The Relationship of Caspase-3, Caspase-9, Matrixmetalloproteinase-9 Protein Expression and C–1562T MMP-9 Gene Polymorphism in Menstrual Blood as the Etiopathogenesis Marker to Clinical Endometriosis Manifestation in the Establishment of its Diagnosis

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ABSTRACT

Endometriosis is an enigmatic disease, with various pathogenesis, uncertain clinical manifestation, invasive and high-risk diagnostic methods, and also various mode of therapy. The endometrium of endometriosis patients shows abnormal protein presentation for protein involved in pathogenesis of endometriosis itself, such as caspases (which is important in apoptosis), and matrix metalloproteinases (which is important in tissue damage, penetration and proliferation of endometriotic lesion, and also in gene polymorphism). The menstrual reflux containing viable endometrial cells affect the development of endometriotic lesion. The aim of this study is to discover non-invasive diagnostic method of endometriosis using menstrual blood, and also to reveal clearer understanding in pathogenesis of endometriosis.

A case control study involving 149 women who visited the Reproductive Endocrinology and Fertility Clinic, FKUP/RSHS and RSHS network hospitals was performed, from February 2007 to February 2008. Screening for suspected endometriosis was performed by history taking, physical examination, and additional examination. Diagnostic laparoscopy or laparotomy and biopsy were performed afterward. The immunocytochemical examination on caspase-3, caspase-9, MMP-9, and mmp-9 gene polymorphism of menstrual blood were performed. Based on the microscopic confir-mation of histopathological result, the relationship of endometriotic and non-endometriotic clinical manifestation with menstrual blood biomolecular marker was assessed.

Sixty-three (42.28%) endometriosis cases and 86 (57.78%) non-endometriosis cases were found. From those subjects, 34 endometriosis cases and 48 non-endometriosis cases with complete data were enrolled in this study. The endometrial cells were successfully isolated using preservative solution, and with immunocytochemical assay, all samples from 34 endometriosis subjects could be analyzed for the expression of caspase-3, caspase-9, and MMP-9. The decreased expression of caspase-3 and caspase-9, and increased expression of MMP-9 in endometriosis group were higher than those in non-endometriosis group (82.4% vs 77.1%, p=0.562; 97.1% vs 87.5%, p_{E-F}=0.129; and 85.3% vs 85.4%, p=0.988 respectively). The frequency of allele C in -1562T region of mmp-9 gene was significantly increased in endometriosis group (p<0.039). There is a significant relationship between minimal response to pain treatment and menstrual abnormalities (menorrhagia, polymenorrhea, and menometrorrhagia) with MMP-9 biomolecular marker (p=0.006 and 0,050 respectively). At least 4 from 12 symptoms and 1 from 5 physical signs have strong relationship with the occurrence of endometriosis (OR 3.0 and 2.65 respectively).

The results of this study lead to a conclusion that minimal response to treatment of pain, and menstrual abnormalities (menorrhagia, polymenorrhea, and menometrorrhagia) have diagnostic values for establishing diagnosis of endometriosis. There are no significant relationship between frequency of allele C and allele T in -1562 region of mmp-9 gene with MMP-9 expression. Immunositochemistry analysis of the menstrual blood endometrial cells can be applied as a non-invasive method for establishing diagnosis of endometriosis in daily practice.

Keywords: MMP-9, caspase-9, caspase-3, C-1562T mmp-9 gene polymorphism, menstrual blood, non-invasive endometriosis diagnostic methods.

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INTRODUCTION

Endometriosis is a progressive gynecological disease, which is characterized by the presence of tissue resembling endometrial glands and stroma in the pelvic peritoneum, and other extrauterine tissue, which can stimulate a chronic inflammatory reaction. Endometriosis lesions will proliferate and implantinto the tissues around, causing hardening of tissue (fibrosis), adhesions and organdistortion.[1-3]

Epidemiologically endometriosis often experienced by women of reproductive age, all ethnic and social groups who suffer from chronic pelvic pain, infertility, and disorders of both the intra-abdominal organs and intra-pelvic. Internal organ disorders occur because of adhesions, suppression and obstruction of the gastrointestinal tract, urinary tract and reproductive organs. This disease shows a high recurrence (75%) after treatment was discontinued because it is progressive, so that often interfere with daily activities, lower labor productivity, causing stress in weight, which all ended with decreased quality of life of sufferers.[1-3]

The prevalence of the disease is not known precisely due to definitively diagnose endometriosis surgery procedures required to be followed by verification histopathologically, whereas in fact such diagnostic procedures for this are not easy to do. Based on the prevalence of endometriosis disease around the world by 10%, then the suspected more than 70 million women worldwide suffer from endometriosis. Endometriosis is attacked more than 10% of the population aged premenopausal women; but in fact the disease is first diagnosed more often in women of reproductive age (25 to 29 years old). In addition, generally the disease was detected too late, because sufferers often only come after complaining of infertility. Most endometriosis (3 to 4.1%) was found by chance when laparoscopy for sterilization in women without complaints and symptoms. Findings higher (around 20%, range 2-78%) when laparoscopy in women with complaints of infertility, and even higher (24%, range 4-78%) in women with pelvic pain.[1-3]

On the other hand, the incidence of endometriosis increases from year to year. In the developed countries, endometriosis is a health problem that is quite prominent. In the United States the disease is found in more than seven million women, including teenagers. Furthermore, it turns out that endometriosis is a third order of gynecological diseases are often an indication for major surgery (laparotomy) with or without hysterectomy, which takes care for a long time and spend a high cost. The annual fee for the management of this disease in 1995 is 2-6 million US dollars and continued to increase to reach 22 million US dollars a year in 2002.[2] Thus it appears that endometriosis is a disease with many problems, ranging

from etiopatogenesisnya (allegedly multifactorial), clinical manifestations, to how diagnostic and therapeutic invasive and risky.[4] Therefore, in the present and the future endometriosis will also weigh on the economy of developing countries such as Indonesia.

