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Role of L-Selectin Gene P213S Polymorphism and Macrophage Profiles (M1, M2) in Cases of Endometriosis

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Abstract :
Objective: To determine the presence of an L-selectin gene P213S polymorphism in association with the pathogenesis of endometriosis.**Design** **:** Comparative analytical study using a 2 population case-control design.**Setting** **:** Department of Obstetrics and Gynaecology at Haji Adam Malik General Hospital, Dr. Pirngadi Medan Hospital, networking hospitals and private clinics throughout Medan from Januray 2013 - March 2014. **Population:** Reproductive aged women complaining menstrual pain, abdominal located bulging, and/or infertility. Through consecutive sampling, patients suspected with endometriosis and meeting the inclusion criteria were taken as samples. The case group comprised of women diagnosed with endometriosis whereas the control group consisted of non endometriosis women.**Methods :** Visiting gynecology outpatient clinic patients were taken a complete history, and underwent physical/gynecological, sonographic examinations to determine an indication to perform a laparoscopy/laparotomy and, if present, a laparoscopy was scheduled to take place during the proliferation phase. Presence of endometriosis was assessed during the procedure, and further confirmed by histopathologically reviewing peritoneal specimens. Patients diagnosed with endometriosis were categorized into the case group, after which severity degrees were determined based on the Revised Classification of the American Sociey for Reproductive Medicine 1997. Non endometriosis patients were categorized into the control group based on inclusion and exclusion criterias. Peritoneal tissues were sampled and 5 cc of blood taken. DNA isolation and genotyping was then performed, whereas peritoneal tissue samples were histopatahologically and immunohistochemically examined. Data were univariately and bivariately analyzed. Descriptive data was univariately analyzed.**Main Outcome Measures :** CD68, CD 163, M1 and M2.**Results** **:** S allele subjects had a 2,893 (95% CI; 1,135-7,373) higher risk of developing endometriosis than P allele subjects (p<0,05). PS and SS genotyped subjects had a 3,556 higher risk of devolping endometriosis than their PP genotyped counterparts (95% CI; 1,049-12,052, p-value 0,041). CD163 was more frequently expressed than CD68 in endometriosis lesions (60,870 ±28,939 vs 34,783 ±18,194, p-value 0,0001). Positive immunohistochemical scorings revealed CD68 macrophages were less frequently expressed (69,96%) than CD163 macrophages (100%) (p-value 0,0001).**Conclusion** **:** L-selectin gene P213S polymorphism is associated with endometriosis. M2 Macrophage has a more significant role in developing endometriosis than M1 macrophage.

INTRODUCTION

Endometriosis is a benign disease defined as an extra uterine presence of endometrial glands and stromas, and is associated with pelvic pain and infertility. Clinical manifestations are usually broad spectrum and highly progressive and with high recurrence rates, and frequently cause treatment complicating issues for both patients and clinicians.[1] 10% of reproductive aged women, of all ethnic and social groups, are diagnosed with this disease.[2] Approximately 20-40% infertile women are diagnosed with endometriosis.[3,4]
Endometriosis causes serious physical, mental, and social implications[5,6] and is also an economic burden to the society. Simoens et al estimated a US national cost of approximately 22 billion USD in 2002.[7] Hummelshoj et al reported that 78% of women diagnosed with endometriosis in UK lost an average of 5,3 working days due to this disease.[8]
However, the pathogenesis is not fully understood.[9] A theory by Sampson in the mid 20s was unable to explain as to why endometriosis only occurs on a few amount of women. Most women experience retrograde menstruation (76-90%) that flows into the peritoneal cavity, however, endometriosis is only evident in 5-10% of these cases.[10] Therefore, endometriosis may not only involve retrograde menstruation but also other factors on the molecular level, including genetic defects and immune system or both, eg. endometrial cell adhesion and invasion, proliferation, angiogenesis, and immune system dettachment.[11]
Endometriosis has several genetic predispositions.[1] Several data indicate that endometriosis is associated with subclinical peritoneal inflammation characterized by increased peritoneal volume, peritoneal white blood cell concentration (especially increased macrophage activities), and increased inflammatory cytokines, growth factors, and angiogenic supporting substances.[12]
Due to potencial T cell regulatory changes, T cells, B cells, mast and dendritic cell and macrophages are altered,

from which ectopic lesions may occur, affecting the events of endometriosis and it's progresivisity.[13] Several studies have shown altered immune cell function in endometriosis women, indicated by decreased cytoyoxic T cell and Natural killer cell activities, cytokine secretion by T cell helpers, and autoantibody production by lymphocyte B. [14]

