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RESEARCH ARTICLE

mRNA Expression and DNA Methylation of *CXCL16* in Menstrual Blood and Endometrium Tissue of Subjects with Endometriosis and Pelvic Pain

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Abstract

BACKGROUND: The cytokine chemokine ligand 16 (CXCL16) plays an important role in the pathophysiology of endometriosis by regulating the inflammatory response and contributing to the pain-associated endometriosis. Despite this, the impact of epigenetic modifications, such as DNA methylation, on *CXCL16* has yet to be fully understood. Therefore, this research was conducted to assess both the mRNA expression and DNA methylation levels of the proinflammatory gene *CXCL16* in the endometrium tissue and menstrual blood of patients with and without endometriosis.

METHODS: Thirty-five women with and without endometriosis were involved in this research. Subjects' menstrual blood samples were collected using filter paper pads, meanwhile the endometrium tissue were collected by performing biopsy, from which DNA and RNA were extracted. The DNA methylation levels of the *CXCL16* were measured using the pyrosequencing method following bisulfite conversion treatment. Meanwhile, the mRNA expression level was measured using the quantitative polymerase chain reaction (qPCR) method and analyzed with the Livak method.

RESULTS: The mRNA expression of *CXCL16* in menstrual blood of endometriosis subjects was 2.42 times higher compared to control group (p=0.030). Furthermore, the expression of *CXCL16* in menstrual blood was identical to that in endometrial tissue (p=0.173). DNA methylation analysis showed that *CXCL16* in the menstrual blood of endometriosis subjctes had lower methylation levels compared to controls (p=0.004), indicating hypomethylation.

CONCLUSION: Increased mRNA expression and hypomethylation of *CXCL16* in the menstrual blood of endometriosis patients could serve as a direct marker for diagnosing endometriosis. However, further study to validate these findings and explore the potential of *CXCL16* as a diagnostic tool, and additional research involving larger patient for the cohorts study is necessary.

KEYWORDS: CXCL16, DNA methylation, endometrium, menstrual blood, mRNA expression, pain

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Introduction

Endometriosis is a chronic, estrogen-dependent inflammation caused by ectopic growth of endometrial tissue, which usually characterized by the presence of tissue resembling the endometrium outside the uterine cavity.(1, 2) Endometriosis often occurs in women of reproductive age, though it is still possible to occur in premenopausal, menopausal, and postmenopausal women.(3) Endometriosis

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affects approximately 10% of women of reproductive age or 176 million people globally.(4,5) Meanwhile, in Indonesia, year-on-year increase in endometriosis prevalence was identified, with a frequency range between 13.6-69.5%.(6) While the precise pathophysiology of endometriosis remains elusive, it is currently regarded as a type of chronic inflammatory disease.(7) The earliest theory explaining endometriosis is the retrograde menstruation, referring to the condition when menstrual blood and cellular debris enter the Fallopian tube and the peritoneal cavity. In women with endometriosis, this scenario triggers an inflammatory response, leading to the activation of macrophages. This inflammatory reaction, characterized by the persistence and proliferation of endometrium-like cell debris, is a defining feature of endometriosis. Macrophages secrete inflammatory mediators including tumor necrosis factor (TNF)-α that can induce an inflammatory gene chemokine ligand 16 (CXCL16). CXCL16 is a small cytokine produced during the inflammation that relates to pain sensation which is expressed in epithelial cells and stromal cells in endometriosis.(8,9)

Menstrual blood, composed of blood, cervical fluid, and endometrial cells, serves as a viable source of nucleic acids, including DNA and RNA samples. This characteristic is significant for research purposes, especially in the field of epigenetics. Epigenetic mechanisms are implicated in numerous chronic diseases including endometriosis. In cases of endometriosis, one of the notable epigenetic alterations is the change in DNA methylation patterns within gene expressions.(10-12) In epigenetic mechanism and DNA methylation, nerve growth factor (*NGF*) gene undergoes hypomethylation in menstrual blood. Furthermore, NGF mRNA expression is found higher in menstrual blood.(10) The *NGF* and *CXCL16* are associated with the pain experienced in endometriosis. The pro-inflammatory gene CXCL16 has not yet been studied in epigenetics. The standard method for diagnosing endometriosis involves the visualization of endometriosis lesions through laparoscopy or by conducting a histopathological examination of the tissue. However, this procedure is invasive, costly, and requires the expertise of experienced professionals. Numerous studies have focused on evaluating a non-invasive diagnostic test for endometriosis. Utilizing menstrual blood as a DNA sample emerged as an initial step in identifying biomarkers that are both effective and efficient in terms of cost and time. In this research, mRNA expression and DNA methylation of the pro-inflammatory gene *CXCL16* were examined as a potential proliferation marker of endometriosis-related pain.