In Indonesia, endometriosis has also been a matter of reproductive health and show a similar pattern of findings, ie especially in women of reproductive age and premenopausal (range 18-49 years), with a chief complaint of dysmenorrhea and infertility. Of the 33 million women of childbearing age turned out 17 million of whom are teenage patients of whom 3.3 million (10%) and an estimated one-third of infertility associated with endometriosis. The incidence of infertility groups according to the three major hospitals in the three big cities in Indonesia ranges from 13.6% to 65.5%. When the prevalence of endometriosis in the world extrapolated to the amount of 10%, then the number of endometriosis sufferers in Indonesia until now estimated at more than 11 million people (with an estimated population of Indonesia at 220 million people).[4]

It is known that endometriosis showing symptoms vary and are not always typical, from asymptomatic, minimal to severe. The results of physical examination, visual inspection when the operating and histopathological findings are often not appropriate. Thus, the way the diagnosis is not always easy, but getting an early diagnosis can be established sooner treatment begins so that the severity of the disease can be reduced.[5-6] Until now, clinical examination, laparoscopy, ultrasonography (USG), computerized tomography scan (CT scan), magnetic resonance imaging (MRI) and examination carcinoantigen-125 (CA 125) is a way to diagnose the disease. Followed laparoscopic biopsy lesions have been used to get to know and look for lesions and perform staging which provides accuracy of the results of histopathology of 50-67%.[7-8]

Although laparoscopy is regarded as a diagnostic method commonly used up to now, but this way is not always easy to do and not always accepted by the female suspect suffered from endometriosis since it is an invasive action. As a result, there is often a delay in diagnosis, and even let loose detection of disease.[1, 6, 9] Investigations such as ultrasound, CT scan, MRI, and CA-125 is a diagnostic method that is non-invasive, but these methods only useful for diagnosing severe abnormalities.[1, 6-9]

Aetiopathogenesis has been proposed for a long time, but the theory of implantation of Sampson remains the main theory used to underlie the pathogenesis concept at this time.Immunological factors, genetic, hormonal, and inflammation are also extensively covered the incidence of the disease through the theory.[2-3, 5-6]

Implantation of endometrial cells were separated when menstruation is thought to occur because the immune system defects,[2, 5-6] and the intrinsic molecular aberrations due to dysfunction of endometrial,[3] so that endometrial tissue is able to implant on the network that are not supposed to. Thought that the endometrial cells attached to the peritoneum must be the cells are viable longer; that is to say, the cells are impaired in the process of apoptosis.[10-11]

Apoptosis is a series of molecular events that started with a couple of different ways and all ended activate caspases. An enzyme caspase proteins essential as implementing the regulation of cell death program. Deviation display caspase lead to failure of apoptosis process, so that presents a viable endometrial cells, as occurs in the development of neoplasms.[12-13]

Implantation of cells occurs due to local degradation of extracellular matrix tissue (ME) by proteolytic enzymes when endometrial cells attached to the ME. Two groups proteolytic enzyme thought to play a role, namely fibronectin and laminin (a serine protease), and matrix metalloproteinases (matrix metalloproteinases, MMPs). MMPs are influenced by several factors, such as the specific tissue inhibitor of metalloproteinases (TIMPs) which act as mediators of inflammation and hormonal processes. Suspected estrogen plays a role in the maintenance of MMP production, while progesterone acts opposite to lower production of MMP. In this disease there is a change of humoral immunity; peritoneal fluid of endometriosis patients experienced an increase in the number of immune cells. This situation is believed to further facilitate the development of the disease rather than prevent it. Nevertheless, abnormal immunological response is not yet clearly revealed.[6] On the other hand, the pattern of clinical manifestations are influenced by environmental factors and genes. Environmental factors will affect the genotype led to different phenotypes. The influence of genetic factors on the pathogenesis of endometriosis is seen in the fact that this disease occurs 6-9 times more frequently in female first-generation derivative.[14] Clinical manifestations of endometriosis are very diverse, and how invasive diagnostic laparoscopy, as well as making handling difficult thought to look for ways diagnostic other options that are not invasive, early, safe, accurate, and affordable cost. Thus thought also that disease progression to a more severe following concomitant impact will be prevented.

Based on the pathogenesis of endometriosis is caused by regurgitation of menstrual blood, it is unthinkable that the way other diagnostic non-invasive can be done by predicting proteins from endometrial cells are able to live and exist in the menstrual blood. These proteins similar to those found in the cells of endometriosis. Of the various types of aberrant proteins turned out that already found in the endometrial tissue is caspase proteins and matrix

metalloproteinases (MMP). Caspases have been known to play a role in apoptosis, whereas MMP plays a role in the process of implantation of cells into tissues of the body.[14-15] Both play an important role at the beginning of the formation of endometriosis lesions. As already known, the process of cell apoptosis takes place following the pattern of the complicated cascade, involving caspase-9, and the end of all of this by activating caspase-3. This compound is a doubt-there cell death by destroying the DNA. On the other hand, from the air turns every kind MMP is MMP-9 protein at the transcription stage can be triggered on a large scale by many compounds in the body cells, and is only shown by trophoblast cells, osteoclasts, leukocytes, and their precursors. Any deviation protein display, including caspase-3 and MMP-9 is closely related to genetic variations, or polymorphisms of the gene. This variation is different in each ethnic.[12-13, 15] The MMP-9 gene polymorphism in the promoter region are involved in the transcription process.[15-17]

Given that these proteins play an important role in the onset of disease, it is thought that the analysis of caspase-3, caspase-9, MMP-9 and MMP-9 polymorphisms in menstrual blood can be used as markers to diagnose endometriosis. Until now generally immunocytochemistry technique is still difficult, especially for the isolation of endometrial cells in menstrual blood.[18] If the endometrial cells in menstrual blood can be isolated and abnormal protein can be detected by immunocytochemistry technique then this method can clarify the pathogenesis of the disease and are also able to diagnose the disease is non-invasive. It is expected to be developed another option diagnosis means that non-invasive. This method can connect the clinical manifestations of endometriosis with a view caspase-3, caspase-9, and MMP-9 following its gene polymorphism in menstrual blood. These findings on the future thought may provide opportunities to develop ways of early diagnosis, management, and prevention of disease in addition to clarify the pathogenesis.