Macrophages are normally located in the peritoneum, the amount and activities of which are markedly increased in endometriosis women.[1] Study on rats indicate that alternatively activated macophages (M2) dramatically increase growth of endometriosis lesions, whereas inflammatory macrophages (M1) effectively protect them from endometriosis.[15]

L-selectin functions in the initital recruitment of circulating leucocytes in to peripheral inflammatory sites, known as rolling leucocytes followed by activation, marked adhesion, and leucocyte transmigration to interstitial tissues.[16]

A immunohistochemical study comparing L-selectin locations in rat model endometriosis tissues and human counterparts revealed that L-selectin was more dominantly expressed in interstitial spaces, including macrophages and lymphocytes compared to endometriosis epithelium, and concluded that L-selectin plays a significant role in the pathogenesis of endometriosis.[17]

No study has previously assessed the association between L-selectin gene Polymorphism with endometriosis. This study was therefore conducted to assess the association between P213S polymorphism with events of endometriosis, and to observe the role of alternatively activated macrophages (M2) compared to scavenger macrophages (M1) in the pathogenesis of this disease.

METHODS

This comparative analytical, two population case-control study was approved by the health research ethical committee of North Sumatera c/o Medical School, Universitas Sumatera Utara and was conducted at the Department of Obstetrics and Gynecolocy at Haji Adam Malik General Hospital, Dr. Pirngadi Medan Hospital, Networking hospitals and private clinics throughout Medan from January 2013 - March 2014 until the minimum required amount of samples was obtained. Reproductive aged women, complaining menstrual pain, abdominal located bulging, and/or infertility, visiting the Gynecology outpatient clinics at Haji Adam Malik General Hospital, Dr. Pirngadi Medan Hospital, satellite hospitals and private hospitals throughout Medan, comprised the study population, from which patients suspected with endometriosis based on a history taking, physical and sonographic examination and meeting the inclusion criteria were taken as samples through consecutive sampling. Sample size was statistically determined ($\alpha = 0,05 \rightarrow Z_\alpha=1,96$ dan $\beta = 0,20 \rightarrow Z_\beta=0,84$), from which the case and control group were both allocated with 22 samples each. The case group comprised of women diagnosed with endometriosis whereas the control group consisted of non endometriosis women.

This study aimed on determining an association betweeen L–selectin gene P213S polymorphism with cases of endometriosis, using M1 and M2 macrophage profiles/ratios as intermediating variables between the two. Previous studies showed that potentially disrupting factors include history of ischemic stroke, spinal muscular atrophy, and celiac disease and were consequently considered exclusion criterias.

Two hypotheseses were proposed. The first, L–selectin gene P213S polymorphism was hypothesized to be associated with the events of endometriosis, whereas the second was to propose a role discrepancy between M1 and M2 macrophages in the pathogenesis of endometriosis.

Twenty to 45 year old women, laparascopicsally/laparatomically and histopatahologically confirmed diagnosed with endometriosis, with irregular menstrual cycles and also willing to participate after filling a written informed consent form, were included in the case group. Twenty to 45 year old women, laparascopicsally/laparatomically and histopatahologically confirmed not diagnosed with endometriosis, eg., cases of self requested tubectomy, regular menstrual cycles, and also willing to participate after filling a written informed consent form were assigned to the control group.

Gynecological disorders, including non endometriosis ovarian cyst, ovarian tumor, and uterine myomas, endocrine disorders, currently on hormonal medication for minimally 3 months prior to recruitment, with a previous pelvic surgery, history ischemic stroke, muscular spinal atrophy, and Celiac disease, or patients who self withdrew, were excluded from the study.

Visiting patients at the gynecology outpatient clinic were taken a complete history, and then underwent physical/gynecological and sonographic examinations to determine an indication (including history of infertility, dysmenorrhea, self requested tubectomy, endometriosis cyst, etc) to perform a laparoscopy/laparotomy, and if present, a laparoscopy was scheduled to take place during the menstrual cycle of the proliferation phase.

The presence of endometriosis was assessed during laparoscopy/laparotomy and was further confirmed by histopathologically examining peritoneal tissues. Laparoscopy is an endoscopic procedure that directly visualizes the peritoneal cavity using a small telescope. Endometriosis was defined as an extra uterine ectopic endometrial glands or stromas, the presence of which were confirmed laparascopically or histopathologically. Patients diagnosed with endometriosis were included in the case group, after which severity was assessed based on the

Revised Classification of Endometriosis, American Society For reproductive Medicine (ASRM), 1997 (see fig. 1), which is as follows : stage I (minimal degree) : ASRM score 1-5, stage II (mild degree) : ASRM score 6-15, stage III (moderate degree) : ASRM score 16-40, and stage IV (severe degree) : ASRM score >40.
Non endometriosis patients were categorized in to the control group based on inclusion and exclusion criterias. During the procedure (laparoscopy/laparotomy), peritoneal tissues were sampled and 5 cc blood taken by venous punctures from the ante cubiti vein area. DNA isolation and genotyping was performed on these samples, whereas peritoneal tissue samples were histopathologically and immunohistochemically examined by semi-quantitative percentage of cells (Score 0: 0%; 1: 1-25%; 2: 26-50%; 3: 51-75%; 4: >75%).