Methods

Subjects Recruitment

Thirty-five subjects were consecutively selected from the Reproductive Immuno-Endocrinology Polyclinic Division at the Department of Obstetrics and Gynecology, Cipto Mangunkusumo Central General Hospital/Faculty of Medicine, Universitas Indonesia, Jakarta and the Obstetrics and Gynecology Polyclinic Harapan Kita Women and Children Hospital, Jakarta. The sample size was determined using a proportion estimation formula based on the data obtained from various hospitals. Inclusion criteria were women aged 20-45 years with a menstrual cycle of 25-30 days. Subjects who experienced cyclic pelvic pain, diagnosed with endometriosis via ultrasound and requiring a biopsy as determined by a team of obstetricians, and who did not receive any treatment were assigned in the case group. Whereas, asymptomatic subjects with infertility who underwent endometrial sampling were assigned into the control group. Exclusion criteria were unmarried and pregnant women, and those with a suspected history of endometrial cancer, ovarian cancer, hydatidiform mole, or any other obstetric and gynecological malignancies.

Sample Collection

Menstrual blood samples were collected using a filter paper pad on the second or third day of menstruation using Whatman filter paper sewn onto the pads. The specimens were immediately sent to the laboratory for analysis. The filter paper was first dried and then separated from the cloth dressing before being cut into small sizes and stored. The DNA and RNA were extracted from the menstrual blood and endometrium tissue. Endometrial biopsies were performed to obtain endometrium tissue, which was then stored in 1.5 mL microcentrifuge tubes at -20°C for preservation. The protocol of this study was approved by the Ethics Commission of the Faculty of Medicine, Universitas Indonesia (Protocol No. 22-01-0076).

The Numeric Rating Scale (NRS) was used to measure the pain level from mild (score 1-3), moderate (score 4-7) and severe (score 8-10). The research subject's most severe pain experienced during the previous three months was used to determine the degree of the pain.(13)

DNA Extraction

Extraction of menstrual blood DNA was carried out using a Qiagen DNA Extraction Kits (Qiagen,Venlo, Netherlands). Fifty mg of the specimens were placed in a 1.5 mL tube,

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followed by the addition of 360 µL of ATL buffer, and then incubated at 85°C for 30 minutes. After incubation, the specimens were centrifuged and 20 µL of proteinase K was added, and then vortexed. Subsequently, the specimens were incubated at 56°C for 2 hours, centrifuged, and 400 µL of AL buffer was added and vortexed. The material was then incubated at 70°C for 10 minutes, after which 400 µL of absolute ethanol was added. The mixture was transferred to a spin column and the spin column was transferred to a new collection tube. Subsequently, 500 µL of AW1 buffer was added to be centrifuged. This step was repeated once. Five-hundreds µL of AW2 buffer was then added, and the column was centrifuged again, which step was done twice. After adding 50 µL of AE buffer, the column was incubated at room temperature for 3 minutes and centrifuged. This step was repeated once. The eluted DNA was analyzed to measure the concentration and purity using a Nanodrop and stored at -20°C.

DNA was extracted from the endometrium using the Geneaid DNA Isolation Kit (Geneaid, New Taipei, Taiwan). Initially, 10 mg of the endometrium tissue sample was combined with 200 µL of GST buffer and 20 µL of Proteinase K. Then, it was incubated at 60°C overnight. In the post-incubation, 200 µL of GSB buffer was and 200 µL of absolute ethanol were added. The sample was then transferred into the GS column using a 2 mL collection tube before being added with 400 µL of W1 buffer. The GS column was then treated with 600 µL of wash buffer and centrifuged. After discarding the filtrate, the column matrix was dried through centrifugation. The spin column was subsequently transferred to a new microtube, onto which 100 µL of pre-warmed elution buffer was added to the center of the column matrix and allowed to stand for 3 minutes. A final centrifugation was conducted, after which the elution was analyzed for DNA concentration and purity using a Nanodrop.