METHODS

Subjects

The subjects were female patients suspected of suffering from endometriosis who came to the Polyclinic-Fertility Reproductive Endocrinology, Gynecology Clinic FKUP / RSHS, and the hospital network of RSHS. All patients with suspected endometriosis women who came went to the Polyclinic of Fertility Reproductive Endocrinology, Gynecology Clinic FKUP / RSHS and hospital networks RSHS much as 150-200 patients for a period of one

year. Women with external endometriosis, endometriomas been diagnosed with endometriosis or externally by histopathology results. Controls were women with histopathological results not endometriosis.

Exclusion criteria were pregnant women. Being in the treatment of hormone, for at least three months, unless GnRH after nine months from the last administration. Other gynecologic disease, such as infection, malignancy, and other serious illnesses. Suffering from other diseases that complicate research, like other systemic diseases. The number of study participants who collected is ninety-two.

Clinical examinations

Anamnesis, examination, and physical ultrasonography performed by researchers in an attempt to make a diagnosis. In the surgery is performed classification, then taken of the sample tissue. In patients who had surgery, surgery of data collection to determine the degree of lesion.

Immunohistochemistry

Endometriosis by histopathologic criteria is the presence of epithelial cells of endometrial glands and stroma in the endometrial tissue examined.In this study, staining cells / tissues in immunohistochemistry using diamino benzene (DAB), and the comparison is used hematoxylin eosin staining (HE) so that the stromal cells which express MMP-9 and caspase-3 and caspase-9 will be stained brown with blue background.

PCR RFLP to analyze MMP-9

DNA samples isolated from whole blood (300 □).DNA isolation was performed usingWizard® Genomic DNA Purification Kit-Promega (CAT-A1620). Primers used (Promoter region -1809 until -1374) were *Forward*:5'-GCCTGGCACATAGTAGGCCC-3'; and *Reverse*:5'-GATGCCGGCTGGCTAGGAAG-3'. The resulting PCR product of 436 bp. Restriction enzymes used arePaeI (5'GCATGC 3'). Polymorphism (allele T): cut (result in 193 bpand 243 bp fragments). Normal (allele C): uncut (436 bp).

The data analysis research conducted descriptive and analytic. Age grouping is intended to eliminate confounding incidence, and sort out cases that occur during adolescence (under 20 years), young adults (20-24 years), the peak age first diagnosed (25-35), and in

perimenopause.Descriptive calculation is made by calculating the amount of the number and percentage, whereas analytical calculations were made with the statistical test adapted to the hypothesis to be tested.

RESULTS

The study lasted for a year (February 2007 to February 2008) in Reproductive Endocrinology Clinic-Fertility and Gynecology FKUP / RSHS and Hospital Network RSHS. Through history, physical examination and other investigations carried out screening of patients with endometriosis, then performed a diagnostic laparoscopy or laparotomy, which ended with a biopsy of the lesion. Biomolecular examination caspase-3, caspase-9, MMP-9 and regio-1562 polymorphism in the gene MMP-9. Based on the results of microscopic histopathology as ascertainment, assessed the relationship of clinical manifestations of endometriosis cases and not endometriosis with menstrual blood biomolecular markers. Found 63 (42.28%) cases of endometriosis, while 86 (57.72%) classified as not endometriosis.

Table 1: Characteristics of Research Subjects

Characteristics	Groups				Significance
	Endometriosis (n = 63)		Non Endometriosis (n=86)		
	N	%	N	%	
Age					***0.166**
17-20	5	7.93	7	8.14	
20-24	7	11.11	9	10.46	
25-34	20	31.75	29	33.72	
35-48	31	49.21	41	47.67	
Mean(SD)	35(6.8)		32(8.6)		
Median	35		36		
Range	20-55		16-47		
BMI					****0.426**
Mean(SD)	22.3(3.5)		27.7(4.3)		
Range	15.4-31.4		16.2-30		

Notes: *) Independent T test; **) Mann-Whitney test

Characteristics of research subjects in Table 1 shows that all variables either age, occupation, marital status, social status and BMI was not found significant differences between groups endometriosis and not endometriosis. Based on the homogeneity of the data, the two groups can be compared.

Table 2: Endometriosis Symptoms in Women with Endometriosis Compared with the group without endometriosis

Symptoms	Groups				Nilai p*)
	Endometriosis (n = 63)		Without Endometriosis (n = 86)		
	N	%	N	%	
1. Menoragia	24	38,1	25	29,1	0,247
2. Polimenorea	10	15,9	10	11,6	0,453
3. Menometroragia	40	63,5	42	48,8	0,076
4. Dismenorea	42	66,7	59	68,6	0,802
5. Premenstrual syndrome	41	65,1	56	65,1	0,996
6. Pelvic pain	33	52,4	34	39,5	0,119
7. Minimal response to analgesia administration	24	38,1	24	27,9	0,189
8. Dispareunia					
9. Diskezia	17	27,0	9	10,5	*0,009*
10. Diarrhea, hematokezia	15	23,8	6	7,0	*0,003*
11. Hematuria	6	9,5	7	8,1	0,767
12. Infertility	3	4,8	1	1,2	0,311
	27	42,9	26	30,2	0,11
Scor history of endometriosis**)					
- Median	4		3		Z_{M-W} = 2,439
- Range	1-12		0-12		*P=0,015*

Notes: *)Chi Square or Fisher-exact for expected cells<4

Table 2 shows that diskezia, dyspareunia, a statistically significant difference (p <0.05) in both groups. Two main groups of symptoms of menstrual disorders (menorrhagia, polimenorea, me-nometroragia) and chronic pelvic pain (dysmenorrhea, premenstrual syndrome, pelvic pain) did not differ significantly. The combined 12 symptoms were statistically significant (p = 0.015).