Histopathological dan Imunohistochemical Examination of Peritoneal Tissues

Peritoneal tissue samples were examined at the Pathology laboratory, Medical Faculty, University of Sumatera. During the procedure, peritoneal tissues of endometriosis and non endometriosis samples were biopsied and fixated using 10% buffer formalin. Fixated tissues were dehydrated by alcohol for 1 hour and 30 minutes, and then cleared using xylene, from which paraffin block were made. These blocks were then cut into 4 µm thick pieces, inserted into a water bath, and subsequently placed on a previously glycerine spreaded object glass. Specimens were then defarafinized using xylol, rehydrated with alcohol, rinsed with streaming water, and stained using Haemotoxyline-Eosine. These specimens were histopatahologically examined under a light microscope with a 400 time magnification, immunohistochemically proccessed using monoclonal mouse anti-human-CD68 (Clone PG-M1) and anti-CD163 (Dako) (BD Pharmingen) staining, and then rinsed using Tris Buffered saline (TBS) pH 7,4. DAB was then combined with a chromogene solution and was diluted with the following ratio : 20 µL DAB : 1000 µL substrate. On cleaning with streaming water, specimens were counterstained using haematoxylin and 5% lithium carbonate, and cleaned again with streaming water, after which samples were dehydrated with alcohol and cleared with xylol. Samples were then microscopically examined. Immunoprecipitated areas of M1 and M2 macrophage cells [with anti-human-CD68 mouse monoclonal markers (Clone PG-M1) and anti-CD163 presented peritoneal tissue slices] were calculated in antigen percentages.
Macrophage (M0) is defined as a monocyte originated macrophage. Main types include: peritoneal and alveolar macrophages; hystiocytes; liver Kupffer cells, and osteoclasts. These macrophages function in non specific (innate) and specific (cell-mediated) immunity systems and by fagositizing cellular and pathogen debris, acting as both a stationary or mobile cell, and also stimulating lymphocytes and other immune cells to respond against pathogens. M1 macrophage is a macrophage induced by the classical profile activation triggered by microbial products, including LPS or TH1 cytokines (IFN--γ and TNF-α). M1 macrophage synthesizes and releases pro-inflammatory factors, including IL-1β, IL-6, IL-12, and TNF-α, producing high concentrated NO and reactive oxygen intermediates (ROIs), and type-1 chemochins, such as CXCL-10, CXCL-11, and CCL5. M1 macrophages are associated with microbial activities, tumor resistance, and cytotoxic tissue damage.
Under stimulation by specific cytokines, eg. IL-4, IL-10, IL-13, and non GM-CSF M-CSF, LPS, INF, etc, M2 macrophages alter the inflammatory and adaptive immune proccess, thus promoting cell proliferation by producing growth factors and arginase pathway products, engulf debris by expressing scavenger receptors, and support angiogenesis, tissue remodelling and regeneration, suppress the adaptive immunity system and support endomteriosis growth. CD-163 is a Hemoglobin/haptoglobin complex scavenger receptor, expressed dominantly by M2 macrophages. CD-68 (Clone PG-M1): M1 macrophage surface marker.

DNA Isolation

During laparoscopy, 500 µL peripheral blood samples were taken from case and control groups by venous punctures from the antecubiti vein, from which DNA was extracted and analyzed. Genom DNA was extracted using a High Pure PCR Templete Preparation Kit (Roche Applied Biosystem). and then restored using an EDTA anticoagulant at 4°C, until used for analysis.
DNA is a polimere of deoxyrybonucletide units. A nucleotide consists of a nitrogen base, one glucose and one more phosphate sequences. The genome is defined as the most comprehensive complement of human DNA.