DNA Methylation Analysis

DNA samples were subsequently subjected to bisulfite conversion using the EZ DNA Methylation-Lightning Kit, (Cat. No. D5031, Zymo Research, Irvine, CA, USA). Following the conversion, polymerase chain reaction (PCR) amplification of the DNA samples was visualized using the electrophoresis technique, while methylation levels were measured using the pyrosequencing method. For this purpose, primers were designed using the PyroMark Assay Design 2.0 software (Qiagen).

In the process, three primers were employed: a forward primer, a reverse primer, and a pyrosequencing primer (Table 1). It is essential for one of the primers to be biotin-labelled on one of the strands to facilitate subsequent steps. Following amplification, the PCR product underwent purification and denaturation, allowing the pyrosequencing primer to efficiently anneal to the biotin-labelled single-stranded template. During the operation of the pyrosequencer, light emissions from each well were captured by a charge-coupled device (CCD) camera and quantitatively represented as pyrogram peaks. These peaks, indicating the presence of "C" for methylated CpG sites and "T" for unmethylated cytosine, allowed for the determination of methylation status. The height ratio of these peaks indicated the methylation percentage at a specific CpG site or location. The overall percent DNA methylation for a given gene was calculated from the average methylation value across all analyzed CpG sites or locations.(14)

RNA Extraction

RNA from menstrual blood and endometrium tissue samples was extracted using Quick-RNA Miniprep (Zymo Research). A 50 mg sample of menstrual blood was added to 600 µL RNA lysis buffer and crushed using a rotor until homogeneous. Thirty µL proteinase K and 60 µL PK digestion buffer were added, and the mixture was incubated at 56°C for 20 minutes. Six-hundred µL of RNA lysis buffer was reintroduced, followed by vortexing and centrifuged. The resulting supernatant was transferred to a spin-away filter and centrifuged. Absolute ethanol was then added to the supernatant in 1:1 ratio. After that, 400 µL of RNA wash buffer was added to the spin column and centrifuged before the supernatant was discarded. Then, 5 µL of DNAse I and 75 µL of digestion buffer were added to the green spin column, which was then incubated for 15 minutes at

Table 1. Primer design of *CXCL16*.

	Sequence
CXCL16 Forward	TGGAGAAGATTATTTAGGGATGAGATG
CXCL16 Reverse	bio/ATACCCCACAAACCCATCCAAAATACTTCC
CXCL16 Pyrosequencing	GTTTAAATTAAGGTTAGAGAGGA

room temperature. Following this, 400 μL of RNA prep buffer was added and the column was centrifuged. After the supernatant was discarded, 700 μL RNA wash buffer and 400 μL RNA wash buffer were added and centrifuged. The green spin column was then transferred to a 1.5 mL microcentrifuge tube to be added with 50 μL of RNA-free water before being centrifuged. The green spin column was then discarded, and the supernatant/filtrate was measured using a Nanodrop and stored at -80°C.

Endometrium tissue samples of 20 mg were processed by adding 300 μL of RNA lysis buffer and crushing until the mixture became homogeneous. Following this, 15 μL of proteinase K and 30 μL of PK digestion buffer were added, then the mixture was incubated at 56°C for 3 hours and centrifuged. The mixture was then transferred into a spin-away filter for centrifugation. A total of 600 μL of absolute ethanol was added, vortexed, and the mixture was transferred to a spin column placed in a collection tube. The RNA purification stage proceeded with the addition of 75 μL DNAse digestion buffer and 5 μL DNAse I, followed by incubation for 15 minutes. The washing was done by adding 700 μL of RNA wash buffer I and a second wash with 400 μL of RNA wash buffer II, with the resulting supernatant being discarded. The spin column was then transferred to a 1.5 mL microcentrifuge tube. To elute the RNA, 50 μL of RNA-free water was added and the tube was centrifuged. The concentration and purity of RNA were measured using a nanodrop and the isolated RNA was stored at -80°C.

cDNA Synthesis and mRNA Expression Analysis

Using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan), cDNA synthesis was performed from whole RNA. Using Thunderbird™ SYBR qPCR Mix (Toyobo), the cDNA samples were amplified by RT-qPCR. Housekeeping gene used was *GAPDH*. *CXCL16* and *GAPDH* primers for RT-qPCR were designed and obtained from the Blastprimer website (*https://www.ncbi.nlm.nih.gov/tools/primer-blast/*) so that the target gene primer sequences were obtained (Table 2). Quantitative relative expression of *CXCL16* mRNA was quantified using the Livak method.