Table 3: Physical Examination Results of Women with and without Endometriosis

Physical Examination	Group				P value*)
	Endometriosis (n = 63)		Without Endometriosis (n = 86)		
	N	%	n	%	
1. Tenderness	35	55,6	29	33,7	*0,008*
2. Pelvic Mass	45	71,4	40	46,5	*0,002*
3. uterosacral ligament nodules	30	47,6	18	20,9	*0,006*
4. Impression attachment	27	42,9	25	29,1	0,081
5. Tractus obstruction GI/UG	14	22,2	6	7,0	*0,007*
Physical Examination Scores					
- Median	2		1		Z_{M-W} = 3,650
- Range	0-5		0-4		p<0,001

Notes**:** *) Chi Square test; Z_{M-W} = Mann-Whitney test.
GI: gastrointestinal, UG: urogenital

The physical examination abdominal tenderness and bimanual examination, the presence of a pelvic mass, uterosacral ligament nodules, the impression of the gastrointestinal tract obstruction and urinary tract in two different groups was statistically significant (p <0.05) (Table 3).

Table 4: Relationship between Score and Physical Symptoms with Endometriosis

Examination Score*)	**Groups**				**P value*)**	**OR (95% CI)**
	Endometriosis (n = 63)		**Without Endometriosis (n = 86)**			
	n	**%**	**n**	**%**		
1. Symptoms:						
≥ 4	31	49,2	21	24,4	0,002	3,0
< 4	32	50,8	65	75,6		(1,41- 6,41)
2. Physic						
≥ 1	42	66,7	37	43,0		2,65
< 1	21	33,3	49	57,0	0,004	(1,28 – 5,52)

Key : *) Chi-Square. OR (95 % CI) = *odds ratio* (95 % *confidence interval*)

Scores of complaints / symptoms, and physical examination in both groups were significantly different (p = 0.002 and p = 0.004) with respective odds ratios 3.0 and 2.65 (Table 4).

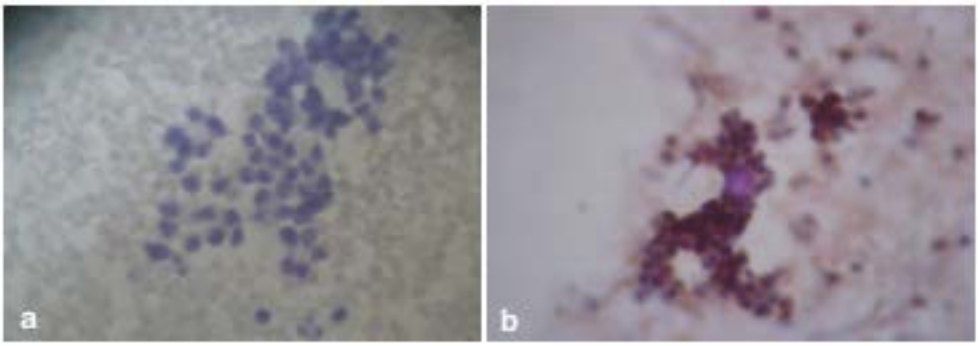

Figure 1. The expression of caspase-3 / caspase-9 in endometrial cells from menstrual blood.

a. Endometrial cells from menstrual blood without expressionsof caspase-3 / caspase-9 (magnification 200 times)

b. Endometrial cells from menstrual blood with expressions of caspase-3 / caspase-9 (magnification 400 times)

Table 5.The expression of caspase-3 and caspase-9 in the group and not Endometriosis Endometriosis

Variables	Groups					OR (95% CI)
	Endometriosis		Without Endometriosis			
	n'	%	n'	%		
Caspase-3 :						
- Negative	28	82,4	37	77,1	X^2 = 0,336	1,43
- Positive	6	17,6	11	22,9	p = 0,562	(0,42-5)
Caspase-9 :						5,46
- Negative	33	97,1	42	87,5	p_{E-F} =	(0,62-124)
- Positive	1	2,9	6	12,5	0,129	

Notes : p_{E-F} = ExactFisher

Table 5 shows the negative expression of caspase-3 and caspase-9 tends to be higher in women with endometriosis compared to without endometriosis, but the differences between the two groups was not statistically significant.

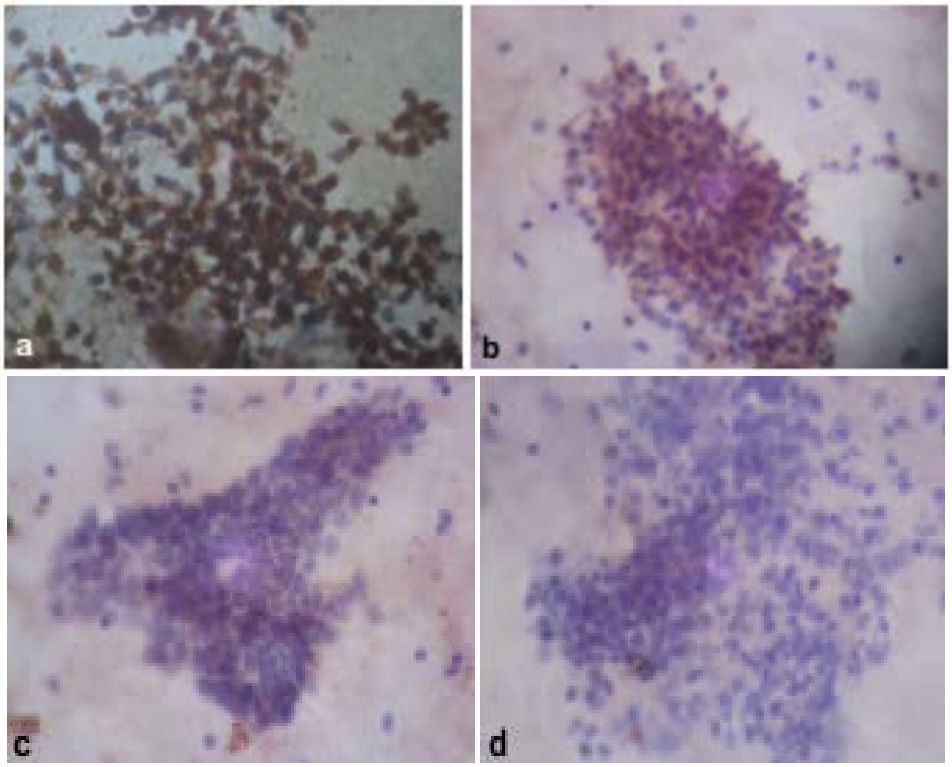

Figure 2.Expression of MMP-9 in endometrial cells of menstrual blood.

a. Endometrial cells in menstrual blood to the expression of MMP-9 (+3) (magnification 400 times)
b. Endometrial cells in menstrual blood to the expression of MMP-9 (+2) (magnification 200 times)
c. Endometrial cells in menstrual blood to the expression of MMP-9 (+) (magnification 200 times)
d. Endometrial cells in menstrual blood to the expression of MMP-9 (-) (magnification 200 times)

Figure 2 a,b,c,d respectively shownexpression of MMP-9 (+3), (+2) and (+) and (-)/ without expression in endometrial cells of menstruation blood, the darker the color, the stronger the destruction power.