Genotyping

L-selectin gene P213S polymorphism genotyping was examined using the PCR-RFLP method. PCR is a technique used to amplify small fragments or DNA areas into sufficiently larger particles adequate for analysis, using electrophoresis and blotting. These fragments were amplified in a Perkin Elmer Gene Amp PCR system 2400, in 25 µL reaction volumes containing 200 ng genome DNA, 200 µM for every dATP, dCTP, dGTP, dTTP, 50 mM KCl, 10 mM Tris-HCl, 3mM $MgCl_2$, 0,5 µM from each primary, and 1 unit Taq polimerase (FastStart Taq DNA Polymerase, dNTPPack, Roche Applied Biosystem). The primary sequence was as follows: forward 5'-TGATTCAGTGTGAGCCTTTG-3' and reverse 5'-CTTGACAGGTTGGTTCTG-3'. The PCR reaction was performed in the following condition: 2 minutes at 94°C, 30 cycles for 1 minute at 94°C, 50 seconds at 59°C, 40 seconds at 72°C and 1 minute at 72°C.
Gen polymorphism was defined as inter various gene DNA sequences between individuals, groups, or populations. L-selectin gene P213S polymorphism gene is an L-selectin gene polymorphism, in which the

cytokine base is converted into thymin on codon number 213, causing the conversion of proline (P) into Serine (S).

PCR products (length 186 base pairs) were verified using agarosa gel electrophoresis (2%) and then digested for 3 hours using a 5 5 U/reaction Hph I enzyme (New England Biolabs, Inc. Beverly, USA), according to manufacturer instructions. On ethidium bromida staining, restricted products were visualized on the 8% polyacrilamide gel under ultraviolet light. DNA were isolated and genotyped at the Medan Prodia Laboratory in cooperation with the Eijkman Molecular Biology Institute, Jakarta.

Data were univariately and bivariately analyzed. Descriptive data were univariatelt analyzed. Using bivariate analysis, variables were analyzed using a chi-square test (a p value<0,05 was considered statistically significant) to compare genotype distribution of polymorphism and macrophage profiles between case and control groups.

RESULTS

On histopatahologically confirming endometriosis in patients who previously underwent laparotomy/laparoscopy at Haji Adam Malik General Hospital, Permata Bunda Hospital, and Stella Maris Hospital from Januray 2013-March 2014, 23 subjects were enrolled and assigned to the case group. Whereas, 23 non endometriosis subjects, previously undergoing a laparoscopic tubectomy at the Mantap Sterilisation Clinic, Medan and confirmed with no peritoneal or reproductive organ lesions of endometriosis, were assigned to the control group. Blood samples were taken preoperatively and only non endometriosis tissues were histopatahologically and immunohistochemically examined.

Subjects Demographic Characteristics

Demographic characteristics are shown in table 1. Endometriosis and non endometriosis subjects were averagely aged 32,83 and 35,48 years old, respectively. The case group was dominated by stage 4 endometriosis patients (69,9%). No cases of stage endometriosis were encountered. Ethnicity was diversed as subjects came from various areas.

Table 1. Subject Demographic Charecteristics

Charecteristics	Endometriosis	Non Endometriosis
Age, Mean (±SD)	32,83 (±6,827)	35,48 (±5,877)
Ethnic, n (%)		
Acehnese	2 (8,7)	2 (8,7)
Bataknese	9 (39,1)	5 (21,7)
Javanese	8 (34,8)	11 (47,8)
Indian	1 (4,3)	-
Malay	1 (4,3)	2 (8,7)
Nias	1 (4,3)	1 (4,3)
Tionghoa	1 (4,3)	-
Minang	-	2 (8,7)
Endometriosis Stage, n (%)		
Stage 1	-	-
Stage 2	2 (8,7)	-
Stage 3	5 (21,7)	-
Stage 4	16 (69,6)	-

Distribution and Association between Genotype and P213S L-selectin allele

Table 2 displays genotype frequency rates of L-selectin gene P213S polymorphism in endometriosis and non-endometriosis subjects. 30,4%, 56,5%, and 13% endometriosis subjects had a PP, PS, and SS genotype, respectively whereas 60,9%, 39,1%, and 0% non-endometriosis subjects had a PP, PS, and SS L-selectin gene P213S polymorphism genotype, respectively. Chi-square tests showed that genotyping significantly differed between endometriosis and non endometriosis subjects, with a p value of 0,048.

Tabel 2. Association between genotype and cases of endometriosis

Genotypes	Subjects		p*
	Endometriosis	Non Endometriosis	
PP	7 (30,4%)	14 (60,9%)	0,048
PS	13 (56,5%)	9 (39,1%)	
SS	3 (13%)	0 (0%)	
Total	23 (100%)	23 (100%)	

**Chi-square test*

This study focused on the P and S allele (of L-selectin gene P213S polymorphismgene) and the possible associated role in developing endometriosis, assuming that the P coversion to S would increase incidence rates of this disease. Results from table 3 show that the S allele has higher frequency rates in endometriosis subjects than non endometriosis subjects (41,3% vs 19,6%). The P and S allele were correlated with the diagnosis of

endometriosis, with correlation coefficients of 0,363 (pvalue 0,48) and 0,383 (p value 0,034), respectively.