Statistical Analysis

The Shapiro-Wilk test was utilized to evaluate the distribution of the data. Comparisons were analyzed using the Student's t-test or Mann-Whitney U test when appropriate. Proportions were compared using the chi-square test or Fisher's exact test. Spearman's rank correlation was employed to evaluate the correlation between mRNA expression and DNA methylation of *CXCL16*.

Table 2. *CXCL16* and *GAPDH* primer design.

	Sequence
CXCL16 Forward	CCAGATCTGCCGGTTCATTAT
CXCL16 Reverse	TAACAGCCTGGGCAACATAG
GAPDH Forward	CCCTTCATTGACCTCAACTACA
GAPDH Reverse	ATGACAAGCTTCCCGTTCTC

Results

Characteristics of the Subjects

A total of 35 subjects met the inclusion and exclusion criteria, consisting of 18 subjects with endometriosis and 17 subjects without endometriosis (control). The characteristics of the subjects of this study include data on age, body mass index, disease history, menstrual cycle, and other questions (Table 3).

DNA Methylation Level of *CXCL16*

DNA methylation of each CpG site was quantified and calculated as an average across all CpG and analyzed to determine the differences between the endometriosis group and the control group. In the *CXCL16*, 2 CpG sites were found in each sample. Representative DNA methylation results of the *CXCL16* in the form of a pyrogram with 2 CpG sites found from menstrual blood and endometrium tissue samples of endometriosis subjects as well as menstrual blood, endometrium tissue in the control of one of the study subjects (Figure 1).

Median levels of DNA methylation of *CXCL16* at the CpG location in the menstrual blood among subjects with endometriosis was 23.7% and in the control group was 27.5% (p=0.004). DNA methylation in the menstrual blood was significantly higher than in the endometrium tissue (p<0.001) in both groups of subjects. (Figure 2).

mRNA Expression Level of *CXCL16*

There was a significant difference in the RNA expression in the menstrual blood of endometriosis group compared to that of the control group (p=0.030). In patients with endometriosis, there was no difference in mRNA expression in menstrual blood compared to endometrium tissue (p=0.173) (Figure 3).

Correlation between mRNA Expression Level and DNA Methylation of *CXCL16* in Endometriosis

Compared to the control group, there was no significant correlation between mRNA expression level and DNA

Table 3. Characteristics of subjects with or without endometriosis.

Variable	Endometriosis (n=18)	Control (n=17)
Age (years old), mean±SD	35.2±5.9	33.0±5.8
Body mass index, n (%)		
Obesity: ≥30 kg/m^2	2 (11%)	3 (18%)
Overweight: ≥25 kg/m^2	7 (39%)	6 (35%)
Normal weight: 18.5-24.9 kg/m^2	6 (33%)	5 (29%)
Underweight: <18.49 kg/m^2	3 (17%)	3 (18%)
Menstrual cycle (days), mean±SD	28.2±2.2	27.7±1.8
Duration of menstruation (days), mean±SD	6.8±0.9	6.6±1.5
Mean age of menarche (years), mean±SD	12.3±0.7	12.6±0.9
Family history of endometriosis, n (%)	3 (15%)	0 (0)
Smoking	1 (5%)	0 (0)
Pelvic pain (NRS)		
Severe pain (8-10)	0 (0)	17 (100%)
Mild pain (1-3)	0 (0)	0 (0)
Moderate pain (4-7)	7 (40%)	0 (0)
Severe pain (8-10)	11 (80%)	0 (0)
Dysmenorrhea, n (%)	18 (100%)	0 (0)
Dyspareunia, n (%)	9 (45%)	0 (0)
Dyschezia, n (%)	2 (10%)	0 (0)

methylation of the *CXCL16* in the menstrual blood of subjects with endometriosis (*p*=0.461), but it showed a negative correlation (r=-0.186). Likewise, the level of mRNA expression and DNA methylation in the endometrium tissue of subjects with endometriosis and the control group was similar (*p*=0.321), with negative correlation (r=-0.248) (Figure 4).

