Peritoneal cytokines in patients evaluated for infertility.

Hilde JØRGENSEN, MD [a,] Peter FEDORCSAK [a, c], MD, PhD[a], Keith Isaacson[b], MD, Abby HILL[b],Erik QVIGSTAD, MD, PhD [a, c], Douglas Lauffenburger[b] , PhD, Linda Griffith[b], PhD

[a] Department of Gynecology, Oslo University Hospital, Ullevål, Norway

[b] Center for Gynecopathological Research (CGR), Massachusetts Institute of Technology(MIT), USA

[c] Faculty of Medicine, University of Oslo, Norway.

Corresponding author: Hilde Jørgensen, E-mail: hildesen5@gmail.com

Department of Gynecology, Oslo University Hospital, Ullevål, Pb 4965, Nydalen, 0424 Oslo, Norway, Telephone number: + 47-22 11 98 00

Abstract

Objective: We investigated inflammatory pathways in peritoneal fluid derived from infertile women with a high incidence of endometriosis with the aim of delineating cytokine signatures that can be related to specific clinical phenotypes, thus potentially revealing mechanistic insights into the clinically heterogeneous disease of endometriosis.

Design: Prospective cohort study

Setting: A collaboration study between Department of Gynecology at Oslo university Hospital and Center for Gynecopathological Research (CGR), Massachusetts Institute of Technology (MIT). Surgery and collection of clinical data were performed in Norway. All cytokines assays and quality control analyses were done at CGR.

Population: Patients undergoing laparoscopy for infertility assessment (n=107).

Interventions: Peritoneal fluid and endometrial biopsies were collected during surgery. All patients answered pain questionnaires (Brief Pain Inventory short version) and clinical parameters were registered preoperatively, after 6 months and after 12 months.
Main outcome measures: We determined the concentration of 48 different cytokines from the peritoneal fluid with multiplex immunoassays. Univariate analyses were done to identify correlations between individual cytokines and clinical findings. To identify clusters of patients with common cytokine profiles we performed an unsupervised multivariate analysis.
Results: Concentration of MCP-1 and SCGF-β were significantly higher in the endometriosis group compared to infertility patients without endometriosis . Concentrations of IL-6 and IL-8 were higher in severe versus no endometriosis. IL-8, IP10 and SCGF-β concentrations positively correlated to pain scores. Multivariate analysis identified 3 clusters of patients with different covariation of 10 cytokines in the PF, but because of low reproducibility with different methods we considered this as not biologically significant. Disuss with Manu et al
Conclusions: Peritoneal fluid MCP-1, SCGF-β, IL-6 and IL-8 may be useful indicators for endometriosis and disease severity in patients with infertility. IL-8, IP-10 and SCGF-β concentrations are correlated to pain intensity.
Keywords: Cytokine, endometriosis, infertility, peritoneal fluid

Introduction

Endometriosis is a common gynecologic disease affecting up to 10% of all women in reproductive age (1). The disease is defined by the presence of endometrial tissue outside the uterine cavity. Apart from infertility, the most common symptoms associated with endometriosis are chronic pelvic pain, dyspareunia, dysmenorrhea, heavy uterine bleeding, dyschezia, and dysuria (2). The disease often has significant negative impact on quality of life, work ability and educational career (3,4).

There are several hypotheses regarding the etiology of endometriosis. Sampson's theory of retrograde menstruation is widely accepted for peritoneal endometriosis (5). Endometrial cells repelled during menstruation are thought to implant on the peritoneal surface. The implants elicit an inflammatory response accompanied by angiogenesis, nerve sprouting, adhesions, fibrosis and

scarring (6,7). The theory does not explain why 95% of women experience retrograde flow of menstruation fluid through the Fallopian tubes while only 10 % of women in the reproductive age suffer from the disease.

Endometriosis has been scored in different ways according to degree of adhesions, distribution of lesions and depth of invasion. The most widely used classification was developed by the American Society of Reproductive Medicine (ASRM) (8). The ASRM staging system divides the disease into four stages based on visual appearance during laparoscopy. Paradoxically, many women with endometriosis, independently of stage have few or no symptoms (9). In spite of decades of research in this field, we have no predictable markers for the disease intensity and long-term outcome (10), nor do we understand why the presence of ectopic endometrium is symptomatic in some women and not in others.

