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Nerve Growth Factor (NGF) Gene Analysis in Menstrual Blood of Endometriosis: DNA Methylation and mRNA Expression Levels

[1,2]Ocktariyana, [3]Nurul Hikmawati, [4]Raden Muharam, [4]Andon Hestiantoro, [5]Muhammad Luky Satria Syahbana Marwali, [5]Agus Surur As'adi and [1,6]Asmarinah

[1]Doctoral of Biomedical Sciences, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia
[2]Departement of Midwifery, Politeknik Kesehatan Kemenkes Palembang, Palembang, Indonesia
[3]Master Program in Biomedical Sciences, Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia
[4]Department of Obstetrics and Gynecology, Faculty of Medicine Universitas Indonesia, Cipto Mangunkusumo, Indonesia
[5]Department of Obstetrics and Gynecology, Fatmawati Hospital, Jakarta, Indonesia
[6]Department of Medical Biology, Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia

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Corresponding Author:
Asmarinah
Department of Medical Biology, Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia
Email: asmarinah.si@gmail.com

Abstract: Menstrual blood flow is reversed through the fallopian tubes during the menstrual process and implants outside the uterus, which is the theory of pathogenesis in endometriosis sufferers. This study aims to analyze the level of DNA methylation and mRNA expression of the Nerve Growth Factor (NGF) gene in menstrual blood in endometriosis patients and analyze the correlation. The study was conducted among 20 women in each group with and without Endometriosis (controls) of reproductive age. Filter paper pads were used to collect the menstrual blood sample, while the eutopic endometrium was collected by biopsy. Following that, NGF DNA methylation analysis of menstrual blood was performed using Methylation-Specific PCR (MS-PCR) and Pyrosequencing. Additionally, the study of NGF mRNA expression was conducted by RT-qPCR. Data analysis using independent T-test or Mann-Whitney, SPSS 22, and $p<0.05$ was considered statistically significant. We found that the DNA of NGF in the menstrual blood of endometriosis patients was hypomethylated; conversely, its mRNA expression was increased compared to that of women without Endometriosis. There was a statistically significant difference in the level of NGF DNA methylation and the relative mRNA expression level of the NGF gene in the menstrual blood of endometriosis patients compared to women without Endometriosis ($p<0.05$). However, there was no correlation between DNA methylation levels and NGF mRNA expression levels in menstrual blood samples of women with Endometriosis ($p>0,05$). Furthermore, the increased expression of NGF mRNA in the menstrual blood of women with Endometriosis is not driven by a decrease in DNA methylation. However, hypomethylation and an increase in mRNA expression of the NGF gene in menstrual blood have the potential to be a biomarker for the early diagnosis of non-invasive Endometriosis.

Keywords: Endometriosis, DNA Methylation, mRNA Expression, Menstrual Blood, Nerve Growth Factor

Introduction

Endometriosis is a gynecological disorder characterized by the growth of endometrial-like tissue in the form of glands and stroma that are located outside the uterine cavity, especially on the ovaries, pelvic peritoneum, rectovaginal septum, and are very sensitive to hormones (Superman, 2012; Machairiotis *et al*., 2013). Suparman (2012); Machairiotis *et al*. (2013) Endometriosis often occurs in women within their

reproductive age by 5-10% incidence, with complaints of dysmenorrhea 60-80%, chronic pelvic pain complaints 40-50%, infertility complaints 30-50% and irregular menstruation 10-20%. Bulletti *et al*. (2010), the pathophysiology of endometriosis pain is modulated by dysregulation of the hormone estrogen, cross-communication of inflammatory reactions between endometriotic cell neurotrophy and peritoneal fluid and hyperinnervation of primary sensory nerve fibres. This leads to the formation of nociceptive signals sent to the Central Nervous System (CNS). Nociceptive signals are integrated centrally via secondary sensory neurons in the spinal cord. Peripheral nerve sensitization involves a decrease in ion channel thresholds and an increase in nociceptor expression. During inflammation, NGF molecules secreted from macrophages then stimulate the activation of nociception receptors. Neuroimmune interaction regulated by estrogen sensitizes peripheral innervation, causing endometriosis pain (Kobayashi *et al*., 2014).

Endometriosis is a multifactorial disease caused by the interaction of genetic factors, steroid hormones, the immune system and the environment. Kobayashi *et al*. (2014) Per sophisticated pathogenesis aetiology, it is generally accepted to include retrograde menstruation, coelomic metaplasia, stem cells, disease induction through inflammation, oxidative stress and immune system dysfunction and dissemination through the vascular and lymphatic systems (Giudice *et al*., 2012; Gupta *et al*., 2015).

Bulun *et al*. (2015) emphasized that ectopic endometrial stromal cells of endometriosis women exhibited the alteration of characteristics and function compared to normal female eutopic endometrial cells. Epigenetic changes in CpG methylation affect several genes' functions that contribute to hormonal and immune factors (Giudice *et al*., 2012; Monsivais *et al*., 2015).

Epigenetics, as a regulator of gene expression, can alter malignancy-related characteristics, such as growth, migration, invasion and angiogenesis. Epigenetic changes in Endometriosis involve changes in the levels of DNA methylation of gene promoters, which include DNA methylation, histone modification and micro-RNA (miRNA). It is also known that the pathogenesis of Endometriosis is implied by Steroidogenic Factor 1 (SF-1) and aromatase by regulating an increase in DNA methylation (hypermethylation) at the progesterone-B (PR-B), HOXA-10 and E-cadherin receptors and a decrease in the level of DNA methylation (hypomethylation) of the estrogen-receptor (Koukoura *et al*., 2016; Nasu *et al*., 2011).