Table 3. Association between L-selectin P213S allele and endometriosis

Allele	Subjects		R	p*
	Endometriosis	Non Endometriosis		
P	27 (58,7%)	37 (80,4%)	0,363	0,048
S	19 (41,3%)	9 (19,6%)	0,383	0,034
Total	46 (100%)	46 (100%)		

**Chi-square test*

Assuming that the S allele is associated with increased rates of endometriosis, table 4 shows that S containing genotypes (PS and SS genotype) are more frequently encountered in the case groups than the control group (69,6% vs 39,1%). This fact also applies for endometriosis subjects, with higher incidence rates of S containing genotypes (PS and SS, 696%), than the P containing type (30,4%). Increased S allele containing genotype rates (PS and SS) in cases of L-selectin P213S gene polymorphism was significantly associated with events of endometriosis (p = 0,038).

Table 4. Association between PP and PS+SS L-selectin gene P213S polymorphism genotypes and endometriosis

Genotypes	Subjects		p*
	Endometriosis	Non Endometriosis	
PP	7 (30,4%)	14 (60,9%)	0,038
PS + SS	16 (69,6%)	9 (39,1%)	
Total	23 (100%)	23 (100%)	

**Chi-square test*

Table 5 displays the extent of association between S alleles and events of endometriosis, from which the presence of this allele is associated with a 3 times higher risk of developing endometriosis (OR 2,893; IK 95% 1,135 - 7,373, p value 0,026). The results of this table also show that S allele carrier containing genotypes (SS and PS) have a 4 times higher risk than the PP genotype (OR 3,556; IK 95% 1,049 - 12,052, p value 0,04).Table 6 shows that PP, PS, and SS containing genotypes were dominantly staged 4 (37,5%, 43,8%, and 18,8% respectively), whereas all SS genotyped subjects had stage 4. Only 5 PS genotyped subjects had stage 3, whereas one PP and PS genotyped subject each were staged 2. On classifying endometriosis into two groups, minimal-mild and moderate-severe, no significant association was apparently established between severity degrees and P213S L-selectin gene genotypes, the results of which are displayed in table 7 (p value 1,0).

Table 5. Association between S allele and L-selectin gentype with cases of endometriosis

Subjects	p*	OR*	IK 95%
S Allele	0,026	2,893	1,135 – 7,373
PS + SS Genotype	0,041	3,556	1,049 – 12,052

**Mantel-Haenszel Common Odds Ratio Estimate*

Table 6. Distribution of endometriosis stages based on L-selectin P213S genotypes

Subjects	Genotype		
	PP	PS	SS
Stage 2	1 (50%)	1 (50%)	0 (0%)
Stage 3	0 (0%)	5 (100%)	0 (0%)
Stage 4	6 (37,5%)	7 (43,8%)	3 (18,8%)

**Kolmogorov-Smirnov Z test*

Table 7. Association between severity degrees of endometriosis and L-selectin P213s genotypes

Severity degrees of Endometriosis	Genotype			p*
	PP	PS	SS	
Minimal – Mild	1 (50%)	1 (50%)	0 (0%)	1,0
Moderate – Severe	6 (28,6%)	12 (51,7%)	3 (14,3%)	

Distribution and Association Between Macrophages and Endometriosis

Specimens were blindly immunohistochemically examined by two pathologists, from whom the clinical features of each group were with held. Table 8 shows kappa values of 92,6% and 100% for CD68 and CD163, respectively, indicating a high inter observer uniformity in assessing CD68 and CD 163 expression.

Table 8. Kappa value to Observer 1 and Observer 2

Observer Compatibility	Value	p value
CD68	0,926	0,0001
CD163	1,000	0,0001

Semi-quantitative immunohistochemical scoring shows that 21,72%, 26,1%, and 21,7% endometriosis subjects express CD68 +1, +2, and +3 macrophages, respectively (overall 69,6% expressed CD68), with a remaining 30,4% non CD68 expressing subjects, with eta coefficient was 0,647 (table 9). Only 8,7% of non endometriosis subjects expressed CD68 macrophage, with a IHC score of +1. Contradictively, all endometriosis subjects expressed CD163 macrophages, with the following incidence rates: 21,7%, 30,4%, and 47,8% subjects expressing +1, +2, and +3 CD 163 macrophages, respectively, the results of which are displayed in table 10. Whereas only 8,7% of non endometriosis subjects expressed CD163 macrophages.