Since endometriosis currently requires surgery and preferably biopsy for a definite diagnosis, it is difficult to obtain a true estimate of the prevalence of the disease. Adding to this difficulty is the fact that the endometriotic lesions vary in size, shape, color and depth of invasion (11).

Several studies have described the peritoneal and endometrial cellular environment in endometriosis (12, 13). Protein and cytokine analyses of the peritoneal fluid partly explain adhesion formation and cellular invasion (14,15). However, there are still missing links before we know how the pelvic cavity and the endometriotic cells interact in a microenvironment to yield the clinical outcome. A systematic biologic approach to examine the different immune, hormonal and inflammatory pathways interact, will give us a better understanding of the clinical diversity of endometriosis (16). By collecting information and samples from different study samples and study them with similar methods, we can obtain comparable results for larger scale international studies. The World Endometriosis Research Foundation published a series of articles in 2014 with protocols for standardized methods for collection of tissue samples and clinical data (17).

In a recent study (18), 50 cytokines from the peritoneal fluid of women undergoing surgery for various gynecological conditions, including endometriosis, were measured simultaneously in multiplex assays. Unsupervised multivariate analysis sorted the endometriosis-cases into two classes depending on cytokine profile. The profiles were then linked to established protein-expression databases and related to clinical characteristics. This way of studying endometriosis, through network behavior, was a novel approach.

The aim of the present study is to correlate patient characteristics and clinical symptoms that are associated with endometriosis with molecular profiles in the peritoneal fluid of a group of women with infertility as the primary symptom. By utilizing similar methods and protocols on different patient populations, we can compare results across study groups and countries and eventually develop a more comprehensive understanding of the pathophysiology of endometriosis. Ultimately, our goal is to understand why endometriosis develops, why it produces unpredictable symptoms, why it recurs after treatment, and most importantly, help clinicians to target therapy to a specific patient profile with a predictable outcome. Amen! But can you give a foreshadowing of the 1-year follow up study so that the "recurrence prediction" is put in context of this study?

The study was performed as collaboration between Department of Gynecology, Oslo University Hospital and Center for Gynecopathological Research (CGR), Massachusetts Institute of Technology (MIT).

Materials and Methods

Study design and patient selection

The study was designed as a prospective cohort study with a one-year follow up after inclusion. Patients evaluated for inclusion, were all undergoing infertility assessment by laparoscopy from September 2013 until November 2014. All women signed an informed consent approved by Oslo University Hospital and The regional ethical committee (REK). The study was approved by REK before the inclusions started. The patients already had a gynecological examination before referral to surgery. On the day of surgery, we collected clinical data from all participants. Cycle phase was calculated from the last menstrual period. Exclusion criteria were irregular cycles (< 25 days or >35 days), hormonal therapy during the last three months, other intrabdominal diseases or inability to understand written consent or written follow-up questionnaires.

Sample size, was determined after a power analysis based on a 80% test power was performed and concluded that at least 60 patients was needed to detect significant different levels of cytokines in the peritoneal fluid between the endometriosis group and the control group. This is in concordance with a similar study (18) performed on a different patient sample.

Sample Collection and Processing

Before surgery and before the diagnosis of endometriosis was made, the patients completed questionnaires about clinical characteristics and pain. We used the Norwegian language version of the Brief Pain Inventory (BPI) (19). The BPI contains four items of pain severity and seven items of pain interference with numeric rating scales. The BPI was originally made for cancer pain but has been used for endometriosis previously (20). The patients answered the BPI (short form) before surgery, 6 months after and 12 months after surgery. Information about clinical characteristics and fertility outcomes were also collected after 6 and 12 months for a later follow up study.