Table 6. Expression of MMP-9 in endometrial cells of menstrual blood in patient with and without Endometriosis

Variables	Group					OR (95% CI)
	Endometriosis N	%	Without Endometriosis n	%		
1. MMP-9 :						
- Positive	29	85,3	41	85,4	$X^2 = 0,000$	0,99
- Negative	5	14,7	7	14,6	p = 0,988	(0,25-4,05)
2. MMP-9						
- (++)	18	62,1	20	48,8	$X^2 = 1,209$	1,72
- (+++)	11	37,9	21	51,2	p = 0,272	(0,59-5,08)

Notes : p_{E-F} = Exact Fisher.

Expression of MMP-9 in the two groups was not significant (Table 6). Expression of MMP-9 (++) in the endometriosis group larger than non endometriosis.

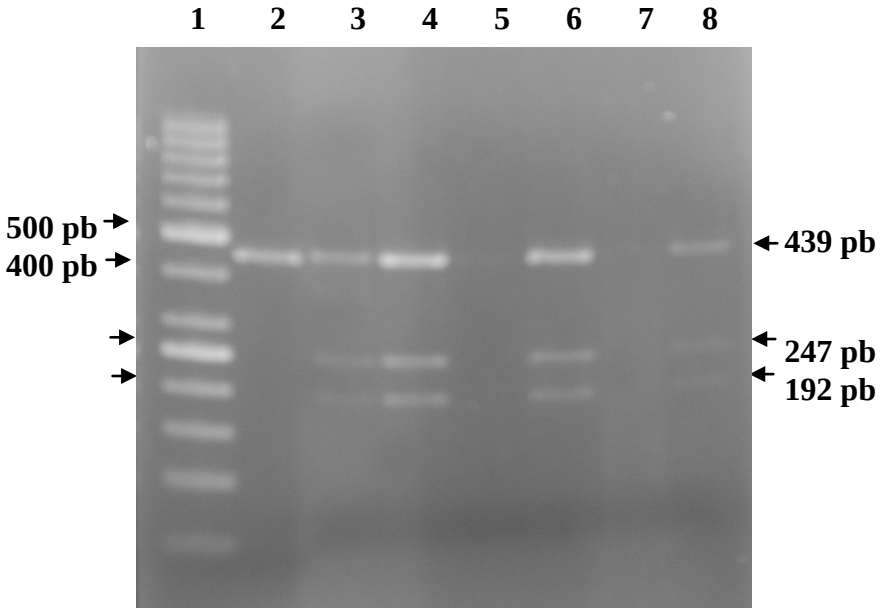

Figure 3. Electrophoresis Results of Restriction Enzymes PaeI.

Lane 1. DNA Ladder 50
Lane 2. PCR product (*Uncut*)
Lane 3,4,6,8. Results of digestion, shown fragment of 439 pb, 247 pb, and 192 pb (CT genotype)

Results PaeI digestive enzymes, agarose electrophoresis was performed using 2%, then visualized under UV and photographed Transilluminator. The results are shown in Figure 4.5, that there are three bands of DNA, which consists of 439 bp, 247 bp and 192 bp (restriction enzyme cutting results).

Table 7 Correlation between expression and genotype of MMP-9 in women with Endometriosis

Variable	Genotype of MMP-9						P value
	CC		CT		TT		
	n	%	n	%	N	%	
MMP-9 (IHC)							
- Positive	22	88,0	7	77,7	0	0	P_{E-F} = 0,591
- Negative	3	12,0	2	22,2	0	0	

Notes : p_{E-F} = Exact Fisher

Table 8. MMP9 gene allele frequency in Patients with Endometriosis Compared to controls

Polymorphism	Group				Significancy
	Endometriosis		Non Endometriosis		
	n	(%)	n	(%)	
Allele T	8	11,76	19	19,79	Z= 0,2209 p = 0,039
Allele C-1562T	60	88,23	77	80,20	

Notes: p = Mann-whitney test

T allele frequency of endometriosis and non-endometriosis group found respectively 11.76% and 19.79%. C allele frequency in the endometriosis group and not endometriosis by 88.23% and 80.20%. The difference was statistically significant (p = 0.039).

Table 9. MMP9 genotype frequency in Patients with Endometriosis Compared to controls

Genotype	Group				Significancy
	Endometriosis		Non-Endometriosis		
	n	(%)	N	(%)	
CT	9	26,5	20	41,7	Z = 1,418
CC	25	73,5	28	58,3	p = 0,078

CC genotype frequencies, and CT in the two groups did not differ significantly (p = 0.078). TT genotype was not found.

DISCUSSION

Symptoms collected main symptoms include chronic pelvic pain[1-2, 5-6] namely dysmenorrhea, premenstrual syndrome, pelvic pain, minimal providing analgesic response, symptoms of menstrual disorders, namely menorrhagia, polimenorea, menometrorrhagia,[19] dispareunia, diskezia, diarrhea, hematoskezia, hematuria andinfertility. These symptoms often complained of by patients with endometriosis.[1, 6, 19] The symptoms are assessed to determine its characteristics in patients with endometriosis in Bandung and West Java in general.

This study found that dyspareunia and diskezia significantly different between the two groups of cases and comparison (not endometriosis). Dyspareunia also a significant symptoms in this study. This situation is closely related to deeply endometriosis and nodules ligament sakrouterina drawn significantly on the results of a physical examination uterosacral ligament nodules.

