio) showed no differences in the estradiol and allopregnanolone levels among the three groups (healthy controls and women with endometriosis who were or were not receiving hormone treatment). However, the serum progesterone levels were lower in women with endometriosis not receiving hormone treatment than in the other two groups ($P < 0.001$ for both). Furthermore, the allopregnanolone/progesterone ratio was greater among women with endometriosis who were not receiving hormone treatment compared to both patients receiving hormone treatment ($P = 0.01$) and controls ($P = 0.02$) (Table 3).

Associations between hormone levels and endometrial nerve fiber density

In women with endometriosis, the serum levels of the analyzed hormones (estradiol, progesterone, allopregnanolone, and the allopregnanolone/progesterone ratio) were not significantly associated with the endometrial nerve fiber density, regardless of their hormone treatment status (Table 2). Moreover, the hormone levels (estradiol, progesterone, and allopregnanolone) were not associated with VAS score among women with endometriosis, regardless of hormone treatment (Supplemental table 2). However, an increased allopregnanolone/progesterone ratio was associated with greater VAS score in women with endometriosis who were not receiving hormone treatment ($\beta = 20.549$ [95 % CI: 0.267, 40.830], $P = 0.047$). This association remained after

Table 1
Baseline characteristics of patients with endometriosis and healthy controls. Mann-Whitney *U* test was used for significance testing between two groups of continues data. Chi-Square test was used for significance testing between two groups of categorical data.

	Controls (n = 24)	Endometriosis (n = 76)	*P*-values
Age, median [IQR]	37.0 [33.5 – 39.0]	34.5 [31.0 – 38.0]	0.062
BMI, median [IQR]	25.1 [23.0 – 26.8]	24.0 [22.6 – 26.0]	0.3
Nulligravida, % (n)	0 % (n = 0)	12 % (n = 9)	0.073
Nulliparous, % (n)	0 % (n = 0)	16 % (n = 12)	**0.037**
Hormonal treatment, % (n)	–	43 % (n = 33)	
Endometrial nerve fibers / mm^2, median [IQR]	1.0 [0.0 – 1.0]	2.0 [2.0—4.0]	**<0.001**
VAS, median [IQR]	–	6.0 [5.0 – 7.0]	–
EHP 30 – Pain, median [IQR]	–	35.0 [13.3 – 43.0]	–
EHP 30 – Control and powerlessness, median [IQR]	–	33.0 [4.0 – 39.0]	–
EHP 30 – Emotional well-being, median [IQR]	–	38.0 [13.0 – 46.0]	–
EHP 30 – Social support, median [IQR]	–	38.0 [6.0 – 50.0]	–
EHP 30 – Self-image, median [IQR]	–	33.0 [0.0 – 42.0]	–

BMI, Body mass index, VAS, Visual analogue scale, EHP 30, Endometriosis health profile 30, IQR, Interquartile range.

adjusting for age ($\beta = 20.662$ [95 % CI: 0.202, 41.121], $P = 0.048$).

Discussion

In this case-control study, the main findings were that women with endometriosis had a greater nerve fiber density in the functional layer of the endometrium than healthy controls. This greater nerve fiber density was associated with more severe pain in these women. Additionally, both groups of patients (i.e., who were or were not receiving hormone treatment) had a greater endometrial nerve fiber density than healthy controls, without a significant difference between the two subgroups. The allopregnanolone/progesterone ratio was greater in women with endometriosis who were not receiving hormone treatment than women receiving hormone treatment and the healthy controls. This greater ratio was associated with more severe pain. However, hormone levels (estrogen, progesterone, and allopregnanolone) were not associated with the endometrial nerve fiber density.