Altered methylation levels may lead to immunological and inflammatory disorders. One clear example in the research of Ocktariyana *et al*. (2019) was reported the DNA methylation status of the P2RX3 nociceptive receptor gene promoter in endometriosis samples, 100% unmethylated band pattern existed on the gene promoter in Endometriosis peritoneal tissue and correlated with pain severity in endometriosis women (Ocktariyana *et al*., 2019). Apart from this, Tokushige *et al*. (2006) and Morotti *et al*. (2014) suggested that the density of unmyelinated C-type nerve fibres was found in endometriotic lesions as well as in the endometrium of women with Endometriosis and correlated with pain severity and endometriosis stage (Tokushige *et al*., 2006; Tokushige *et al*., 2006a-b).

Owing to the activation of nociceptive receptors at the end of primary sensory nerve fibres, releasing Nerve Growth Factor (NGF) neurotrophin mediator by macrophages leads to nerve fibres' survival, development and function (Tokushige *et al*., 2006b). Nerve Growth Factor (NGF) is released from macrophage cells and forms a complex with the high-affinity TrkA receptor on neuronal cells, which is redistributed to somatic neuron cells (Morotti *et al*., 2014).

Peng *et al*. (2018) investigated the role of NGF with its receptor (TrkA/p75NTR) in endometriosis patients with dyspareunia in the posterior pelvis (cul-de-sac/uterosacral). Endometriosis was confirmed by endovaginal palpation and ultrasound. The results of this study prove that there is a higher immunointensity reaction in NGF with TrkA receptors in stromal cells and endometrial epithelial cells in endometriosis women with deep dyspareunia compared to women without dyspareunia. However, at the p75NTR receptor, the same reaction did not occur. According to Peng, immunoreactivity in nerve growth factors in stromal cells is significantly related to nerve fibre density and pain intensity of dyspareunia. In endometrial stromal cell culture, NGF was also reported to be significantly correlated with the increase in PTGS-2/COX-2-2 mRNA and PGE2 secretion. This correlation could be mediated by high nerve fibre density and COX-2/PGE2 stimulation via the Trk receptor. The NGF/Trk signalling pathway may play a role in endometriosis pain, particularly in dyspareunia (Peng *et al*., 2018).

Mu *et al*. (2020) analyzed the relationship between pain levels in patients with deep infiltrating Endometriosis (DIE) and the expression of Nerve Growth Factor (NGF) through Magnetic Resonance Imaging (MRI). The results found that in patients with deep infiltrating Endometriosis (DIE) in uterine fibula ligaments, vagina, uterine rectum, rectum and ureters correlate to pain. Increased expression of NGF with its receptors manifests as a pain signal. This increase in expression is essential in the diagnostic and follow-up methods after endometriosis surgery (Mu *et al*., 2020).

Research Out Smart Endometriosis (ROSE) developed a method for diagnosing Endometriosis through menstrual blood. Early diagnosis through

menstrual blood will help in the treatment at an early stage in preventing the spread of endometrial lesions to other organs and preventing unnecessary hysterectomy. Menstrual blood as DNA samples is used as an initial study to identify diagnostic biomarkers that are effective and efficient in terms of cost and treatment time (MacDonald, 2020).

Menstrual blood is a complex biological fluid consisting of blood, vaginal secretions, immune cells, and endometrial cells from the uterine wall. Menstrual blood was selected as a tissue sample in this study to be a preliminary study to identify biomarkers in the development of early diagnostics. Menstrual blood is also used to identify and evaluate biomarkers through spectrometric analysis techniques. Yang *et al*. (2012) state that many proteins are expressed in the shedding of endometrial cells during menstruation. Proteins that prepare for embryo implantation include integrins, Matrix Metallo Proteinases (MMPs), Galectins (GAL), Glucose-Regulating Proteins (GLUT), Interleukins (ILs), proteolytic enzymes, cytokines, apoptotic regulatory proteins, several proteins from various immune cells, which is an integral part of menstruation. This protein in menstrual blood can be a non-invasive biomarker in diagnosing endometrial pathology and infertility (Yang *et al*., 2012).

Materials and Methods

Samples Collection

In this study, samples were taken from research subjects willing to participate by signing an informed consent form at Fatmawati Hospital and Dr Cipto Mangunkusumo Hospital, RSUPN, Jakarta. Menstrual blood samples were collected from 20 women with Endometriosis and 20 without Endometriosis.

The inclusion criteria for endometriosis cases were married infertile women aged 20-45 years. Exclusion criteria included pregnant women, unmarried women, women with endometrial cancer, ovarian cancer, and endometritis. Subjects without Endometriosis were elected as controls, as well as infertile married women between 20-45 years old who underwent endometrial micro curettage examination at Dr. Cipto Mangunkusumo, Indonesian Centre Hospital, Jakarta, and without indication of Endometriosis.

DNA Methylation and mRNA Expression were Measured

Menstrual blood samples were collected using sanitary filter paper modified with sanitary napkins. Menstrual blood is taken on the second to third day of menstruation. If the filter paper has been collected, dried, cut into small pieces (6 mm in diameter), and weighed 50 mg for nucleic acid extraction.