Peritoneal fluid (PF) was collected from the cul de sac at the beginning of the laparoscopic surgery after insertion of the trocars and before any manipulation of the pelvic organs. We used a thin suction cannula or a suction tube for aspiration of undiluted PF. Patients were evaluated for the presence of endometriosis and staged according to ASRM criteria. The diagnosis was confirmed with peritoneal biopsy in 73% of the cases. For the remaining 27% the diagnosis was made by visual inspection of endometriotic lesions. The PF was stored on ice up to 45 minutes before it was centrifuged on 300g for 5 minutes to pellet cells. The supernatant was transported to the laboratory on wet ice. The cell pellet was resuspended in phenol red-free Dulbecco's modified Eagle`s medium supplemented with 10% charcoal stripped bovine serum and penicillin/streptomycin right after centrifugation. The fluid was stored in aliquots at -80C. Fifty μl of the cell suspension was saved for cell count with Scepter automated cell counter from Millipore(Sigma-Aldrich. St. Louis, MO). We used a 60uM sensor to count leucocytes only. The suspension was then centrifuged again on 300g and resuspended in the same medium supplemented with 10% dimethyl sulfoxide (DMSO) until a cell concentration of 1 mill cells/ml. The cells were cryo-preserved in 1 ml aliquots and stored at -80C.

All samples were shipped from Norway to USA for analyzes and processing.

The sample collecting protocol and analysis methods we used were developed and validated at CGR on a different patient population (18). To examine if we had comparable samples, 20 of the Oslo PF samples were analyzed in a pilot experiment for validation and compared with the

samples collected locally in Newton, MA, USA. The variation in PF volume, distribution of cycle phase, PF protein content and leukocyte composition were similar in the two study groups. The total cytokine concentration in the PF was well correlated (r=0,81) between samples collected in the U.S and Oslo, indicating that the protocol gave comparable results.

Multiplex Cytokine Immunoassay

The concentration of 48 different cytokines, chemokines and growth factors in the peritoneal fluid were measured with Bio-Plex 200 system (Bio-Rad Laboratories) and data collected with Bio-Plex Manager Software. The cytokines are all included in human cytokine panel I and Π from Bio-Rad.The cytokines were measured in triplicate aliquots of undiluted PF samples.
The mean of median fluorescence intensity (MFI) in 10 parallel aliquots of standard diluents was used as a measurement for the background MFI. The lowest limit of detection (LoLD) was defined as the background plus 2 SD. The LoLD was subtracted from the MFI values before the average (MFI) was converted to absolute concentrations LoLD via calibration to nine- point standard curves. All values below the LoLD were replaced with zero.

Statistical Analysis

Patient characteristics, symptoms and sample parameters were described and evaluated for differences between patients with and without endometriosis. They were also stratified according to different stages of endometriosis. The chi-square test was used for categorical variables and the t-test for continuous variables.
The significance of nonequivalence in BPI scores between the groups was tested with Mann Whitney-U test.

Correlations between BPI scores and individual cytokines were tested with Spearman’s correlation coefficient. The differences in individual cytokine concentrations between the patients grouped according to clinical symptoms were tested with Mann Whitney-u test.

A two-sided p-value <0,5 was considered as significant association. SPSS software was used for statistical analyses.

For multivariate analysis different the cytokine concentrations were log-transformed. Non negative matrix factorization (NMF) was used to classify the cytokines into clusters of covariation. Class assignment stability was quantified by the cophrenic correlation coefficient comparing observed and permutated covariations.

Results

Of the107 patients recruited, four were excluded before sample processing; two patients because of irregular menstrual cycles, one patient had ascites and liver cirrhosis and in one additional case a complication during surgery impeded collection of peritoneal fluid. Of the remaining103 patients, 99 had sufficient volume of peritoneal fluid (PF) collected for Luminex assays. Dataset from two patients with low PF volume were later excluded because of suspected technical errors with the assay.

The clinical characteristics and peritoneal fluid composition of the 97 patients are summarized in Table 1. The primary diagnosis in all patients was infertility. Patients were divided into two groups according to the absence or presence of endometriosis. In 39 women, there was no visual or histological sign of endometriosis. Endometriosis was diagnosed for 58 women. The diagnosis was confirmed by histology of peritoneal biopsies in 70 % and by visual inspection during laparoscopy in 30 % of the cases. The endometriosis disease was classified as mild-to moderate in 78% of the cases.

The occurrence of endometriosis-associated symptoms, including dysmenorrhea, dyspareunia, dysuria, bowel symptoms and infertility, were similar in women with and without endometriosis (Table I). Patients with endometriosis were significantly younger than the ones without the disease. Similar proportions of women were in the follicular and luteal phase of the menstrual cycle at the time of surgery.