Table 9. Association between CD68 macrophage expression and endometriosis

	IHC SCORE				r*	p**
	0	+1	+2	+3		
Endometriosis	7 (30,4%)	5 (21,7%)	6 (26,1%)	5 (21,7%)	0,647	0,001
Non endometriosis	21 (91,3%)	2 (8,7%)	0 (0%)	0 (0%)		

*Eta Correlation, **_Chi-square test_

Table 10. Association between CD163 macrophage expression and endometriosis

	IHC SCORE				r*	p**
	0	+1	+2	+3		
Endometriosis	0 (0%)	5 (21,7%)	7 (30,4%)	11 (47,8%)	0,936	0,001
Non endometriosis	21 (91,3%)	2 (8,7%)	0 (0%)	0 (0%)		

*Eta Correlation, **_Chi-square test_

Results from table 11 shows that macrophages from CD68 expressing endometriosis lesions significantly differ to their counterparts from CD163 expressing lesions, with positive CD68 and CD163 cell expressions of 34,783 ±28,939 and 60,870 ±18,194, respectively (p<0,0001). Alternatively, activated CD163 macrophages were more frequently encountered than CD68 counterparts Immunohistochemical testing reported that CD68 and CD163 macrophages infiltrate endometriosis lesions, although non endometriosis specimens also revealed similar findings.

Table 11. Macrophage positive cell percentages: a comparison in endometriosis

Marker	Mean Positive cells (±SD)	CI 95%	p*
CD68	34,783 (±28,939)	22,27 – 47,30	0,001
CD163	60,870 (±18,194)	53,00 – 68,74	

*_Independent sample t-test_

Results from table 12 show that CD68 expression did not significantly differ between degrees of severity (p-value 0,995). Expression of lesion infiltrating macrophages did not differ in moderate-severe cases, with 28,6%, 23,8%, 28,6% and 19% endometriosis subjects expressing CD68 IHC scores of 0, +1, +2, and +3, respectively. Mild-minimal degree subjects also showed a similar tendency. Although expression rates was increased in moderate-severe cases (23,8%, 28,6%, and 47,6% subjects expressed +1, +2 and +3 CD163), CD163 expression also did not significantly differ between degrees of endometriosis, as shown by results displayed in table 13 (p-value 1,0).

Table 12. Association between severity degrees of endometriosis and CD68 expression

Severity degrees of Endometriosis	Immunohistochemical scoring of CD68				p*
	0	+1	+2	+3	
Minimal – Mild	1 (50%)	0 (0%)	0 (0%)	1 (50%)	0,995
Moderate – Severe	6 (28,6%)	5 (23,8%)	6 (28,6%)	4 (19%)	

*_Kolmogorov-Smirnov Z test_

Table 13. Associaton between severity degrees of endometriosis expression of CD163

Severity degrees of Endometriosis	Immunohistochemical scoring of CD163			p*
	+1	+2	+3	
Minimal – Mild	0 (0%)	1 (50%)	1 (50%)	1,0
Moderate – Severe	5 (23,8%)	6 (28,6%)	10 (47,6%)	

*_Kolmogorov-Smirnov Z test_

Table 14 displays that S allele containing (PS and SS) genotypes was encountered in 68% of CD163 macrophage expressing specimens, indicating that CD163 expression (M2) was significantly associated with certain genotypes (p-value 0,043).

Table 14. Association between L-selectin gene P213S polymorphism genotype on expression of CD163 and CD68

Genotype	Expression of CD163		p*	Expression of CD68		p*
	Positive CD163	Negative CD163		Positive CD68	Negative CD68	
PP	8 (38,1%)	13 (61,9%)	0,043	7 (33,3%)	14 (66,7%)	0,46
PS + SS	17 (68%)	8 (32%)		11 (44%)	14 (56%)	

**Chi-square test*

However, contradictive results are shown, indicating that P213S genotypes of L-selectin genes were apparently not significantly associated with CD68 expression. CD68 was found positive in 33,33% and 44% of PP and PS+SS genotypes, respectively. The expression of CD 163 is displayed in figures 8 and 9.

Hypothesis Testing

Hypothesis I

Bivariate analysis shows that S allele subjects had a 2,893 (95% CI; 1,135-7,373) higher risk of developing endometriosis than P allele subjects, with a p value of 0,026 ($p<0,05$). Results also revealed that PS and SS genotyped subjects had a 3,556 higher risk of developing endometriosis than their PP genotyped counterparts (95% CI; 1,049-12, 052, with a p-value 0,041), thus indicating that L-selectin gene P213S polymorphism is associated with the incidence of endometriosis.

Hypothesis II

Analysis through differentiating average positive cells expressed from CD68 and CD163 antigenes (designated as M1 and M2 respectively) showed that CD163 was more frequently expressed than CD68, in endometriosis lesions (60,870 ±28,939 vs 34,783 ±18,194) with a p-value 0,0001. Positive immunohistochemical scorings revealed that CD68 macrophages was less frequently expressed (69,96%) than CD163 macrophages (100%) with a p-value 0,0001, indicating different roles of M1 and M2 macrophages in the pathogenesis of endometriosis.