DNA isolation from menstrual blood samples was performed using the QIAamp® DNA Mini Kit (Qiagen) following a dried blood spot DNA purification protocol. DNA was also extracted from 20-25 mg of eutopic endometrial tissue using the gSYNCTM DNA Extraction Kit (Geneaid, Taiwan). The resulting DNA extraction was then converted into a bisulfite solution using the Epitect Bisulfite Kit from Qiagen, Germany, to examine DNA methylation levels using MSP and pyrosequencing.

The Methylation-Specific PCR (MSP) method. We used specially designed primers from MethPrimer as follows: methylated specific primers (M)(F) 5'-cgtttcgaagaaaaggagtagtc-3' and (R) 5'-aaccgactaactaaaactaaacgaa-3', while unmethylated specific primers (U) (F) 5'- ggtgttttgaagaaaaggagtagtt-3' and (R) 5'-aaccaactaactaaaactaaacaaa-3'. The MSP results were subsequently electrophorized using 2.4% agarose gel with 100 volts for 45 minutes and analyzed using ImageJ software to measure the percentage of band intensity.

Meanwhile, DNA methylation level was measured by pyrosequencing, and we only used 10 menstrual blood samples for endometriosis and non-endometriosis. The primer design software PyroMark Assay Design 2.0. Three CpG sites in the NGF gene promoter were analyzed. Briefly, each PCR mixture contained My Taq HS Red Mix, 2' (12.5 µL), 1 µL forward and reverse primers (10 pmol/L each PCR primer), 8.5 µL ddH_2O and 2 µL bisulfite-converted template DNA in a total volume of 25 L. PCR conditions were as follows: Initial denaturation at 95°C for 5 min; 45 cycles at 95°C for 30 sec, 59°C for 30 sec and 72°C for 30 sec; and final extension at 72°C for 3 min resulted in an amplicon length of 150 bp.

The PCR products were visualized with 1% agarose gel electrophoresis. If the results of the electrophoresis band show a single solid band and no primary dimer, in that case, the pyrosequencing reaction can be continued by making a mixture for Plate 1 with the composition: 37 µL Binding Buffer, 3 µL Streptavidin sepharose beads, 25 µL ddH_2O and 15 µL PCR product. In Pyrosequencing Vacuum Preparation Tool (Qiagen, Germany), as much as 15 µL of biotinylated PCR product was immobilized on streptavidin sepharose beads. Then, sepharose beads were purified, rinsed, denatured using 0.2 M NaOH and washed again. The final concentration of 0.3 mM pyrosequencing primer was annealed to single-strand PCR products, and pyromark Q96 2.5.7 software was used (Qiagen, Germany). The pyrosequencing reaction will run automatically.

Total RNA from menstrual blood samples was extracted using the Quick-RNA Miniprep Plus Kit, Zymo. 50 ng of RNA was reverse transcribed into cDNA using Ace qPCR ReverTra RT Master Mix with gDNA Remover from Toyobo. The cDNA samples were then

amplified using Thunderbird SYBR qPCR Mix with primer pairs for the NGF gene. The GAPDH gene was used as a reference gene in this study. Analysis of the relative mRNA expression of the NGF gene using the Livak method (2-ΔΔCt), which was carried out by comparing the relative difference between the Cyclic threshold (Ct) of menstrual blood in women with Endometriosis and without Endometriosis.

Statistical Analyses

Data analysis using SPSS software with the Shapiro-Wilks test to determine the normality of the data distribution. DNA methylation and mRNA expression levels were analyzed using independent T-test or Mann-Whitney. Pearson or Spearman's tests were performed to see the correlation between DNA methylation and mRNA expression. The p-value is considered significant ($p<0.05$).

Results

Characteristic of Participants

The characteristics of women in this study included data on age, body mass index, disease history, pain symptoms that commonly occur in Endometriosis, and other questions related to the criteria in this study. Table (1) summarizes the characteristics of the research subjects from 20 women with Endometriosis and without Endometriosis. The endometriosis group found an average age of 37 years old. Meanwhile, the group without Endometriosis was 34 years old. Body Mass Index (BMI) of women is dominated by women with normal or ideal BMI, with a percentage of 45% of women with Endometriosis and 40% without Endometriosis. In addition, the menstrual cycles in the group of women with Endometriosis were within an average of 28.7±3.802 days with a duration of 7.5±1.02 days and menarche age at 12.8±1.24 years.

The DNA Methylation Level of Human NGF Gene Analyses Used MS-PCR

The result of electrophoresis visualization of MSP product samples of menstrual blood and eutopic endometriosis endometrium compared to a group of normal women as controls is shown in Fig. (1). From this figure, the NGF gene showed that partial methylation occurred or that some bands were methylated and some were unmethylated. The size of the NGF gene amplicon is 150 bp. This study also used a methylated positive control (K+) and an unmethylated negative control (K-) from Epitech Methylated Human with an amplicon size of 200 bp.