It has been proven that dyspareunia will be felt before menstruation at deep penetration during sexual intercourse (deep dyspareunia); This situation associated with lesions in the cul-de-sac (pouch of Douglas) and rectovaginal septum, and more frequently in the rectovaginal septum lesions.[20]

Diskezia symptoms also show significant results. This means that the implantation of endometriosis lesions occur in organs such as the uterus around the colon and rectum, as illustrated by the results of the physical examination was significant, among others, the

impression of adhesions and obstruction of the gastrointestinal tract and urinary tract. In addition extra pelvic endometriosis can be accompanied by symptoms of cyclic diverse because the lesion involves many organs, namely abdominal scar lesions, gastrointestinal and urinary tract, diaphragm, pleura, and peripheral nerves.[6] Endometriosis of the colon associated with painful bowel movements, diarrhea, or hematokezia when menstruation. In addition dysuria, pelvic pain or hematuria when menstruation in connection with endometriosis in the ureter and urinary bladder.

Risk Factors of Endometriosis

Risk factors such as family history of endometriosis, smoking, alcoholism, drink coffee, eating saturated fat and immune diseases do not show significant results. Thus these factors do not greatly affect the incidence of endometriosis in women's groups. This situation that has been presented in accordance with other researchers, that the factors which are less consistent support of endometriosis are age, social status, race, use of oral contraceptives, family history of endometriosis, smoking, alcoholism, drink coffee, and eating saturated fat is excessive, IMT, dioxins, hormonal, immune diseases such as rheumatoidarthritis, SLE, hypothyroidism, hyperthyroidism, multiple sclerosis, and non-Hodgkin's lymphoma.[19]

Physical Examination of Patient with Endometriosis

Tenderness on bimanual examination showed lesions that have berimplantaso to other organs in the pelvis. In addition to the activity of cytokines and local bleeding lesions, the infiltration and irritation of the lesions is the mechanism that results in pain.**Error! Bookmark not defined.**Intrapelvic palpable mass is also a symptom that strengthens the case toward the diagnosis of endometriosis. Palpable nodules in uterosacral ligament, the impression and the impression adhesions gastrointestinal disorders and urinary tract have been described earlier.

Pain is thought to occur because (1) there is activity of cytokines in the peritoneal cavity, (2) the efficacy of direct and indirect local bleeding that comes from the lesion; (3) direct infiltration irritation and lesions of the nervous system organs of the pelvic floor. This event is the mechanism that many agreed. The strength of the pain associated with deep penetration and proximity to or directly on the implantation of nerve fibers.[6]

Relationship between symptoms and physical examination with Diagnosisof Endometriosis

Physical symptoms of endometriosis compared with the group not endometriosis showed highly significant relationship (p = 0.002 and p = 0.004), so it may be possible score. Scores of complaints / symptoms> 4 is finding four or more symptoms that are risk factors for patients with suspected endometriosis, while the physical findings score> 1 was the finding of one or more physical symptoms that are risk factors for endometriosis.

Odds ratios respectively 3.0 and 2.65. That is, if there are at least four complaints of 12 symptoms in women who are suspected of suffering from endometriosis, then these women have three times the risk of suffering from endometriosis than women who have a complaint under five. Similarly, for women with endometriosis alleged that on examination findings obtained one or more physical example of tenderness in bimanual examination, the mass intrapelvik, sakrouterina ligament palpable nodules, lesions of endometriosis adhesions impression, and the impression of the gastrointestinal and urinary tract obstruction, then the woman has a risk of 2.65 times that of women with endometriosis compared to normal.

Almost 75% of patients with endometriosis in this study is the degree being (III) and heavy (IV). On the degree of advanced disease is already happening impact of, among others, clearly palpable masses and adhesions with surrounding tissue lesions due penyusukan, with all its consequences such as pain and symptoms of obstruction. At a minimum degree lesions and light, which on examination found no obvious abnormalities, symptoms such as dysmenorrhea, pelvic pain, the symptoms of PMS, and minimal response to drug treatment of dysmenorrhea or pelvic pain relievers can be used as a handle beginning to suspect endometriosis.

Expression caspase-3, caspase-9 and MMP-9 in Endometrial Cells in Menstrual Blood

Physiologically apoptosis was detected in endometrial glandular epithelium which is at the end of the secretion phase and menstrual phase, while little apoptosis was detected during the proliferative phase or initial phase of secretion. Eutopic endometrium of endometriosis patients change like ectopic tissue. The changes are not found in eutopic endometrium of women without endometriosis. This fact has led to the view that there is a primary defect in endometriosis in eutopic endometrium. The elements of cells and tissues derived from the change eutopic endometrium and overflow into the peritoneal cavity allegedly high potential for implantation and grow on the surface of the peritoneum, developing into endometriosis.

The reduced apoptosis in cells suspected endometriosis is an important factor in the development of this disease.[6, 12-13]

In this study, patients with endometrial cells of endometriosis based disorders endometrial response to progesterone expected found intact and alive because of impaired apoptosis, so that they can live longer. As a regulator of apoptotic caspase enzymes in endometriosis experiencing irregularities, thus weakening apoptosis and caspase shown decreases in endometrial cells from menstrual blood.

Results of this study confirms what previous researchers claim that endometrial cells can be found either in the network endometriosis (ectopic endometrium), an abundance of menstrual blood and peritoneal fluid.[13, 21] It has been thought that the analysis of cells through menstrual blood can not be done because it is already necrosis and only a few cells are found so a lot of research endometriosis use of scrapings / endometrial biopsy or make menstrual blood culture.

Results showed that there was no difference in appearance ratings protein caspase-3, caspase-9 and MMP-9 in the endometriosis group than non endometriosis. The preparation can not be analyzed is the preparation that does not contain endometrial stromal cells, while other cells there looked good (it can be analyzed) although the amount is too little. This condition is thought that menstrual blood is taken after the expiration of the first days of menstruation, because it is important ascertained menstrual blood storage time is right, namely the first or second day of menstruation. Thus, this study managed to alienate menstrual blood and endometrial cells also displayed satisfactorily managed and can be analyzed properly so the chances mendiag-prognosis non-invasive endometriosis can be further developed.