Cytokines in the Peritoneal fluid .

We measured the concentration of 48 different cytokines in the undiluted peritoneal fluid using multiplex immunoassay. Six cytokines (IL-17, TNF-ß, IL-1α, IL-15, MIP-1α1, and GM-CSF) concentrations were below the level of detection in all samples. For 23 additional cytokines (IL-2, IL-4, IL-5, IL-7, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-1ra, IL-1ß, TNF-α, RANTES, LIF, M-CSF, PDGFbb, MIP-1ß, MCP-3, TRAIL, IFNγ, GROα, FGFbasic and G-CSF), fewer than 10% of the patients had concentrations above the level of detection. The median concentration of IFNa2, VEGF, and IL-3 was zero in one or more of the compared groups.

We compared the concentrations of the remaining 16 cytokines in women with and without endometriosis (Table 2). The concentration of MCP1 and SCGF-ß were significantly increased in patients with endometriosis. MCP1 and SCGF-ß were higher in more advanced stages of endometriosis when comparing stage III/IV to stage I/II disease (Table 3). The concentration of IL-8 and IL-6 were significantly increased in women with stage III/IV endometriosis compared to women without endometriosis (stage 0).

The effect of age and phase of the menstrual cycle on cytokine concentrations was examined by comparing respective groups pairwise, and no significant difference was found (Supplemental data Table 1).

Unsupervised multivariate analysis identified 3 clusters, but the the cophrenic scores of the observed clusters were not significantly different from the permutated. One of the clusters had significantly more patients with advanced stage endometriosis. We performed the multivariate analysis from two independant places. Both identified one out of three clusters with higher incidence of advanced endometriosis but the cytokines that showed covariation within the clusters were not corresponding.

Pain and Pain interference scores

In order to describe endometriosis-associated pain, we used the worst pain, the mildest pain, and the average pain items form the Brief Pain Inventory (BPI). Pain interference items of BPI described how the pain was interfering with general activity, mood, walking ability, normal work, social relations, sleep, and enjoyment of life. The BPI metrics did not differ significantly between

patients with and without endometriosis (Supplemental data Table 2). When classified according to ASRM stage, there were no differences in the scores when comparing between no endometriosis, mild/ moderate endometriosis and severe endometriosis.

We then divided the scale of maximal pain into none-to-mild pain (NRS= 0-3) and moderate-to-severe pain (NRS=4-10). The same cut off was applied to the pain iterference metrics for work ability and social relations. There was no significant difference for the 3 metrics between women with or without endometriosis, but we observed increased pain burden, work inability and impacted social relations according to endometriosis stage (Supplemental data Table 3).

To investigate the association between pain and peritoneal fluid cytokines, we calculated the correlation coefficient between BPI metrics and concentration of 16 cytokines (see above). There was a significant positive correlation between maximal pain the patients experienced during the last 4 weeks and the concentration of IP-10, IL-8, and SCGF-ß (Table 4). The concentration of IP-10, IL-8, and SCGF-ß also differed significantly when the patients were divided into subgroups of none-to-mild and moderate-to-severe pain (Table 5). Hilde, is this all patients or just patients with endometriosis.

Discussion

Among patients with unexplained infertility, the prevalence of endometriosis is reported to be up to 50% (1,21). We studied 97 patients with infertility as the primary diagnosis and found endometriosis in 60% of the cases. With multiplex assay we measured a panel of 48 different cytokines from the peritoneal fluid collected during laparoscopy. The primary aim of the study was to find markers to differentiate patients with and without endometriosis. Of all the cytokines, two (MCP-1 and SCGF-ß) were significantly elevated in patients with visual or histological proven endometriosis compared to patients without endometriosis. Another two cytokines (IL-6 and IL-8) were elevated only when comparing moderate/severe endometriosis to no endometriosis. Dysmenorrhea is, in addition to infertility, the most frequent symptom in patients with endometriosis (21,22). The reported pain intensity in this study was positively correlated to the cytokines IP-10, IL-8 and SCGF-ß.