Correlation between *CXCL16* mRNA Expression Level and Pain intensity in Endometriosis

Based on Spearman's rho statistical test, analysis of the relationship between the mRNA expression level of the *CXCL16* and the intensity of pain in the menstrual blood of endometriosis subjects, there was no significant relationship (*p*=0.063) but showed a positive correlation (r=0.444). Likewise, in the endometrium tissue of endometriosis subjects, the results showed that there was no significant relationship between the mRNA expression level of the *CXCL16* and pain intensity with a value (*p*=0.083; r=0.420) (Figure 5).

Discussion

In this study, we found significant differences and showed lower methylation of the *CXCL16* in the menstrual blood of endometriosis subjects compared to the control group. The DNA methylation level of the *CXCL16* in menstrual blood was significantly different compared to endometrium in endometriosis patients. It is possible that this difference is due to differences in the phase of endometrial sampling. Menstrual blood samples are collected during the menstrual phase where estrogen and progesterone hormone levels decrease, while endometrial tissue samples are obtained during the proliferation and secretion phases.(15,16) *CXCL16* DNA methylation increases during the menstrual phase which may reduce the secretion of steroid hormones (estrogen and progesterone) for the regulation of endometrial physiological functions. Differences in the phase of the menstrual cycle when sampling which can cause increased DNA methylation in menstrual blood compared to endometrial tissue, occurred not only in samples from endometriosis patients but also in the control group. Previous studies reported that methylome changes in the female endometrium occur dynamically throughout the menstrual cycle and play a role in endometrial abnormalities through physiological changes in gene expression.(17)

Our results showed a 2.42-fold increase in mRNA expression of the *CXCL16* in the menstrual blood of endometriosis patients compared to controls. The increase in mRNA expression of *CXCL16* in menstrual blood of endometriosis patients compared to controls was