Results showed negative expression of caspase-3 and caspase-9 tend to be greater in patients with endometriosis compared to normal women. The differences of each examination for the two groups was not significant. Odds ratios for caspase-3 is 1.43. This means that women with endometriosis have a display alleged caspase-3 were negative, and 1.43 times likely to have endometriosis compared to without endometriosis. Similarly, when the caspase-9 negative views on women with suspected endometriosis, then the chances of suffering from endometriosis is 5.46 times compared to women without endometriosis.

MMP-9 is a protein that plays a role in the destruction of the extracellular matrix, due to the properties of these cells were able to implant once attached to the peritoneum. Expression of MMP-9 (+3) and the expression of MMP-9 (+2) in both groups the difference was not significant. Have believed that a strong apoptosis disorders prevalent in

endometriosis.[22-23] In this study, the percentage of expression of MMP-9 (+2) more precisely, while all cases of endometriosis showed expression of caspase (+3) and (+2). Ten cases (29%) endometriosis that normal visual assessment showed the expression of MMP-9 (+2).

Odds ratio value of 1.72 states that in this study patients with endometriosis to display its MMP-9 in endometrial cells (+2) and (+3), likely to have endometriosis by 1.72 times higher than normal women.As the results of the ISK for caspase-3 and caspase-9, increased expression of MMP-9 in endometriosis tend to be higher than non endometriosis.

The expression of caspase-3, caspase-9 and MMP-9 menstrual blood endometrial cells in this study shows that clarify the relationship display the pathogenesis of endometriosis is a decrease apoptosis and increasing destruction (degradation) of extracellular matrix. A similar Figure has been widely studied in eutopic endometrial tissue.[22, 24-25] These results, although not significant but showed a tendency that can not be ignored. This phenomenon is evidence that must be taken into account, namely the possibility of subclinical disorder endometriosis can already ditasah first before clinical disorders arise. If this is so then finding molecular markers before the disorder becomes clinically important.

Relations MMP-9 gene polymorphisms with Display caspase-3, caspase-9, and MMP-9 in Menstrual Blood in Patients with Endometriosis.

Further analysis it was found that CC genotype frequency was higher in the positive expression of MMP-9 compared with a negative expression. It gives the sense that the genotype C / C proponents have high activity. CT genotype frequency was higher in the positive expression, although not statistically significant (p = 0.557). Thus polymorphism characterized by the genotype C / T does not affect the display MMP-9 gene in endometriosis.

MMP-9 expression occurs in coronary atherosclerotic plaques, which is the gene MMP-9 amended the base C was changed to T in the region (applicants) -1562 position. However, different effects that result in a weak transcriptional activity genotype CC, but increases the transcriptional activity of CT and TT genotypes.**Error! Bookmark not defined.**Other investigators reported either allele C, T allele, genotype CC, CT, and TT in endometriosis group than not endometriosis is not statistically significant. In the two-locus haplotype analysis study reported that haplotype was significantly anything to do with endometriosis, but the risk of endometriosis has nothing to do with individual SNPs research.[16]

In this research, the discovery of MMP-9 gene variation which is located at the site of the region -1562 applicants, namely alkaline C is replaced by T, so that the resulting two different alleles. CC and CT genotypes encourage the activity of MMP-9 gene applicants. C allele frequency was found endometriosis patients is higher (88.23%) compared with the group not endometriosis (80.20%), whereas allele T in the endometriosis group only 11.76% lower than the group not endometriosis 19.79%. This difference was statistically significant ($p = 0.039$).

This study shows that the T allele had no effect on the incidence of endometriosis, so it is thought that the increase in MMP-9 may be caused by other factors as a driver (enchancer). Factors driving the increased expression of MMP-9 can occur in other promoter region which affects the transcription process, can also in the synthesis of protein molecules phase II, III and even stage IV or may lie in exons and introns.

This study shows the frequency of the CC genotype MMP-9 in endometriosis is higher (71.74%) compared to CT genotype (28.26%). This evidence shows that CC genotype did not affect transcription. This result suggested that the gene polymorphism in this study is not a risk factor for endometriosis. Excessive expression of MMP-9 is due to other factors that affect the production of MMP-9 before the process of transcription or regulation.

Studies comparing the expression of MMP-9 ectopic endometrium and eutopic using ELISA and RT-PCR, also produces display MPP-9 were not significantly different between the two groups, but the ratio of MMP-9 / TIMP-1 was increased significantly. This shows that there is a balance disorder MMP-9 / TIMP-1 which resulted in excessive expression of MMP-9 that endometrial cells capable of implantation.

On the other hand, endometriosis has also been believed to be a multifactorial disease. Display MMP-9 and caspase abnormal can occur due to various environmental factors that influence arousal pathways and apoptosis cascade of MMP-9 in cells. It will display the endometrial cells are able to live and able to perform the implantation of tissue outside the uterus. This situation seems to be amplified by a factor endometrium of endometriosis sufferers who are resistant to progesterone.

Clinical manifestations relationship, Histopathology, and Cellular Biochemistry Endometriosis

The relationship between the complaints and symptoms with the expression of MMP-9, caspase-3, caspase-9, and MMP-9 genotypes showed that the complaints of menstrual abnormalities, MMP-9 positive view, the expression of caspase-3 and caspase-9 CC genotype

negative and more significantly higher than the negative expression of MMP-9, caspase-3 positive, caspase-9 positive and CT genotypes (binomial test: 0.011, 0.011, 0.001 and 0.007). For complaints of dyspareunia found expression caspase-9 significantly negative than positive caspase (binomial test 0.062), while for diskezia found differences in caspase-3 expression was significantly negative compared to caspase-3 positive (0.062 binomial test).