Table 1: Characteristics of participants

Variable	Endometriosis N = 20	Without endometriosis N = 20
Age, mean ± SD	37,05±6,41	34,55±5,48
Body Mass Index, (%)		
1. Skinny (<18.5)	5%	10%
2. Normal (18,5-22,9)	45%	40%
3. Overweight (23-24,9)	30%	20%
4. Obesity (>25)	20%	30%
Menstrual Cycle (days)	28,7±3,80	27,7±1,82
Menstruation Length (days)	7,5±1,02	6,9±0,31
Average age at menarche (years)	12,8±1,24	12,4±1,31

Fig. 1: Electrophoresis visualization results of the MSP product of the NGF gene. K+ = methylation positive control; K- = unmethylated negative control; NTC = Non-Template Control. M = Methylation; U = Unmethylation

Figure (2) is the result of a quantitative analysis of NGF gene gel electrophoresis bands. Based on measuring the surface area of the band using ImageJ software, the NGF gene promoter from the endometriosis group was 52.05% in menstrual blood and 50.85% in eutopic endometrium. Meanwhile, in the ordinary women without endometriosis group, the average DNA methylation percentage was 51.35% in menstrual blood and 51.2% in eutopic endometrium. The statistical analysis results of the Shapiro-Wilk normality test showed that the data was not normally distributed even though transformation had been carried out. So, the non-parametric Mann-Whitney test was carried out with results ($p = 0.262$) for the endometriosis menstrual blood group compared to non-endometriosis. Likewise, the results of the statistical analysis of endometriotic menstrual blood compared with eutopic endometrium have a value of $p = 0.095$ ($p>0.05$). This indicates no significant difference in the level of DNA methylation of the NGF gene promoter in either menstrual blood or eutopic endometrial tissue of endometriotic and non-endometriotic women using the MS-PCR method.

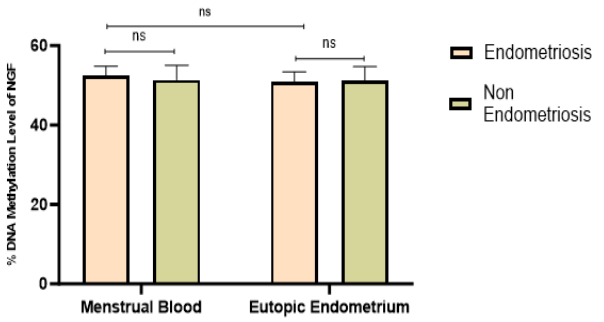


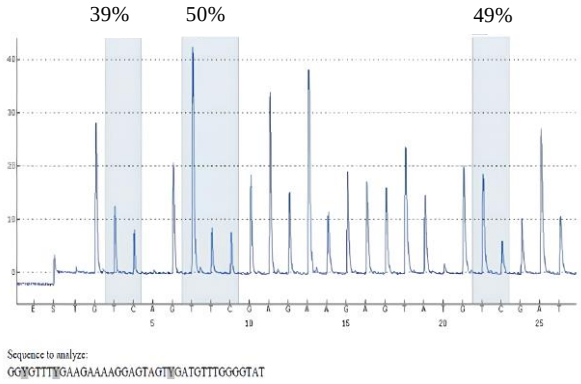
Fig. 2: Differences in DNA methylation of the NGF gene promoter in menstrual blood and eutopic Endometriosis compared to a group of women without Endometriosis using the MSP method (ns = p>0.05)

The DNA Methylation Level of Human NGF Gene Analyses Used Pyrosequencing

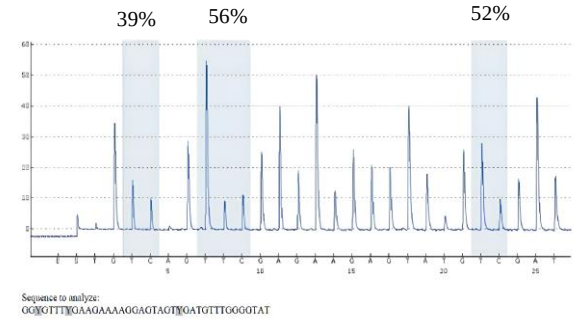
Measurement of DNA methylation of the NGF gene promoter was continued using the quantitative Pyrosequencing method. This method is widely used in research exploration, toxicological testing and pharmacological approaches. PyroMark CpG Assays (Qiagen, Germany; EpigenDX USA) is a software widely used to analyze DNA methylation of a gene based on specific CpG islands.

The methylation of the NGF gene from endometriosis patients and in women without Endometriosis that PyrogramTM showed is depicted in Fig. (3). The mean percentage of NGF gene DNA methylation levels at CpG sites 1-3 from 10 endometriosis menstrual blood samples were 36.8, 42.4 and 38.7%, respectively, with the mean value of CpG sites of NGF gene in the endometriosis group being 39.3%. Meanwhile, from 10 menstrual blood of women without Endometriosis, the average percentage of DNA methylation levels at CpG 1-3 locations was 38.6, 55.2 and 50.6%, respectively, with an average CpG location in the control group of 48,1% Fig. (4).

The results of statistical analysis of the Shapiro-Wilk normality test show that the data is normally distributed. So, an independent parametric T-test was carried out with significant results (p = 0.332; p = 0.009; p = 0.010) for each CpG location, and the average of the 3 CpG locations in the endometriosis group had a significance value of p = 0.025 (p<0.05). There was a significant difference between DNA methylation of the NGF gene in the menstrual blood of the endometriosis group compared to the group without Endometriosis. DNA methylation of the NGF gene in menstrual blood with Endometriosis was lower than in women without Endometriosis. This finding was reported for the first time that DNA methylation of the NGF gene in menstrual blood samples of subjects with Endometriosis was hypomethylated compared to those without Endometriosis (p<0.05).