Symptoms and demographic charachteristics were recorded before laparoscopy. The patients with endometriosis were significantly younger than the ones without endometriosis. Other studies have demonstrated the same distribution (23,24). This can be attributed to symptoms that bring them to medical care earlier than other patients with infertility. The avarage age of women treated with assisted reproduction is 33 years in Oslo University Hospital. The frequency of dysmenorrhea was high within both groups, but there was no significant difference between patients with and without endometriosis. In the Department of Gynecology, laparoscopy is not routinely done on all patients evaluated for infertility. Patients with dysmenorrhea or other pain related symptoms were selected for surgery more often than patients without symptoms and/or higher age. This can explain why we have had a high frequency of pain in both groups.

A recent cross-sectional study by de Oliviera et al (25) found endometriosis in 50% in 1243 patients with infertility We found endometriosis in 60% of the included patients, which is in the upper range of what has been seen in other studies (22, 26). A selection towards patients with pain can be an explanation to the higher prevalence of endometriosis in our study and to why there was no difference in reported pain between patients with and without endometriosis.

Endometriosis stage I and II was diagnosed according to the ASRM classification in 78% of the cases. In a study from the IVF clinic at Oslo University Hospital from 2012 (27), the proportion of stage I and II endometriosis was 68%. This difference could be due to observer variability (28) and a selection of the patients with suspected advanced disease towards surgery in a specialized endometriosis unit.

There was no difference in the frequency of the different pain symptoms between patients with or without endometriosis or between different stages of the disease. The scores on the BPI (Brief Pain Inventory) questionaires were also equivalent between the groups. These observations confirm other studies indicating that there is no correlation between rASRM stage and pain (29,30).

Of the 48 cytokines we measured, only 16 proteins were detected above the background level for a representative number of patients. In the study from Beste et al (18), they detected 47 out of 50 proteins. This can be due to different Luminex kits and calibration methods. The cytokines we measured above the background had concentrations within the same range. We therefore believe that the two studies are comparable.

The concentration of MCP-1 and SCGF-ß were significantly higher in patients with infertility and endometriosis versus patients without the disease.

MCP-1 is produced by a variety of cells and is a chemoattractant for monocytes, T-helper cells and NK-cells. It is one of the key chemokines for regulation and infiltration of monocytes in response to inflammation. MCP-1 is involved in many inflammatory diseases involving monocytic infiltrates. A sytematic review by Borelli et al (31) showed that MCP-1in the PF of endometriosis patients compared to controls is increased in 54% of the included studies. How MCP-1 is affected by other diseases in the pelvis, is not known.

SCGF-ß is a hematopoietic growth factor. In combination with GM-CSF and erythropoietin, SCGF-ß promotes proliferation of erythroid and myeloid progenitor cells in the bone marrow. The cytokine has been studied in serum samples from patients with dilated cardiomyopathy (32) and silent brain infarction (33) Serum concentrations were increased in both conditions compared to controls. Increased serum levels have also been demonstrated after stem cell transplantation (34). SCGF-ß in the PF was also analysed by Beste et al (18) and they found no difference between patients with endometriosis and controls. This is to our knowledge the only time SCGF-ß has been studied in relation to endometriosis. Among the different theories on the pathegenesis of endometriosis is the contribution of endometrial stem cells and bone marrow derived stem cells as origin for ectopic lesions (35,36). If SCGF-ß elevation in endometriosis can be reproduced in other studies, it could add insight into this theory.

Among the patients with advanced stage endometriosis, we found significantly increased concentrations of IL-6 and I-L8, in addition to MCP-1 and SCGF-ß. Only MCP-1 and SCGF-ß were elevated in stage I and II relative to stage 0 (no endometriosis). In the the study from Beste et al (18) no individual cytokines demonstrated any difference when comparing stage I /II with controls. However, IL-8 was significantly elevated when comparing endometriosis in general to controls. These findings are consistent with those found by Borelli et al who described elevated IL-8 and MCP-1in patients with endometriosis compared to controls (24). In both studies the porportion of advanced endometriosis was higher than in our study.

IL-8 is a well studied proinflammatory chemokine that attracts neutrophiles and promotes angiogenesis. Most studies measuring IL-8 in the PF found IL-8 elevated in endometriosis (31). IL-6 is mainly produced by macrophages and has both inflammatory and anti-inflammatory actions and has been found to be elevated in endometriosis in several studies (36,37).

