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Association of endocannabinoids with pain in endometriosis

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Abstract

Endocannabinoid (eCB) levels fluctuate in inflammatory conditions and as such may take part in endometriosis-associated pain or even in endometriosis pathogenesis. In this case–control (23 cases and 19 controls) study, targeted lipids were measured in the serum and peritoneal fluid collected during laparoscopy. Endometriosis was confirmed histologically. Dysmenorrhea, abdominal pain, and dyspareunia were assessed using the Numeric Rating Scale for pain. Steroids, eCBs, and related lipids were quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Tumor necrosis factor alpha, IL-8, PAPP-A, PP14, RANTES, OPG, MIDKINE, MCP-1, VEGF, leptin, and defensins were quantified by ELISA. We found that eCB levels were significantly influenced by both noncyclic and cyclic abdominal pain. Specifically, women suffering from noncyclic abdominal pain were characterized by a higher 2-AG level in the peritoneal fluid throughout the menstrual cycle, whereas women suffering from dysmenorrhea had higher 2-AG levels and lower AEA levels during the proliferative phase alone. In addition, 2-AG positively correlated with prostaglandin E2 (PGE2), and the ratio AEA/2-AG positively correlated with defensins, suggesting a possible link between endocannabinoids system and inflammatory pain. The results of the current study indicate that the eCB system may play a role in endometriosis-associated pain, but additional studies are needed to investigate the causal relationship.

Keywords: Endometriosis, Dysmenorrhea, Abdominal pain, Dyspareunia, Endocannabinoids, Prostaglandins, 2-AG, Peritoneal fluid, Serum

1. Introduction

Endometriosis is a hormone-dependent disease in which endometrial tissue grows outside the uterus. For many women with endometriosis, pain, commonly dysmenorrhea, noncyclic pelvic pain, dyspareunia, and dyschezia, represents the most important symptom.[13] Dysmenorrhea, especially, is reported in more than 80% of the women with endometriosis.[14]

Dysmenorrhea refers to the presence of recurrent, crampy, and lower abdominal pain occurring during menses. In primary dysmenorrhea, the pain originates from released prostaglandins, essentially prostaglandin E2 (PGE2) and prostaglandin F2 alpha (PGF2 alpha), at the beginning of the menses, leading to nonrhythmic or incoordinate contractions. In light of the role of prostaglandins in pain treatment, changes in arachidonate remodeling and correlations with endocannabinoids (eCBs), which are generally antinociceptive and anti-inflammatory lipids counteracting prostaglandin signaling, are still poorly studied in endometriosis. Endocannabinoids which can be detected in tissue are represented by anandamide (AEA) and 2-arachidonoylglycerol (2-AG). Both are synthesized from phospholipid precursors at the inner leaflet of the cell membrane. Anandamide can be further hydrolyzed by the fatty acid amide hydrolase into arachidonic acid (AA) and ethanolamine, whereas 2-AG can be further hydrolyzed by the monoacylglycerol lipase (MAGL) into AA and glycerol. During inflammatory conditions, eCBs and in particular 2-AG may serve as a reservoir of classic prostaglandin production through release of arachidonic acid, especially in the brain.[21,34] In specific conditions, such as inflammation and reduced activity of the hydrolytic enzymes, eCBs can be directly oxygenated into prostaglandin-like molecules.[42]

In addition to this immunomodulating role, eCBs could potentially act locally on pain through multiple receptors such as the cannabinoid receptor type-1 (CB1) and type-2 (CB2), transient receptor potential vanilloid type 1 (TRPV1),[21] gamma-aminobutyric acid (GABA) type A receptors,[41] glycine receptor, and the peroxisome proliferator–activated receptor gamma (PPAR-γ)[3] which are expressed in the fibers innervating the endometriotic lesions[9] or in the endometriosis foci as reported in deep-infiltrating endometriotic[26] and peritoneal endometriosis.[37] Each of these receptors have been all suspected to mediate pain (visceral, pelvic, and gynecological pains), in animal models or in endometriotic women.[9,20,29–31] Of interest too, clinical studies testing the effect of palmitoylethanolamide (PEA), a major N-acylethanolamine, demonstrated a significant reduction of

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chronic pelvic pain, dysmenorrhea, and deep dyspareunia in women with endometriosis on treatment.[4,23]

The eCB system (ECS) is involved in controlling several functions both at peripheral and central levels, and in recent years, it has become apparent that the ECS including the 2-AG or AEA metabolites is pivotal in the regulation of inflammation[42] and that ECS may be a possible target for prevention and treatment of endometriosis-associated pain. Despite this, little is known about the regulation and action of eCBs on the pathophysiology of endometriosis. In this study, we analyzed the serum and the peritoneal fluid to evaluate whether eCBs are associated with pain and inflammatory markers in women suffering from endometriosis.

2. Materials and methods

2.1. Source and handling of the biological material

The study has been approved by the Swiss Ethic Committee before its start (KEK-BE (149/03, 2003)). The patients involved in this study provided informed consent and were requested to complete a pain questionnaire (Numeric Rating Scale for pain, NRS) covering information on different types of pain including menstrual pain, lower abdominal pain, and dyspareunia. Whole blood was drawn before anaesthesia for serum preparation. The peritoneal fluid was collected at the beginning of the laparoscopic procedure from the pouch of Douglas. The volume of peritoneal fluid was measured for each patient. Sera and peritoneal fluids were clarified by centrifugation at 800*g* for 10 minutes and stored in aliquots at −80°C for subsequent analysis.