(a) PyrogramTM of a menstrual blood sample of case



(b) PyrogramTM of a menstrual blood sample of control

Fig. 3: The DNA Methylation of human NGF gene analyses in menstrual blood of Endometriosis and without endometriosis, used by Pyrosequencing

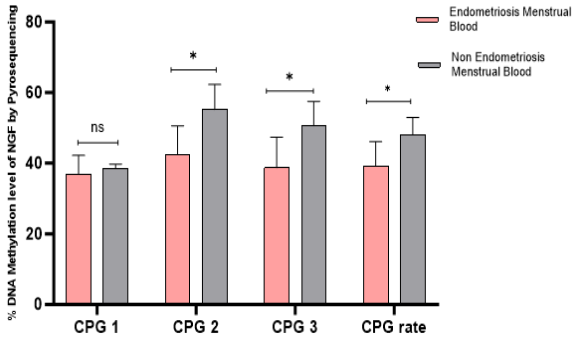


Fig. 4: The differences in DNA methylation levels at 3 CpG sites of the NGF gene by Pyrosequencing and the average methylation levels of the three CpG sites in menstrual blood samples of endometriosis women compared to a group without Endometriosis. (ns = p>0.05; *p<0.05)

The mRNA Expression Level of the Human NGF Gene in Endometriosis

The differences in mRNA expression levels of the Nerve Growth Factor (NGF) gene as a growth factor for nerve fibres in the menstrual blood of endometriosis patients and women without Endometriosis were measured using the quantitative real-time PCR method. The quantitative result is an amplification curve that shows the Cycle threshold (Ct) value. Owing to the Cycle Ct value, the number of cycles captured by the fluorescent substance was subsequently analyzed using the Livak method to determine the relative mRNA expression value of the NGF gene.

Based on the analysis of the Livak method, the average value of the relative expression of NGF gene mRNA increased 4.29 times in the menstrual blood of women with Endometriosis compared to women without Endometriosis. The results of the Mann-Whitney test statistical analysis showed that there was a significant difference ($p = 0.021$) (Fig. 5).

Correlation between DNA Methylation Level and mRNA Expressions of NGF Gene in Menstrual Blood of Endometriosis Patients

Owing to Spearman's rho statistical correlation analysis for DNA methylation levels and relative expression of NGF mRNA in menstrual blood samples in the endometriosis group, a negative and no significant correlation was found ($r = -0.073$ and $p = 0.841$).

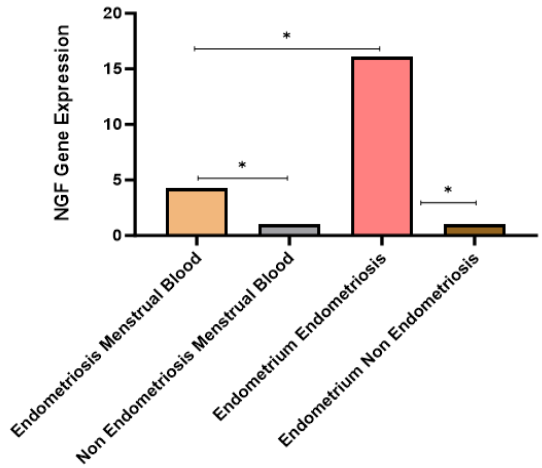


Fig. 5: The differences in the relative expression levels of NGF gene mRNA in menstrual blood of women with Endometriosis compared to menstrual blood without endometriosis *p<0.05, there is a significant difference in the relative expression level of NGF gene mRNA in menstrual blood

Discussion

Endometriosis is considered a form of malignancy in gynaecology. In which the development is often associated with genetic and epigenetic alteration. It is well known that DNA methylation in Endometriosis can drive the expression changes of genes involved in Estrogen Receptor (ER) and Progesterone Receptor (PR) signalling pathways. Alterations in these signalling pathways result in molecular changes in the endometriosis microenvironment, such as the increased activity of NF-B, PGE2, E2 and ER-β, leading to inflammation, local dysregulation of hormonal pathways and pain sensitization (Monsivais *et al*., 2015).

This study reports that women with Endometriosis have an average age of 37.05±6.41 with an age range between 29-45 years as the reproductive age (premenopause). At the same time, the Body Mass Index (BMI) patients with Endometriosis exhibited BMI differences such as a normal BMI of 45, 30 overweight and 20% obese. Although studies of endometriosis populations in Asia are still minimal, research by Liu and Zhang (2017) reported that the epidemiological factors of body mass index in East Asian women influence the severity of Endometriosis. Women with a higher BMI tend to be prone to early or mild symptoms of Endometriosis. At the same time, other factors are influential, such as genetics, menstrual and environmental factors (Liu and Zhang, 2017; Yan *et al*., 2017). Darrow also investigated the increased risk of Endometriosis by emphasizing six days of longer menstrual flow by enlisting specific factors: Heavy flow, severe cramps, and those aged more than 30 years have a susceptibility to Endometriosis (Darrow *et al*., 1993). Abnormal menstrual bleeding is the basis for markers of endometrial pathology (Yang *et al*., 2012).