In the intraperitoneal microenvironment ,immune cells and resident tissue cells engage in complex interactions where cell-secreted cytokines play an important role. Cytokine profiles may therefore reflect more fundamental pathophysiological processes and define disease states than individual cytokines. To further evaluate possible cytokine patterns related to symptoms or patients demografics , we performed a multivariate cluster analysis. Because of inconsistent results depending on methods and different normalizations we are not confident that the clusters we identified give us any biological information about the patients.

Little is known about the mechanisms behind pelvic pain and severe dysmenorrhea in endometriosis. At least 25% of patients with endometriosis are free of symptoms and only one-third of patients with chronic pelvic pain have endometriosis when evaluated by laparoscopy (38,29). The pain expressed by the patients appears to have no relation to ASRM stage of endometriosis or location of lesions (38). We asked the patients before surgery about dysmenorrhea, dyspareunia, non-cyclic pelvic pain, dyschesia and dysuria. There was no difference between the patients even if we stratified according to ASRM stage. Many different pain assassment scales have been used for endometriosis (20). We used the Norwegian translation of the Brief Pain Inventory (short version). We analysed all the scores and found no relation to endometriosis or stage of endometriosis for any of the pain items in the questionaire. When we split the patients into two groups according to NRS score ≥4 on the BPI scores we did see a significant difference between mild and severe endometriosis. These observations indicate that there are some assosiations between anatomical disease severity and moderate to severe pain and pain modalities in contrast to other studies describing no correlation between disease severety and pain symptoms (29). Dysmenorrhea or NRS scores was not a good indicator to sort out patients with endometriosis in this patient-population with a high percentage (60%) of endometriosis where 78% were classified as mild/moderate.

All pain modality scores were correlated to the individual cytokines. There was a significant positive correlation between the maximum pain the patients experienced during the last four weeks and the cytokines IL-8, IP-10 and SCGF-ß. When we sorted the patients into 2 groups where pain max >3 was used as the limit for moderate to severe pain (20) ,we found a significant different concentration of IL-8, IP-10 and SCGF-ß. in the two groups.

Few other studies have looked at the relation between inflammatory markers and pain intensity in endometriosis. One study from 2014 by Neziri et al found correlation between altered central

pain process in patients with endometriosis and MCP-1 and TNFα. No correlation was found between pain and IL-8 or IP-10 in this study (39).

It is believed that the pain is a result of altered activity in the central nervous system as a response to sensitization of peripheral nocicreeptors (29). Angiogenesis and nerve sprouting are important factors (30). Il-8 and IP-10 are both cytokines involved in angiogenesis. Il-8 is a strong promotor of angiogenesis and IP-10 is a inhibitor of neovascularization. IP-10 was not correlated to endometriosis in general or disease stage in this population of patients with infertility.

Conclusion

In a patient population with infertility as the primary symptom, we measured a wide range of cytokines in the peritoneal fluid. We found two cytokines, MCP-1 and SCGF-ß, with significantly higher concentrations in patients with endometriosis compared to patients without the disease. There was a high proportion of pain in both groups, and the intensity of pain was not related to endometriosis in general but patients with more severe disease reported more often moderate to severe pain that affected work and soscial life. The cytokines IL-8, IP-10 and SCGF-ß were related to pain intensity.

A marker or a panel of markers for endometriosis in infertility patients would help us sorting out patients who can benefit from surgery. PF is not an easy accessible material for diagnosis. A next step would be to evaluate the potential markers in serum and/or endometrial biopsies in a well stratified patient population.

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Table 1. Patient and peritoneal fluid (PF) characteristics.
Data are mean (range) or proportion (percent).

Characteristics	**Endometriosis**		
	present	**absent**	P (#)
	N= 58(60%)	N= 39(40%)	
Age	32 (24-39)	34 (27-41)	0.04*
Reproduction			
Pregnancies	0.5 (0-4)	0.5 (0-2)	0.68
Infertility (months.)	33 (12-100)	33 (12-100)	0.95
Male factor	8 (14%)	3 (8%)	0.15
Tubal factor	5 (9%)	6 (15%)	0.30
Pain Symptoms			
Dysmenorrhea	44 (76%)	24 (62%)	0.13
Dyspareunia	21 (36%)	18 (46%)	0.33
Bowel sympt.	18 (31%)	10 (26%)	0.35
Urinary sympt.	9 (16%)	3 (7%)	0.25
PF aspirates			
PF volume(ml)	11.7 (0.5-50)	11.2 (0.5-44)	0.95
PF cells (x10…)	6.0 (1-14)	5.5 (1.5-9.9)	0.16
Luteal phase	32 (55%)	25 (64%)	0.28
Follicular phase	26 (44%)	14 (36%)	0.28
ASRM classification			
I/Π(minimal/mild)	45 (78%)		
ΠI/Iv(moderate/severe)	13 (22%)		