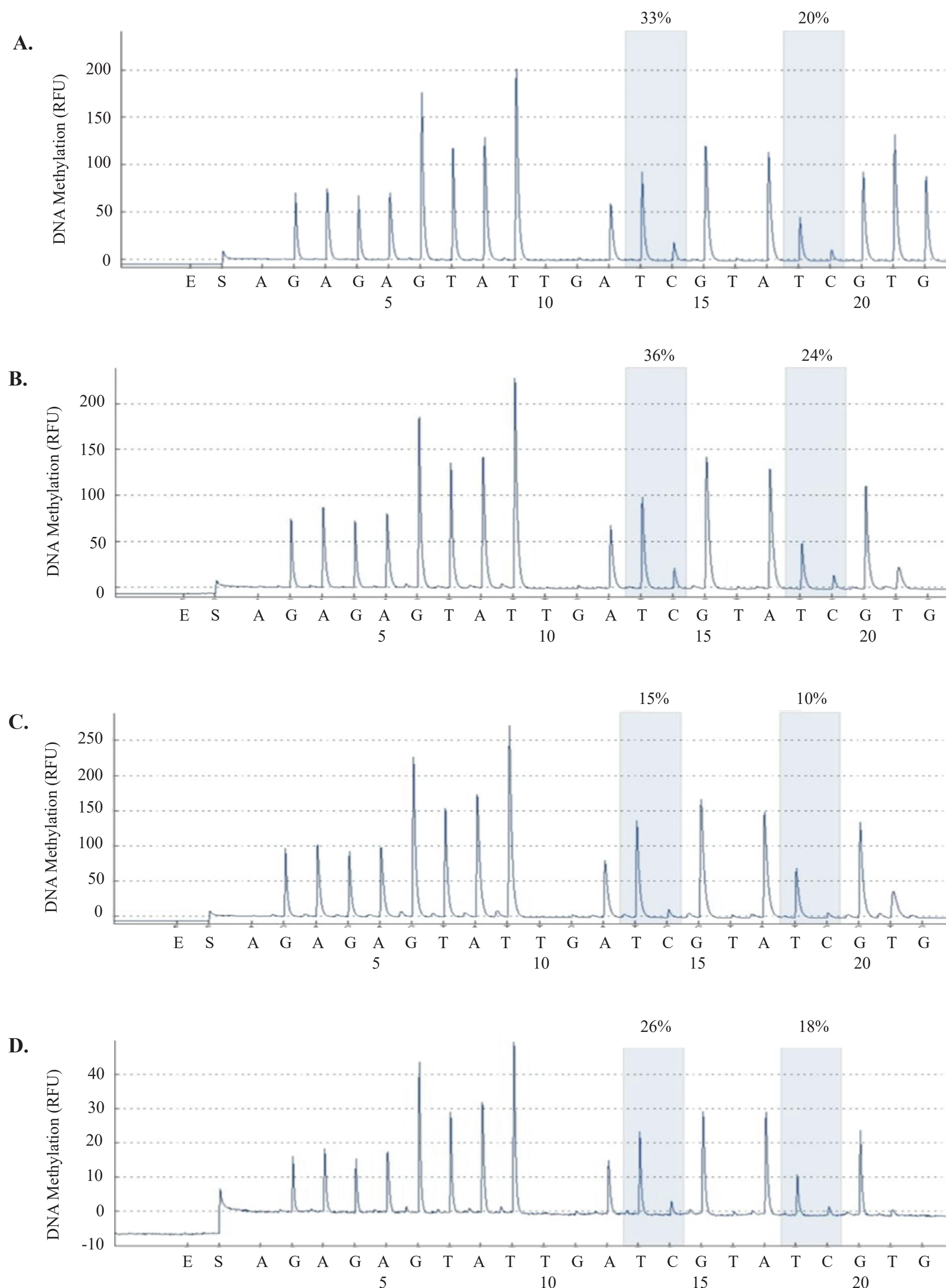

Figure 1. Representative of pyrosequencing results of the *CXCL16* in the form of pyrogram. A: Menstrual blood of an endometriosis subject; B: Menstrual blood of a subject control; C: Endometrium tissue of an endometriosis subject; D: Endometrium tissue of a subject control. RFU: relative fluorescence units.

statistically significant (p=0.030). Our study also showed a negative correlation between DNA methylation level and mRNA expression although not significantly different. According to the theory, DNA methylation contributes to the control of gene expression, where the methylation process can result in a decrease or loss of gene function (gene silencing).(18) It is known that methylation on nucleotides located in gene promoter regions can inhibit the binding of transcription factors to their specific sites on gene promoters. This prevents the transcription factor from inducing the transcription process, thus inhibiting transcription and subsequently reducing the expression of the gene's mRNA.(19) However, DNA methylation is not the only factor affecting changes in mRNA expression, the presence of other epigenetic mechanisms, including histone modifications and microRNA may play a role in gene expression.(20) These histone modifications, such as acetylation, methylation and ubiquitination, alter the interaction between DNA and histone proteins, thereby affecting transcription.(21) Research into gene promoter methylation patterns has revealed that although few methylation differences are significantly associated with changes in gene expression, some methylated genes have been identified as playing a role in the development of

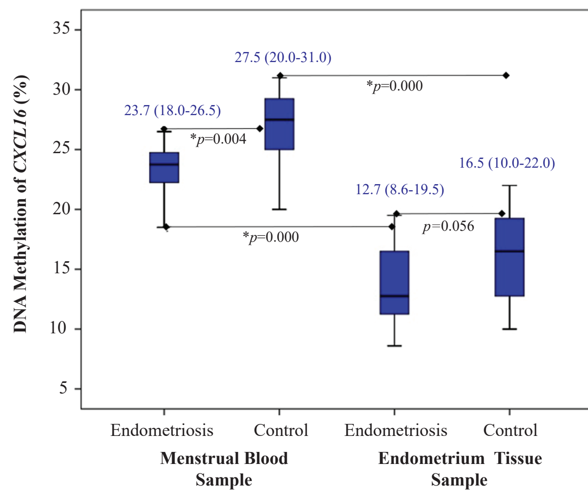

Figure 2. Differences in DNA methylation of the *CXCL16* promoter in menstrual blood and endometrium tissue of endometriosis and control subjects. *p<0.05 shows significant differences between group. n=18 (endometriosis); n=17 (controls).