Endometriosis-associated pain with dyspareunia has been reported by Peng *et al*. (2018), which is stimulated by the nerve fibre growth factor (NGF) signalling pathway with the TrkA receptor. High levels of NGF increase the secretion of cyclooxygenase-2 (COX-2) and prostaglandins (PGE2) (Peng *et al*., 2018). The same correlation results were reported by Mu *et al*. (2020); it is known that patients with Deep Infiltrating Endometriosis (DIE) in the uterine fibular ligament, vagina, uterine rectum, rectum, ureter are closely related to pain during menstruation. This pain signal is modulated by elevated levels of expression of the NGF protein with its receptor (Mu *et al*., 2020). NGF has been associated with several different persistent pain conditions, including Osteoarthritis (OA), low back pain, diabetic peripheral neuropathy, bladder pain syndrome, bone cancer pain and Endometriosis (Kelleher *et al*., 2017).

Ocktariyana (2019) also reported the simultaneous increase in NGF mRNA expression and nociceptive P2RX3 receptor in eutopic endometrial tissue of women with Endometriosis that is correlated with the intensity of pain severity and the increase in the incidence of Endometriosis. The higher the expression of NGF mRNA and P2RX3 receptors, the higher the intensity of pain felt by endometriosis subjects (Ocktariyana, 2019). Owing to the findings, the researchers aimed to prove the expression of NGF mRNA and DNA level methylation in the menstrual blood of patients with Endometriosis. Menstrual blood is considered a reliable sample DNA source for exploring and investigating endometrial cells. Menstrual blood was selected as a sample for a preliminary study to identify biomarkers in the development of early diagnostics. Research using menstrual blood has also been carried out by Madjid *et al*. (2020) in detecting matrix metalloproteinase (MMP-9) protein and inhibitors of metalloproteinase-1 (TIMP-1) in women with Endometriosis and evaluated by immunohistochemistry. As a result, MMP-9 expression was more robust, and TIMP-1 expression was lower in women with endometriosis than in the control group, which had statistically significant results (Madjid *et al*., 2020).

Gene expression is the consecutive transformation of genetic information of a gene into functional ones. Messenger RNA (mRNA) is a product of gene expression at the transcriptional level, while protein is a product of gene expression at the translational level. Analysis of gene expression can be detected at the mRNA and protein levels. Dysregulation of gene expression plays a crucial role in disease development. Defining the characteristics of each gene is the first step toward developing new therapeutic strategies (Ping *et al*., 2016). Our study has shown overexpression of NGF mRNA in menstrual blood patients with Endometriosis. This finding is important for the advancement of the detection of Endometriosis (Ping *et al*., 2016).

Manconi *et al*. (2018) proved an increase in NGF levels in endometrial lesions and intense immunoreactivity in endometriotic glands, which was assumed to stimulate neurogenesis (Manconi *et al*., 2018). Barcena de Arellano *et al*. (2011) also reported that the overexpression of NGF in peritoneal fluids of endometriosis patients was analyzed using Western methods, such as blot and cell culture staining (Barcena de Arellano *et al*., 2011). High levels of NGF were able to promote the expression of nociceptors and neurogenesis of sensory neurons, which influence pain by nociceptive mechanisms (Manconi *et al*., 2018). The mRNA expression of the NGF gene in eutopic endometrium has been reported to exhibit a positive correlation with increased expression by the nociceptor P2RX3 (Ocktariyana, 2019).

Alterations in gene expression are often associated with epigenetic changes, such as DNA methylation in Endometriosis, which is often associated with pathological conditions. Hypermethylation and hypomethylation in the promoter region of the CpG islands result in aberration of gene expression. Hypermethylation results in decreased expression, and hypomethylation results in increased expression (Monsivais *et al*., 2015).

Naqvi *et al*. (2014) stated that a certain amount of DNA methylation is unknown to affect gene expression. Subsequently, it is correlated to several additional regulatory factors and the study extension of cells and tissues. The correlation analysis of DNA methylation and gene expression varies according to the corresponding region of the genome (Liang *et al*., 2018; Naqvi *et al*., 2014).

Borghese *et al*. (2017) stated that the mechanism of epigenetic alteration does not only act on each mechanism in regulating transcription of gene expression. But also their frequent interaction with each other as intra-epigenetic. For example, DNA methylation and histone modification can increase expression (Borghese *et al*., 2017; Yan *et al*., 2015).

The results of pyrosequencing method analysis, DNA methylation of the NGF gene showed a significant difference with a decrease in DNA methylation or hypomethylation in menstrual blood in the group of women with Endometriosis compared to the group without Endometriosis as control. Yuan *et al*. (2020) also investigated the role of NGF in chronic inflammatory pain, which proved the existence of hypomethylation of CpG islands in the NGF gene promoter by administering the Freund Adjuvant Complex (CFA). CFA not only causes hypomethylation but also induces upregulation of NGF mRNA, as well as dorsal root ganglion protein levels in mice (Yuan *et al*., 2020).

Based on this research, an increase in NGF gene mRNA expression also occurred in both menstrual blood and eutopic endometrial tissue of Endometriosis compared to controls. Meanwhile, the results of the correlation test between DNA methylation and mRNA expression of the NGF gene did not show a significant relationship ($p>0.05$). The results of this study cannot prove that changes in hypomethylation of the NGF gene in menstrual blood affect the increase in its mRNA expression. However, hypomethylation and an increase in NGF gene mRNA expression in endometriotic menstrual blood were shown to be much higher than in controls. Hence, we propose that NGF hypomethylation and mRNA expression can be used as non-invasive diagnostic biomarkers for early detection of Endometriosis.