Previous studies reported contradictory results regarding the endometrial nerve fiber density in women with endometriosis [4,13,21,22]. Although these nerve fibers were not exclusive to women with endometriosis, our results strongly support the hypothesis that women with endometriosis and pelvic pain have a greater nerve fiber density in the functional layer of the endometrium. Moreover, we found that a greater endometrial nerve fiber density was associated with more severe pain in these women. This finding supports the idea that nerve fibers within endometriotic lesions as well as nerve fibers within the eutopic endometrium could be involved in the pain reported by women with endometriosis. Our results are supported by those reported by Zhang et al. [5], who found that endometrial nerve fibers were exclusively present in patients with pain symptoms. A more recent study showed a positive correlation between the endometrial nerve fiber density and VAS score in women with endometriosis [23], which also aligns with our findings. The finding that the uterine innervation is related to the pain intensity in women with endometriosis is of clinical importance because it provides an increased understanding of the role of the uterus and the eutopic endometrium in the pain experienced by women with endometriosis. Moreover, this helps explain the benefit of hysterectomy to treat the pain associated with endometriosis[24,25].

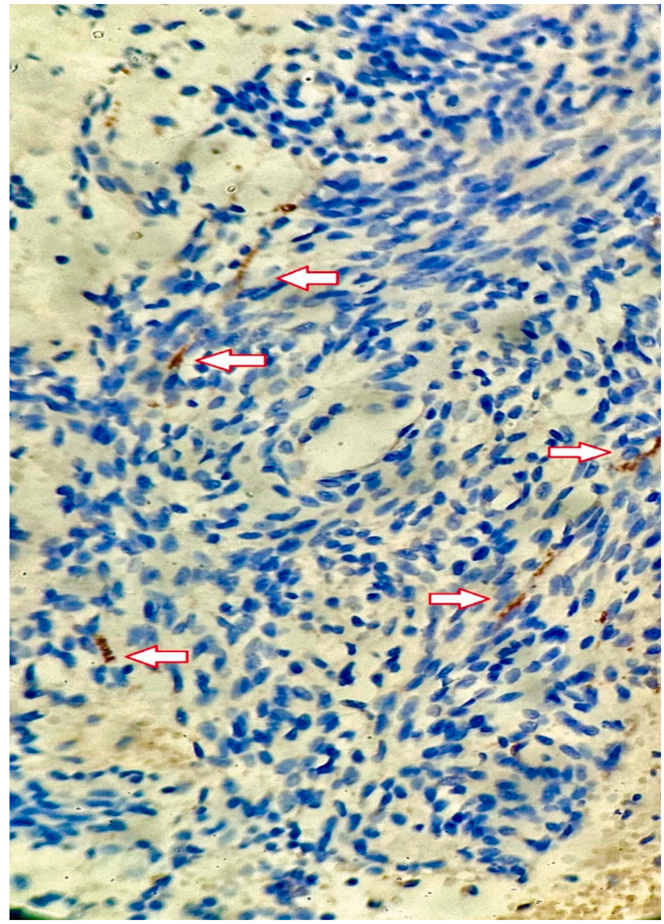

Fig. 2. Expression of nerve fibers in endometrial tissues, in women with endometriosis The manual immunohistochemical procedure was performed using rabbit anti-human protein gene product (PGP) 9.5 as a neuron-specific protein to identify nerve fibers (indicated with an arrow).

Understanding how hormone treatment affects the endometrial nerve fiber density is crucial because it could provide valuable insight into how hormone treatment might alleviate the pain symptoms in women with endometriosis. A previous study found a lower endometrial nerve fiber density in women receiving hormone treatment than in women without hormone treatment [26]. A similar result was seen in women with adenomyosis, suggesting that hormone treatment could reduce the nerve fiber density within the endometrium [27]. Our results do not support this idea because we found no difference between the women who were or were not receiving hormone treatment. This aligns with a recent study by Velho et al., who examined nerve fibers within endometriotic lesions and also failed to show any difference in the nerve fiber density between women who were or were not receiving hormone treatment [28]. Similar to our study, the majority of women in their study suffered from pain symptoms, including those receiving hormone treatment. In contrast, the studies cited above did not report data regarding the pain symptoms experienced by patients[26,27]. This difference in study design could explain the discrepancies in the results.

The idea that hormone treatment with progestins could affect the nerve fiber density through a decreased estrogenic effect is supported by the assumption that estrogen promotes the expression of uterine nerve growth factor, as demonstrated in animal models [7]. Interestingly, we

Table 2
The association between endometrial nerve fibers and symptom severity in women with endometriosis and the association between serum hormonal levels and endometrial nerve fibers in women with endometriosis without and with hormonal treatment, assessed by linear regression.