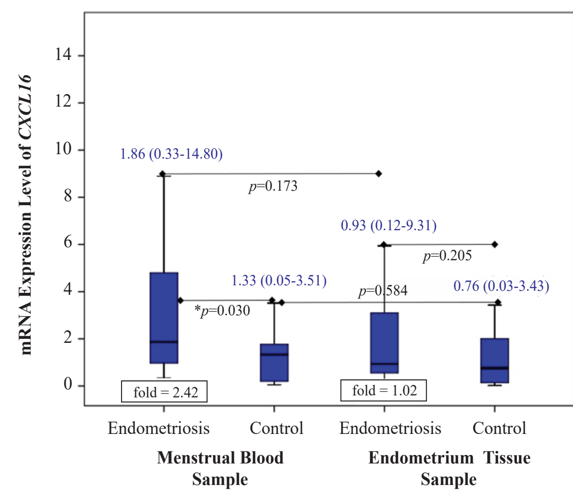

Figure 3. Differences in *CXCL16* mRNA expression in menstrual blood and endometrium tissue of endometriosis and control subjects. *p<0.05 shows significant differences between group. n=18 (endometriosis); n=17 (controls).

conditions such as endometriosis.(22) This is supported by a study that found no significant correlation between DNA methylation and mRNA expression for the *TRPA1* in menstrual blood, in line with the findings of previous studies.(23)

The results of this *CXCL16* mRNA expression study in menstrual blood and endometrium, we can confirm that there is inflammation that causes the state of endometriosis. *CXCL16* acts as a pro-inflammatory cytokine that appears as pain associated with endometriosis which is regulated by the inflammatory mediator TNF-α.(24) Endometriosis can be said to be an inflammatory disorder disease due to the process of reversing menstrual blood flow which carries endometrial cell debris into the peritoneal cavity triggering an inflammatory response resulting in local macrophage activation. The existence of this inflammatory response results in immune system abnormalities that prevent the clearance of menstrual cell debris, thereby increasing the implantation and growth of endometrial tissue in ectopic locations. Finally, endometriosis has high magrophages, because macrophages are the main source of mediators of angiogenic factors in the growth of endometriosis implants in the peritoneal area so that macrophages release interlukin and cytokines.(25,26)

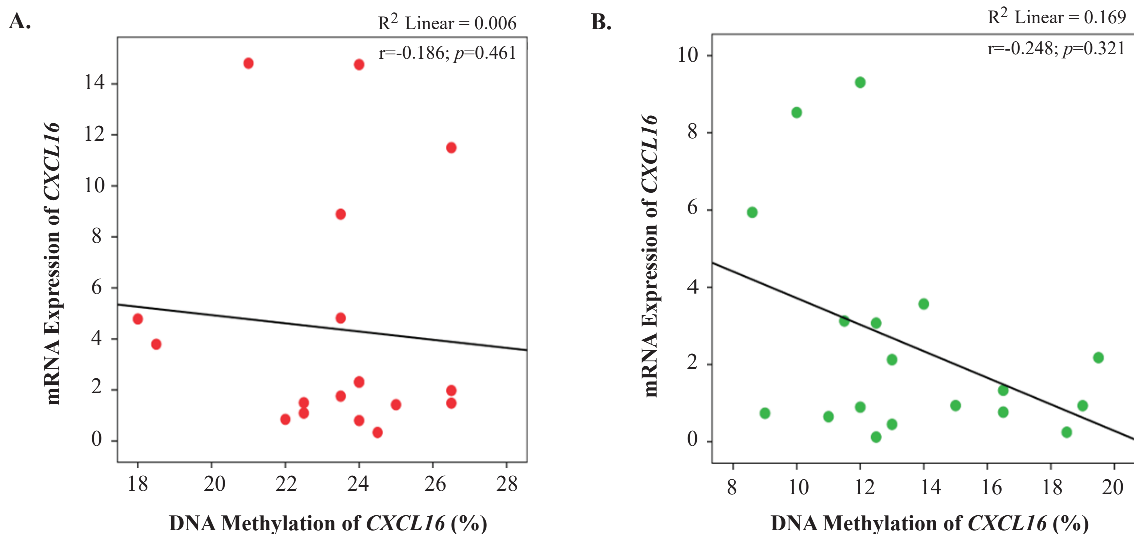

Figure 4. Correlation between mRNA expression and DNA methylation of the *CXCL16* in both samples. A: Correlation in endometriosis subjects' menstrual blood; B: Correlation in endometriosis subjects' endometrium tissue.