The limitation of this research is that it uses a small sample size. This research is a pilot study to identify biomarkers in menstrual blood that represent endometrial tissue in reproductive women. In addition, it is also difficult to obtain eutopic endometrial tissue in the same phase. So, proper protocols are required for sampling.

Conclusion

The increased mRNA expression of the NGF gene in menstrual blood in women with Endometriosis compared to menstrual blood in women without Endometriosis was not due to decreased DNA methylation. However, hypomethylation of the NGF gene has the potential to be a biomarker for the early diagnosis of non-invasive Endometriosis.

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Author's Contributions

Ocktariyana: Collected subjects, performed analysis, and edited manuscript revision.

Nurul Hikmawati: Collected subjects, took measurements, and prepared original draft of the manuscript.

Raden Muharam and Andon Hestiantoro: Supervised work, collected samples, interpreted the results.

Muhammad Luky Satria Syahbana Marwali and Agus Surur As'adi: Supervised work collected samples.

Asmarinah: Idea, design study, revised the manuscript, and gave the final approval.

Ethics

The Ethics Committee of the Faculty of Medicine, University of Indonesia, has approved the study with the number 0126/UN2.F1/ETIK/2018 and addendum number KET-284/UN2.F1/ETIK/PPM.00.02/2020, and the patient signed the consent for analysis.

Reference

Barcena de Arellano, M. L., Arnold, J., Vercellino, F., Chiantera, V., Schneider, A., & Mechsner, S. (2011). Overexpression of nerve growth factor in peritoneal fluid from women with Endometriosis may promote neurite outgrowth in endometriotic lesions. *Fertility and Sterility*, *95*(3), 1123–1126. https://doi.org/10.1016/j.fertnstert.2010.10.023

Borghese, B., Zondervan, K. T., Abrao, M. S., Chapron, C., & Vaiman, D. (2017). Recent insights on the genetics and epigenetics of Endometriosis. *Clinical Genetics*, *91*(2), 254–264. https://doi.org/10.1111/cge.12897

Bulletti, C., Coccia, M. E., Battistoni, S., & Borini, A. (2010). Endometriosis and infertility. *Journal of Assisted Reproduction and Genetics*, *27*(8), 441–447. https://doi.org/10.1007/s10815-010-9436-1

Darrow, S. L., Vena, J. E., Batt, R. E., Zielezny, M. A., Michalek, A. M., & Selman, S. (1993). Menstrual Cycle Characteristics and the Risk of Endometriosis. *Epidemiology*, *4*(2), 135–142. https://doi.org/10.1097/00001648-199303000-00009

Giudice, L. C., Evers, J. L. H., & Healy, D. L. (2012). *Endometriosis: Science and Practice*.

Gupta, S., Harlev, A., Agarwal, A., & Pandithurai, E. (2015). Theories on Endometriosis. *Endometriosis: A Comprehensive Update*, 17–21. https://doi.org/10.1007/978-3-319-18308-4_3

Kelleher, J. H., Tewari, D., & McMahon, S. B. (2017). Neurotrophic factors and their inhibitors in chronic pain treatment. *Neurobiology of Disease*, *97*, 127–138. https://doi.org/10.1016/j.nbd.2016.03.025

Kobayashi, H., Yamada, Y., Morioka, S., Niiro, E., Shigemitsu, A., & Ito, F. (2014). Mechanism of pain generation for endometriosis-associated pelvic pain. *Archives of Gynecology and Obstetrics*, *289*(1), 13–21. https://doi.org/10.1007/s00404-013-3049-8

Koukoura, O., Sifakis, S., & Spandidos, D. A. (2016). DNA methylation in Endometriosis (Review). *Molecular Medicine Reports*, *13*(4), 2939–2948. https://doi.org/10.3892/mmr.2016.4925

Liang, Y., Xie, H., Wu, J., Liu, D., & Yao, S. (2018). Villainous role of estrogen in macrophage-nerve interaction in Endometriosis. *Reproductive Biology and Endocrinology*, *16*(1), 122. https://doi.org/10.1186/s12958-018-0441-z

Liu, Y., & Zhang, W. (2017). Association between body mass index and endometriosis risk: a meta-analysis. *Oncotarget*, *8*(29), 46928–46936. https://doi.org/10.18632/oncotarget.14916

MacDonald, A. (2020). Diagnosing Endometriosis Using Menstrual Blood. *Diagnostics from Technology Network*.