The evidence states that in women suspected of having endometriosis findings of abnormal bleeding is a symptom that needs to be taken into account in strengthening the suspicion of endometriosis. Complaints of dyspareunia and diskezia consecutive symptomatic endometriosis confirming allegations. These results may support previous research which states that complaints of abnormal bleeding is a symptom that consistently supported the diagnosis of endometriosis.[19]

In the results shown that the expression of caspase-9 menorrhagia negative and CC genotypes of MMP-9 was significantly higher than the expression of caspase-9 positive and CT genotypes. Menorrhagia may be associated with hormonal changes due to local production of estrogen that is not supposed to occur in phase secretion. Similarly, in polimenorea display negative caspase-3, caspase-9 negative and CC genotype was significantly higher than see caspase-3 positive, negative and caspase-9 CT genotype (p = 0.31, 0.31 and 0.31), In the same study reported that in addition to polymenorrhea menorrhagia is also a consistent symptom occurs in the case of endometriosis.[19]

The expression of caspase-3 negative, negative caspase-9 and MMP-9 positive, significantly higher on dysmenorrhoea than caspase-3 positive expression, positive caspase-9, and MMP-9 were negative with respective significance p=0.001, 0.007 and 0.001. Dysmenorrhoea incidence in patients with endometriosis epidemiologically quite high.[6, 16, 19]

The result showed premenstrual syndrome and pelvic pain associated with MMP-9, caspase-3 and caspase-9 showed me a meaningful result, except for genotype MMP-9, while the minimal response to therapy only showed significant results when associated with caspase-9.In infertility, positive expression of MMP-9, caspase-3 negative, and negative expression of caspase-9 was significantly higher than the negative expression of MMP-9, caspase-3 positive, and caspase-9 positive. The incidence of infertility is often associated with endometriosis.[3, 6, 9, 19]

Pelvic tenderness, finding a mass in the pelvis, and palpable nodules uterosacral ligament can be a physical sign that confirm the diagnosis of endometriosis. Variations (polymorphisms) of genes that confirm the diagnosis is CC genotype MMP-9.

In this study it has been found a consistent relationship between histopathologic diagnosis of endometriosis by symptoms, physical examination, expression of MMP-9 (++) and (+++), the expression of caspase-3, caspase-9 expression with CC alleles were detected through endometrial cells.

Judging from the degree of endometriosis, it appears that the symptoms and physical signs associated with a high degree of disorder endometriosis (stage III and IV), among other menstrual disorders. This situation can occur due to ovarian enlargement will disrupt the follicles producing steroid hormones, thus giving rise to menstrual disorders. There appears to be differences may not be significant with non-cystic ovarian endometriosis is accompanied by abnormal uterine bleeding. The findings in endometriosis occurs due to the excessive destruction of extracellular matrix due to endometriosis penyusukan power is not shared by other types of cysts.

In endometriosis minimal degree or degrees I found no symptoms and physical disorders, but there is a consistent relationship between the appearance of MMP-9, caspase and CC genotypes.Of the 34 cases of endometriosis (54%) found the number of cases of degree I, II and normal by 12 (35%) cases. The expression of caspase-3 were decreased by 30 (88.23%) cases, while for the decreased expression of caspase-9 was found in 100% of cases. Expression of MMP-9 were increased (++) amounted to 18 (53%) cases, while (+++) by 16 (47%) cases. CT allele expression of MMP-9 were increased was 8 (34%) cases, while the CC genotype expression of 26 (76.55%) cases. Seeing the results of the suitability of the results of histopathologic examination with endometriosis are quite high (the expression of caspase-3 and caspase-9), the caspase-3 and caspase-9 can be used to detect mild cases of endometriosis (minimal lesions).

The above discussion shows a different relationship with the clinical manifestations of biomolecular markers were significant in the group of endometriosis, although the relationship is not consistent. The results of this study can not be extrapolated to the general population, because the difference between biomolecular markers (caspase-3, caspase-9, MMP-9) in endometriosis and non-endometriosis is insignificant.

The relationship between display caspase-3, caspase-9 and MMP-9 menstrual blood with clinical manifestations of the disease in the diagnosis of endometriosis

In this study, the clinical manifestations of endometriosis to be analyzed conjunction with the display of biomolecular markers caspase-3, caspase-9, MMP-9 and C-1652T

polymorphism is a clinical symptom that can be detected in daily practice, among others, the main symptoms of endometriosis are chronic pelvic pain, dysmenorrhoea, PMS pain, and minimal response to the treatment of pain. Other symptoms of menstrual disorders (including menorrhagia, polimenorea, menometrorrhagia), dyspareunia, diskezia, diarrhea, hematoskezia, hematuria, and infertility

The results showed a direct correlation between the expression of caspase-3, caspase-9, MMP-9, genotype and allele gene MMP-9 with the clinical symptoms and it turns out there is a significant correlation between the appearance of biomolecular markers MMP-9 with less pain symptoms responsive to the treatment of pain and menstrual abnormalities (with significance respectively $p = 0.050$ and $p = 0.006$, whereas the sensitivity of 65.5% and 50%). This happened while the endometriosis group on non-endometriosis groups was not significant. Thus the minimal response the treatment of pain and the symptoms of menstrual disorders have diagnostic value for endometriosis.

In practical terms these findings can be used as a support for the non-invasive diagnosis, especially in cases of endometriosis with clinical manifestations that can not be determined by non-invasive method that has been commonly used (ultrasound, MRI, CT-scan and CA-125). These cases usually require proving diagnostic laparoscopy. With that relationship was found to have the value of this diagnostic laparoscopy for patients who refuse non-invasive diagnostic method is advantageous. Besides this significant association can be used an excuse to start hormonal therapy, which until now has not had a basis/rationale.

For women with symptoms of endometriosis that supports the allegations, but without obvious physical examination abnormalities or simply suffer from endometriosis grade I and II, the examination can also be encouraged to strengthen clinicians in starting a non-invasive treatment. One of the benefits of early detection is to prevent the disease in women with endometriosis do not continue with complications. Prevention can be done by eliminating and minimizing the risk of leading to the development of the disease, among others, avoidance of exposure to toxic substances by improving lifestyle, such factors cigarettes, alcohol, hormones, dioxins, or excessive grease. The use of antioxidants (free radical) can avoid developing the disease.

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