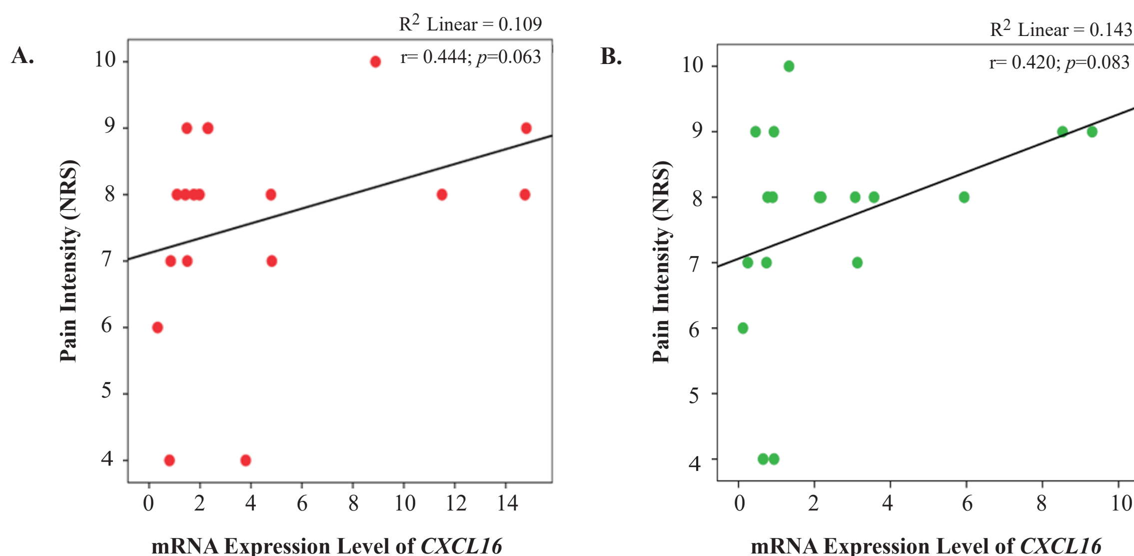

Figure 5. Correlation between the level of *CXCL16* mRNA expression and pain intensity in both samples. A: Correlation in endometriosis subjects' menstrual blood; B: Correlation in endometriosis subjects' endometrium tissue. NRS: numerical rating scale.

We found a positive correlation between *CXCL16* expression in menstrual blood and endometrium and pain intensity in endometriosis patients, although there was no significant association. It can be argued that activation of inflammatory mediators and secretion of cytokines are associated with chronic pain. According to the theory that nerve fibers surround ectopic endometrial tissue and are surrounded by new blood vessels (neuro angiogenesis), so the level of sensitivity is modulated by estradiol and cytokines. Endometriosis pain is a nociceptive pain associated with active inflammation, which secretes proinflammatory neural mediators. These mediators reach the spinal cord, which can engage neurons throughout the brain to cause and perpetuate pain in endometriosis.(27,28) The difference between our findings and previous studies may be related to the sample size used. *CXCL16* expression has been identified on epithelial and stromal cells in endometriosis.(29) TNF-α expression in endometriosis lesions was significantly increased compared to control endometrium, and *CXCL16* mRNA was also detected on stromal cells of ectopic endometrium.(30)

The limitations of our research were related to the relatively small sample size and reliance on clinical diagnoses. While the group diagnosed clinically exhibited chronic endometriosis symptoms, our control group was free from pelvic pain. The analysis of menstrual blood, which is both accessible and potentially inexpensive, is a major strength of our research. This research offers a novelty as it examined menstrual blood as a potentially cost-effective and simple method accessible to a broader population. The results of this research showed that elevated *CXCL16* expression in menstrual blood may serve as a straightforward marker for endometriosis. Therefore, we propose that mRNA expression of *CXCL16* could serve as a biomarker for the development of non-invasive diagnostic tools for endometriosis. Further investigation should be carried out involving larger patient cohorts to gain more comprehensive findings.

Conclusion

The discovery that *CXCL16* expression is higher in the menstrual blood of endometriosis patients implies that it could be used as a simple and direct marker for the disease. More study with bigger populations of patients is required to establish its efficacy and reliability as a diagnostic tool.

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Authors Contribution

All authors were involved in research planning. FE collected subjects, took measurements, performed analysis, and prepared the original draft of the manuscript. AS designed research idea, supervised the work, and interpreting the results. AH supervised the work, interpreting the results, revising, and giving final approval of the manuscript. TT assisted in interpreting the results and assisting the manuscript revision. M assisted in interpreting the results. FS assisted in interpreting the results ang giving critical suggestion to the manuscript. All authors discussed the results and commented on the manuscript.

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