(#)Significance of non-equivalence by independent samples' T-test for continuous data and Chi-Square test for categorical data.

Table 2. Pairwise comparison of cytokine concentrations (pg/mL) between patients with and without endometriosis. Data are medians and interquartile range.

Cytokine	**Endometriosis present** N= 58(60%)		**absent** N= 39(40%)		P(∗)	Q
BNGF	7.75	(6.54)	6.92	(5.82)	0.480	0,591
CTACK	187.13	(82.15)	203.76	(106.55)	0.444	0.591
Eotaxin	27.79	(47.25)	22.92	(44.79)	0.316	0.507
HGF	317.41	(315.01)	248.22	(284.45)	0.051	0.208
IL-16	547.65	(226.59)	513.71	(224.29)	0.059	0.208
IL-18	24.82	(27.03)	25.01	(31.52)	0.965	0.965
IL2Ra	72.63	(65.52)	82.14	(59.77)	0.638	0.729
IL-6	16.37	(27.57)	9.61	(25.43)	0.317	0.507
IL-8	4.25	(4.82)	2.50	(4.34)	0.065	0.208
IP-10	475.90	(494.04)	452.91	(370.94)	0.427	0.591
MCP-1	66.88	(193.68)	19.47	(119.36)	0.006 ∗	0.048*
MIF	1786.48	(2783.84)	1313.60	(1985.49)	0.216	0.494
MIG	464.28	(376.04)	428.94	(233.32)	0.141	0.376
SCF	237.62	(147.71)	223.21	(166.05)	0.300	0.507
SCGF-ß	9484.20	(6422.69)	5826.95	(4954.33)	0.000 ∗	0.001*
SDF-1α	176.51	(130.29)	163.43	(108.42)	0.845	0.901

(∗)Mann Whitney-U test , Q-value after Benjamini-Hockberg correction for multiple comparsion

All cytokines (n=29) with measurable values in less than 10% (n=10) or median concentration = 0 were omitted from the statistical analysis.

Table 3. Pairwise comparison of cytokine concentrations (pg/mL) in patients with different stages of endometriosis. Data are medians and interquartile range.

Cytokine	Stage 0(n=39)		Stage 3-4(n=13)		p
IL-6	9.61	(25.43)	27.65	(232.34)	0.019*
IL-8	2.50	(4.31)	4.80	(63.10)	0.010*
MCP-1	19.47	(119.36)	82.04	(332.91)	0.009*
SCGF-ß	5826.95	(4954.93)	11604.95	(8456.51)	0.001*

	Stage 0 (n=39)		Stage 1-2(n=45)		p
IL-6	9.61	(25.43)	14.91	(20.06)	0.819
IL-8	2.50	(4.31)	4.22	(4.70)	0.248
MCP-1	19.47	(119.36)	61.65	(190.68)	0.022*
SCGF-ß	5826.95	(4954.93)	8383.19	(6156.51)	0.003*

(∗) Significance of nonequivalent proportions by Mann Whitney -U test

All cytokines(n=29) with measurable values in less than 10% (n=10) or median concentration =0 were omitted from the statistical analysis.

Table 4. Correlation between individual cytokine concentrations and Brief Pain Inventory (BPI) scores.

BPI	IL-8	SCGF-ß	IP-10
Pain max.	0.21∗	0.25∗	0.21*
Pain min.	0.11	0.12	0.08
Pain mean	0.10	0,20	0.18
General activity	0.03	0,16	0,10
Mood	0.03	0,18	0.13
Walking ability	0.07	0.03	0.04
Work ability	0.02	0.21∗	0.06
Social relations	0.06	0.24∗	0.09
Sleep	0.04	0.06	0.03
Enjoyment of life	0.16	0.19	0.11

Correlation was tested with Spearman`s correlation coefficient.