	Endometrial nerve fibers in endometriosis (total)		Endometrial nerve fibers in patients without treatment	Endometrial nerve fibers in patients with treatment
VAS	β = 0.130, 95 % CI [0.019, 0.240], P = **0.02**	**Estradiol (pg/mL)**	β = 0.000, 95 % CI [-0.010, 0.010], P > 0.9	β = 0.001, 95 % CI [-0.010, 0.013], P = 0.8
EHP 30 Pain	β = -0.772, 95 % CI [-2.558, 1.014], P = 0.4	**Progesterone (nmol/L)**	β = -0.361, 95 % CI [-1.481, 0.759], P = 0.5	β = 0.018, 95 % CI [-0.481, 0.516], P = 0.9
EHP 30 Control and powerless-ness	β = -0.822, 95 % CI [-2.860, 1.216], P = 0.4	**Allo-pregnanolone (nmol/L)**	β = 17.491, 95 % CI [-36.846, 71.828], P = 0.5	β = -12.632, 95 % CI [-46.466, 21.202], P = 0.5
EHP 30 Emotional well being	β = -0.625, 95 % CI [-2.358, 1.108], P = 0.5	**Progesterone / allo-pregnanolone ratio**	β = 11.334, 95 % CI [-35.979, 58.646], P = 0.6	β = -113.995, 95 % CI [-330.444, 102.454], P = 0.3
EHP 30 Social Support	β = -1.095, 95 % CI [-3.306, 1.116], P = 0.3			
EHP 30 Self image	β = -0.693, 95 % CI [-2.571, 1.184], P = 0.5			

VAS, Visual analogue scale, EHP 30, Endometriosis health profile 30.

Table 3
VAS, endometrial nerve fiber density and serum hormonal levels in healthy controls, patients with endometriosis without hormonal treatment and patients with endometriosis and hormonal treatment. Significance testing between two groups was performed using Mann-Whitney *U* test. Significance testing between three groups was performed using Kruskall-Wallis test and *P*-values were adjusted for multiple testing with the Bonferroni correction.

	Controls (A) (n = 24) median [IQR]	**No hormonal treatment (B) (n = 43)** median [IQR]	**Hormonal treatment (C) (n = 33)** median [IQR]	***P* – values**
VAS		6.0 [5.0 – 7.0]	6.0 [5.0 – 7.0]	B – C: 0.9
Endometrial nerve fibers / mm^2	1.0 [0.0 – 1.0]	2.0 [1.0 – 4.0]	3.0 [2.0 – 4.0]	A – B: < **0.001** A – C: < **0.001** B – C: > 0.9
Estradiol (pg/mL)	77.0 [43.3 – 121.8]	77.5 [24.8 – 133.5]	77.5 [48.3 – 151.5]	A – B: > 0.9 A – C: > 0.9 B – C: > 0.9
Progesterone (nmol/L)	6.1 [5.0 – 8.8]	2.1 [1.4 – 2.5]	6.6 [4.9 – 8.6]	A – B: < **0.001** A – C: > 0.9 B – C: < **0.001**
Allopregnanolone (nmol/L)	0.003 [0.003 – 0.013]	0.003 [0.003 – 0.004]	0.003 [0.003 – 0.032]	A – B: 0.7 A – C: > 0.9 B – C: 0.2
Allo/prog. ratio	0.001 [0.000 – 0.005]	0.002 [0.001 – 0.004]	0.001 [0.000 – 0.006]	A – B: **0.02** A – C: > 0.9 B – C: **0.01**

VAS, Visual analogue scale.

found no association between the circulating estrogen levels and the endometrial nerve fiber density, either in women with or without hormone treatment.

The circulating progesterone levels were significantly lower in women with endometriosis who were not taking hormone treatment compared with those receiving hormone treatment and the healthy controls. However, this difference is probably explained by the variability in cycle day within the secretory phase among the participants because progesterone levels show marked variation across the secretory phase and, in this study, the exact day of the menstrual cycle when the sampling was done was not confirmed. Measured hormone levels among patients on hormone treatment may be influenced by a possible lack of compliance. Compliance in the hormone treatment group could vary, as it was not measured and general compliance to medical therapy is typically low, especially if the treatment does not yield the desired effects [29].