DISCUSSION

This study showed that S allele subjects (with L-selectin gene P213S polymorphismgene) have a 3 time higher risk than P allele subjects to develop endometriosis (OR 2,893; 95% CI; 1,135-7,373 with a p value 0,026). SNP's are various sources of a genom and is generally a mutated base form. They are the most simple and common form of human genetic polymorphism.[18] As they are located on regulator elements (such as gene promotors, enhancers or silencers) and coding regions, synonymous SNPs, may cause amino acid alterations, frame shifts or terminated translations, consequently providing functional effects on protein levels.[11] P213S polymorphism is itself an SNP, in which the 637th base is converted (from Cytosine to Thymine on exon 6), resulting in amino acid coding on codon 213 (from Proline to Serine). Exon 6 located on the L-selectin gene, is known as the domain of the Short Consensus Repeat (SCR1), that functions in cell adhesions, oligomerization, optimizes Epidermal Growth Factor (EGF) and lectin domains. Consequently, exon polymorphism would effect L-selectin gene SCR1 associated functions.[19] In this study, this event is stressed as mostly associated with the events of endometriosis.

This study shows that PS and SS genotypes are more significantly associated with the events of endometriosis than PP genotyped specimens, whereas no non endometriosis subject was SS genotyped. S alleles was more frequently encountered in endometriosis specimens than the control group. However, this study demonstrated that severity degrees of endometriosis was not associated with L-selectin gene P213S polymorphism.

Combining L-selectin with monoclonal and ligand antobodies may cause alterations in cellular morphology, cellular mechanical action patterns, and increase IL-9 levels and expression of TNF-α genes.[20,21] IL-8 is increased in peritoneal fluids of women diagnosed with endometriosis and correlates with disease severity, and may act as an endometrial autocrine growth factor. Consequently, IL-8 may affect endometrial cells and cellular growth. TNF-α is an activated macrophage product that activates inflammatory leucocytes and is directed towards production of other proinflammatory cytokines, including IL-1, IL-6, and additional TNF-αs. As a result, endomterial cellular adhesions are stimulated, inducing MMP expression, and participates in ectopic endometrial tissue implantation to the peritoneum.[22]

Odagiri et al suggested that intertitial cellular located L-selectin was expressed in human endmetriosis, including lymphocytes and macrophages, however, L-selectin was not expressed in epithelial L-selectins. This finding concluded the significant role L-selectin has in endometriosis.[17] In a rat model, endometriois was observed with a 46 time mRNA transciption increase compared to normal endometrial tissues.[23]

Peritoneal macrophage numbers and activated status are increased in women diagnosed with endometriosis. Increased growth factors and cytokines affect ectopic endometrial cellular survival and growth, and may cause receptor function disability. Activating macrophages, through cytokine and growth factor release, may contribute to the concurrent initial formation and development of endometriosis.[22] Monocyte/macrophage recruitment and

activation induces a local inflammatory reaction and is highly affected by macrophage activating factors produced by both eutopic and ectopic endomterial cells, including MCP-1, migration inhibitory factor (MIF) macrophages, and IL-1, RANTES.[24,25,26]

Macrophages are known to regulate antitumor immunity response. In the presence of stromal remodelling related-tumor growth factors and production of proinflammatory molecules as foreign bodies, M1 macrophages (similar to NK cells) responds through IFN-γ dan IL-12 secretions.[27,28] By activating IFN-γ, M1 releases tumor recidual products including reactive oxygen intermediates and nitric oxides that destroy tumor cells.[29] These activated macrophages also produces TNF-α, that also destroys foreign bodies by inhibiting vascular formation.[30]

Macrophage alternative activation (M2) is a key step in the development of endometriosis, from which increased M2 macrophages secrete and increase concentrations of prostaglandin, complement components, and growth factors including TNF-α, IL-6, and TGF-β, IL-4, IL-13, IL-10, IL-1.[33,34] Edometriosis cells entering the peritoneal cavity are normally eliminated by macrophages. This mechanism of aberration in endometriosis results in an ineffectve immunological cleaning system towards foreign bodies. M2 macrophages and increased cytokine levels induce the initiation of endometrial cellular prrogression, growth, and neovascularization.[33] Consequently, M2 macrophages tend to have a beneficial effect on cellular growth or tumors.