∗ $p < 0.05$

All cytokines(n=29) with measurable values in less than 10% (n=10) or median concentration =0 were omitted from the statistical analysis.

Table 5. Pairwise comparison of cytokine concentrations (pg/mL) in patients mild/no pain versus patients with moderate/severe pain. Data are medians and interquartile range.

Cytokine	Painmax≤3 n= 38		Painmax≥4 n= 59		P *
IL-8	2.09	(4.55)	4.70	(5.17)	0,001*
IP-10	342.03	(289.56)	529.44	(603.15)	0,008*
SCGF-ß	5977.75	(5284.29)	9304.40	(6332.06))	0,010*

*Significance of non-equivalence by Mann-Whitney-U test.

Supplemental data Table 1.

Cytokine	**Cycle phase**				P(∗)
	Follicular		**Luteal**		
	N= 58(60%)		N= 39(40%)		
BNGF	6.63	(6.99)	7.80	(5.70)	0.687
CTACK	188.20	(97.87)	189.52	(91.93)	0.949
Eotaxin	28.61	(59.68)	23.17	(36.44)	0.528
HGF	284.97	(328.31)	290.02	(304.71)	0.462
IL-16	537.58	(229.23)	536.40	(220.09)	0.526
IL-18	22.63	(32.93)	27.84	(24.43)	0.159
IL2Ra	82.14	(72.86)	76.42	(50.05)	0.970
IL-6	8.86	(26.18)	15.26	(28.32)	0.222
IL-8	3.91	(4.83)	3.31	(5.11)	0.808
IP-10	475.66	(305.80)	398.58	(611.63)	0.271
MCP-1	31.80	(97.64)	47.18	(186.91)	0.158
MIF	2154.73	(2943.55)	1160.49	(2138.79)	0.097
MIG	431.15	(207.89)	453.21	(441.91)	0.538
SCF	263.83	(147.29)	220.24	(127.41)	0.274
SCGF-ß	8176.17	(6951.57)	7333.52	(6951.57)	0.274
SDF1-α	155.14	(113.85)	189.97	(123.94)	0.132

(∗)Mann Whitney-U test

All cytokines(n=29) with measurable values in less than 10% (n=10) or median concentration = 0 were omitted from the statistical analysis.

Supplemental data Table 2. BPI scores and endometriosis stage. Data are mean (range) .

BPI-item	Stage 0 N= 39	Stage I/II N= 45	Stage III/IV N=13	p(#)
Pain-max	4.2 (0-9)	4.9 (0-10)	6.7 (0-10)	0.087
Pain-min	1.4 (0-4)	1.6 (0-9)	2.6 (0-7)	0.109
Pain-mean	3.0 (0-7))	3.5 (0-9)	5.1 (0-9)	0.070
Daily activity	3.2 (0-10	3.7 (0-10)	4.5 (0.10)	0.547
Mood	3.2 (0-9)	3.0 (0-10)	4.7 (0-10)	0.265
Walk-ability	2.0 (0-9)	2.0 (0-9)	3.5 (0-10)	0.226
Work ability	2.0 (0-10)	2.5 (0-10)	3.6 (0-9)	0.352
Social relations	1.5 (0-9)	1.6 (0-10)	3.4 (0-10)	0.140
Sleep	2.3 (0-9)	1.9 (0-8)	3.8 (0-9)	0.150
Enjoyment of life	1.6 (0-8)	1.8 (0-10)	3.2 (0-9)	0.234

(#)Significance of non-equivalence by oneway ANOVA

Supplemental data table 3: Distribution of patients according to endometriosis stage and pain score.

	Painmax≤3 n= 38	Painmax≥4 n= 59	
Stage 0	19 (49%)	20 (51%)	39
Stage I/II	18 (40%)	27 (60%)	45
Stage III/IV	1 (8%)	12 (92%)	13

Significance of non-equivalence by chi-square test P= 0,032.
p=0.043 when comparing stage I/II and III/VI using Fishers exact test.
P= 0,009 when comparing stage 0 and stage III/IV.
The difference between stage 0 and stage I/II is not significant.