An earlier study that examined the association between the allopregnanolone levels and pain showed that higher allopregnanolone levels are associated with less-severe self-reported pain symptoms in a group of war veterans with chronic pain [11]. This suggests that allopregnanolone might function as a natural pain reliever in the body, a possibility that was also reported in an animal model of post-stroke pain [30]. In our study, we found no significant differences in the serum allopregnanolone levels among the three groups (healthy controls and women with endometriosis who were or were not receiving hormone therapy), and we found no association between the serum allopregnanolone levels and the endometrial nerve fiber density. However, the allopregnanolone/progesterone ratio was greater in women not receiving hormone treatment compared with the other two groups. This suggests that the allopregnanolone/progesterone ratio, a proxy for the metabolism of progesterone to allopregnanolone, increases in patients with endometriosis who are not on hormone therapy. Although this finding, similarly to the finding of decreased progesterone levels among women with endometriosis without hormone treatment, may be affected by a possible variation in cycle days among the study participants, this increase could be a response to their pain, which is supported by our finding of an association between the allopregnanolone/progesterone ratio and VAS score in this group.

Strengths and limitations

The strengths of our study include a well-diagnosed study population, as women with endometriosis and healthy controls underwent laparoscopy to confirm or rule out endometriosis. Although the sample size is likely too small to be representative of different aspects of the condition, it is relatively large compared to many other studies on endometriosis and pain. Moreover, all assessments of nerve fiber density were performed by the same observer, who was blinded to background characteristics. However, the median age of the study population was 34.5 years, and only a minority were nulligravida. This demographic profile may limit the generalizability of the findings to younger, nulligravida patients. We did not systematically assess the severity of symptoms among the women recruited in Sweden, nor were hormone data included for those women, which is a major weakness explained by the fact that Swedish subpopulation had already been examined when it was decided to add data on symptom severity and hormone analysis. The analyses of symptom severity and hormone levels were limited to the subpopulation of women recruited in Turkey, which could affect the statistical power of the secondary outcomes. Moreover, the differences in baseline characteristics between patients recruited in Sweden and Turkey is a potential source of bias, however when we performed the analysis without the Swedish subpopulation the results regarding endometrial nerve fiber densities between groups persisted, moreover, any results regarding hormones as well as the association between endometrial nerve fibers and symptom severity were not affected by this possible bias considering this data was exclusively assessed in the subpopulation recruited in Turkey. Another important limitation is the lack of data on the specific cycle day at the time of blood sampling. Although all participants, except those on hormone treatment, were examined in the secretory phase of the menstrual cycle, the exact day within this phase may influence hormone levels and symptom severity, and could affect the results.

Conclusion

We have shown that women with endometriosis express a greater nerve fiber density in the functional layer of the endometrium, regardless of whether they are receiving hormone treatment, compared with healthy controls. We have also shown that a greater nerve fiber density in the eutopic endometrium is associated with more severe pain in women with endometriosis. In addition, the serum levels of allopregnanolone relative to its precursor progesterone (allopregnanolone/progesterone ratio) were higher in women with endometriosis who were not receiving hormone treatment relative to both patients receiving hormone treatment and healthy controls, and that a higher ratio was associated with more severe pain. Our results suggest that both the intrauterine innervation and the metabolism of progesterone to allopregnanolone are involved in the mechanisms underlying the pain reported by women with endometriosis. These results are clinically interesting because they may suggest that the uterus and possibly also the metabolism of progesterone to allopregnanolone may be possible targets when treating pain in women with endometriosis.

CRediT authorship contribution statement

Anton Sommar: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Pinar Yalcin Bahat:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Ipek Yildiz Özaydin:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Marie Bixo:** Writing – review & editing, Methodology, Conceptualization. **Torbjörn Bäckström:** Writing – review & editing, Methodology, Conceptualization. **Engin Oral:** Writing – review & editing, Methodology, Conceptualization. **Sahruh Turkmen:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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