Machairiotis, N., Stylianaki, A., Dryllis, G., Zarogoulidis, P., Kouroutou, P., Tsiamis, N., Katsikogiannis, N., Sarika, E., Courcoutsakis, N., Tsiouda, T., Gschwendtner, A., Zarogoulidis, K., Sakkas, L., Baliaka, A., & Machairiotis, C. (2013). Extrapelvic Endometriosis: a rare entity or an under diagnosed condition? *Diagnostic Pathology*, *8*(1), 194. https://doi.org/10.1186/1746-1596-8-194

Madjid, T. H., Ardiansyah, D. F., Permadi, W., & Hernowo, B. (2020). Expression of Matrix Metalloproteinase-9 and Tissue Inhibitor of Metalloproteinase-1 in Endometriosis Menstrual Blood. *Diagnostics*, *10*(6), 364. https://doi.org/10.3390/diagnostics10060364

Manconi, F., Fazleabas, A. T., Markham, R., & Fraser, I. S. (2018). Nerve fibre infiltration and expression in peritoneal lesions of Endometriosis in a nonhuman primate model of Endometriosis. *Journal of Endometriosis and Pelvic Pain Disorders*, *10*(4), 198–207. https://doi.org/10.1177/2284026518810594

Monsivais, D., Kakinuma, T., Furukawa, Y., Bernardi, L., Pavone, M., Dyson, M., & Bulun, S. (2015). Molecular Biology of Endometriosis: From Aromatase to Genomic Abnormalities. *Seminars in Reproductive Medicine*, *33*(03), 220–224. https://doi.org/10.1055/s-0035-1554053

Morotti, M., Vincent, K., Brawn, J., Zondervan, K. T., & Becker, C. M. (2014). Peripheral changes in endometriosis-associated pain. *Human Reproduction Update*, *20*(5), 717–736. https://doi.org/10.1093/humupd/dmu021

Mu, L., Wang, M., & Yu, Y. (2020). Correlation Between Pain and Nerve Growth Factor Receptor Expression in Patients with Endometriosis Diagnosed by Transvaginal Color Ultrasound and Magnetic Resonance. *World Neurosurgery*, *138*, 629–636. https://doi.org/10.1016/j.wneu.2020.01.088

Naqvi, H., Ilagan, Y., Krikun, G., & Taylor, H. S. (2014). Altered Genome-Wide Methylation in Endometriosis. *Reproductive Sciences*, *21*(10), 1237–1243. https://doi.org/10.1177/1933719114532841

Nasu, K., Kawano, Y., Tsukamoto, Y., Takano, M., Takai, N., Li, H., Furukawa, Y., Abe, W., Moriyama, M., & Narahara, H. (2011). Aberrant DNA methylation status of Endometriosis: Epigenetics as the pathogenesis, biomarker and therapeutic target. *Journal of Obstetrics and Gynaecology Research*, *37*(7), 683–695. https://doi.org/10.1111/j.1447-0756.2011.01663.x

Ocktariyana, Hestiantoro, A., Rahmala, R., & Asmarinah. (2019). DNA methylation of P2X3 receptor gene encoded pain marker protein in Endometriosis. *Journal of Physics: Conference Series*, *1246*(1), 012031. https://doi.org/10.1088/1742-6596/1246/1/012031

Ocktariyana. (2019). *Analisis Genetik dan Epigenetik Gen Pengkode Faktor Nyeri NGF, Receptor TRPA1, Receptor P2RX3 pada Endometriosis*.

Peng, B., Zhan, H., Alotaibi, F., Alkusayer, G. M., Bedaiwy, M. A., & Yong, P. J. (2018). Nerve Growth Factor Is Associated With Sexual Pain in Women With Endometriosis. *Reproductive Sciences*, *25*(4), 540–549. https://doi.org/10.1177/1933719117716778

Ping, S., Ma, C., Liu, P., Yang, L., Yang, X., Wu, Q., Zhao, X., & Gong, B. (2016). Molecular mechanisms underlying endometriosis pathogenesis revealed by bioinformatics analysis of microarray data. *Archives of Gynecology and Obstetrics*, *293*(4), 797–804. https://doi.org/10.1007/s00404-015-3875-y

Suparman, E. (2012). Penatalaksanaan endometriosis. *Jurnal Biomedik: JBM*, *4*(2), 69–78. https://doi.org/10.35790/jbm.4.2.2012.754

Tokushige, N., Markham, R., Russell, P., & Fraser, I. S. (2006). High density of small nerve fibres in the functional layer of the endometrium in women with Endometriosis. *Human Reproduction*, *21*(3), 782–787. https://doi.org/10.1093/humrep/dei368

Tokushige, N., Markham, R., Russell, P., & Fraser, I. S. (2006). Nerve fibres in peritoneal Endometriosis. *Human Reproduction*, *21*(11), 3001–3007. https://doi.org/10.1093/humrep/del260

Yan, B., Li, X., Johnson, A., Yang, Y., Jian, W., & Qiu, Y. (2015). Chapter 18 - Epigenetic drugs for cancer therapy. *Epigenetic Gene Expression and Regulation*, 397–423. https://doi.org/10.1016/b978-0-12-799958-6.00018-4

Yan, D., Liu, X., & Guo, S.-W. (2017). Nerve fibers and endometriotic lesions: partners in crime in inflicting pains in women with Endometriosis. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, *209*, 14–24. https://doi.org/10.1016/j.ejogrb.2016.06.017

Yang, H., Zhou, B., Prinz, M., & Siegel, D. (2012). Proteomic Analysis of Menstrual Blood. *Molecular & Cellular Proteomics*, *11*(10), 1024–1035. https://doi.org/10.1074/mcp.M112.018390

Yuan, H., Du, S., Chen, L., Xu, X., Wang, Y., & Ji, F. (2020). Hypomethylation of nerve growth factor (NGF) promotes binding of C/EBPα and contributes to inflammatory hyperalgesia in rats. *Journal of Neuroinflammation*, *17*(1), 34. https://doi.org/10.1186/s12974-020-1711-1