M2 macrophages play a more significant role than M2 macrophages in the pathogenenesis of endometriosis. This may be due to genetic, hormonal, and enviromental factors. One study suggested that estrogen increases M2 macrophage activities through estrogen receptors expressed by surface located macrophages. Under these effects of estrogen, M2 macrophages secrete cytokines and growth factors (including VEGF, hepatocyte growth factors, TNF-α), contributing to the developing and persistance nature of endometriosis.[35]

This study indicated that CD68 and CD163 macrophages expressions significantly differed. In endometriosis lesions, CD 163 macrophages were apparently higer than CD 68 macrophages. CD 68 was also observed in eutopic endometrial women diagnosed with endometriosis with various levels in each menstrual phase. Increased CD68 macrophage cells was also present in eutopic endometrial women diagnosed with endmoteriosis during the proliferative phase, whereas CD162 was only observed in endometriosis lesions. Although observed in non-endometriosis peritoneal specimens, immunohistochemical testing showed that CD68 also infiltrates endometriosis lesions.[13,15]

CONCLUSION

L-selectin gene P213S polymorphism is associated with endometriosis. M2 Macrophage has a more significant role in developing endometriosis than M1 macrophage.

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Figure Legends

Figure 1. PCR electrophoresis of three genotypes. NB : L = Ladder 100 bp; Lane 10 PP genotype, visible band on 142 bp and 44 bp; lane 11 PS genotype visible band on 186 bp, 142 bp and 44 bp; lane 12 SS genotype visible band on 186 bp.

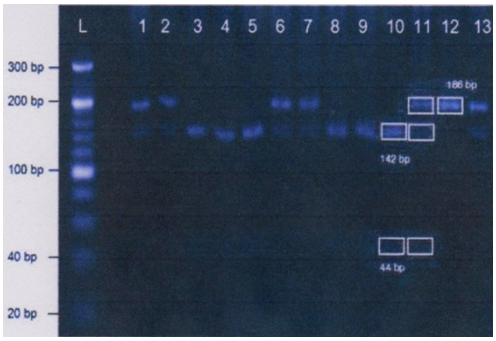


Figure 2. Expression of CD163

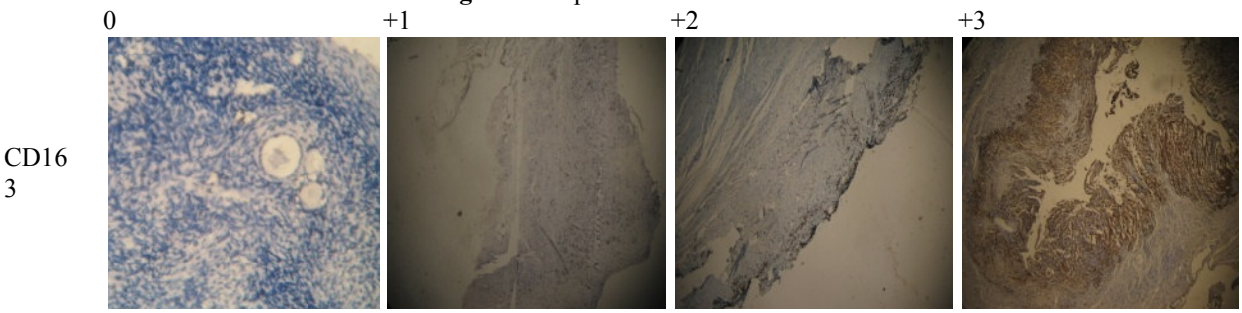
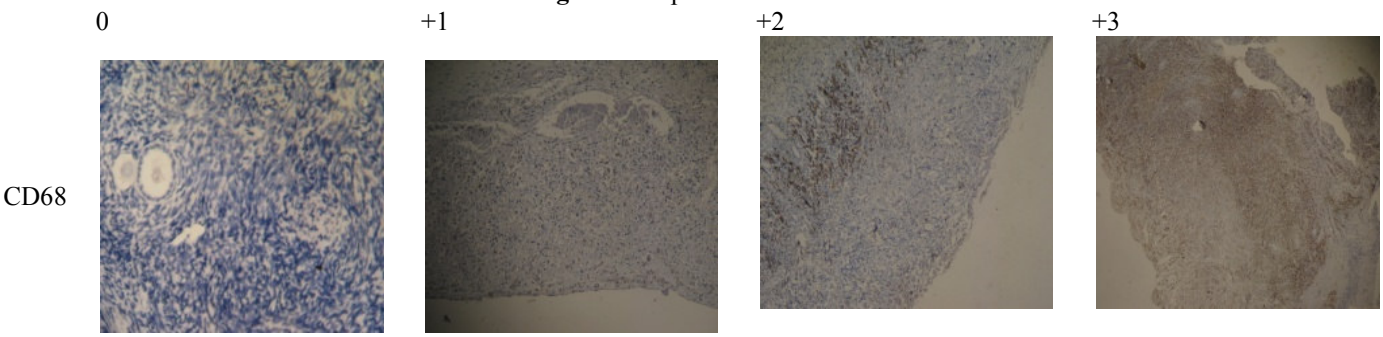


Figure 3. Expression of CD68



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