2.2. Including and excluding criteria

All patients included in this study were operated by laparoscopy. The indications for laparoscopy included abdominal pain, uterine fibromas, ovarian cysts, or tubal pathologies. The peritoneal cavity of all patients was carefully inspected by experienced gynecologists to detect the potential presence of endometriosis lesions. When lesions were visually detected, a score (stage I-IV) was assigned based on the revised American Fertility Society staging system (AFS),[36] and the subtype (peritoneal, endometrioma, or deep infiltrating) recorded. All suspicious lesions were removed during laparoscopy and analyzed by an experienced pathologist to validate the endometriotic nature of the biopsy. The total protein content in peritoneal fluid (PF) was determined using a micro bicinchoninic assay (Quanti-Pro BCA; Sigma-Aldrich, St. Louis, MO) to ascertain absence of dilution with abdominal flushing medium under the procedure. The PF samples with a total protein content below 15 mg/mL or with hemolysis were excluded from the study. Menstrual phase assignment was performed using serum progesterone level and patients self-report in which patients informed about the first day of the last menses. The proliferative phase has been defined as follows: cycle day below 16 and progesterone level below 5 nmol/L. Progesterone was quantified in the serum by a radioimmunoassay (coat-a-count, DPC; Buhlmann Laboratories, Allschwill, Switzerland). The menstrual phase was not confirmed histologically. Patients older than 50, patients with acute pelvic inflammation, and patients treated with either hormonal medication in the past 3 months or nonsteroidal anti-inflammatory drugs in the past 2 weeks were excluded.

2.3. Detection of endocannabinoid ligands in the serum and peritoneal fluid

Samples were blinded before processing. The eCBs AEA, 2-AG; the N-acylethanolamines LEA (N-linoley-), PEA (N-palmitoyl-), OEA (N-oleoyl-), SEA (N-stearoyl-), and the other related lipids including AA (arachidonic acid), SAG (stearoyl-arachidonoylglycerol), PGD2, PGE2, C20:4 phosphatidylethanolamine (C20:4 PE), C18-20:4 phosphatidylcholine (C18-20:4 PC), and C18-20:4 PE were quantified after extraction using liquid chromatography-tandem mass spectrometry (LC-MS/MS) multiple reaction monitoring (MRM) parameters exactly as described in Ref. 6. In brief, the samples were weighed in the bead beater vials. The extraction was performed using 5 volumes of methanol followed by homogenization with the bead beater (Mini Beater 24 Biospec USA, max level 3200 strokes/minute for 30 seconds). Subsequently, samples were centrifuged at max speed for 10 minutes at 4°C in an Eppendorf centrifuge (5415R). Fifty microliters of supernatant were recovered, and 5 μL of internal standards were added to all tubes, and 10 μL of the solution were injected in the LC-MS/MS system. Measurements and validation of the new peptide LC-MS/MS method were performed on a more sensitive 4000 QTrap mass spectrometer equipped with an ESI probe (AB Sciex Concord, Ontario, Canada) and connected to an ExionLC AC UHPLC system (AB Sciex Concord).

2.4. Inflammatory marker quantification by ELISA

Leptin, Monocyte Chemoattractant Protein 1 (MCP-1), Osteoprotegerin (OPG), and Regulated on Activation Normal T cell Expressed and Secreted (RANTES) were determined using the Duo-Set method from R&D Systems (Abingdon, England). Neutrophil defensins (combined alpha defensins HNP-1, HNP-2, and HNP-3) were determined with the assay from Hycult Biotech (Uden, the Netherlands). Pappalysin-1 (PAPP-A) was determined with the double-antibody enzyme immunometric method that was developed in our laboratory and available from Buhlmann Laboratories, Allschwil, Switzerland. MIDKINE was determined by a microplate enzyme immunometric assay manufactured by BioVendor (Modrice, Czech Republic). Glycodelin (PP14) and Vascular Endothelial Growth Factor (VEGF) were determined with an ELISA kit from Bio-Serv (Dispolab, Dielsdorf, Switzerland) and the Quantikine© kit from R&D Systems (Oxford, England), respectively. Peritoneal fluid samples were diluted as 1:2 to 1:11. The assays were performed according to the recommendations of the manufacturers with modifications.[1,2,32,39]

2.5. Statistical analyses

The sample size was not based on a power analysis in this exploratory study. A paired Student t test was used to compare the systemic values with the peritoneal values. The correlations between the systemic values and the peritoneal values were further assessed by a basic linear regression. An ANOVA was performed to compare the means of every metabolite in function of the menstrual cycle, the pain, or the disease. A 2-way analysis of variance (ANOVA) was performed to analyze the metabolites in function of dysmenorrhea. In these analyses, a pain threshold was set up arbitrary at NRS = 5. Women with NRS < 5 were included in the group "low pain" and the others formed the group "high pain". Women were distributed to these groups regardless of their endometriosis status. To assess the crude association between pain-associated metabolites and selected inflammation markers, a basic linear regression was performed. Values were considered statistically significant at $P < 0.05$. The statistical analyses were performed using JASP and GraphPad Prism softwares. Serum levels of PGD2, PGE2, and corticosterone were found to be below the detection limit in 27, 18, and 13 measurements, respectively (over 42 patients). Similarly, the PF level of corticosterone was below the detection limit in 31 measurements. To improve the chance to detect an eventual effect of pain, cycle, or disease, an arbitrary value corresponding

to the detection limit has been attributed and all samples were used for the analysis.

3. Results

3.1. Population characteristics

Endocannabinoids and related lipids were measured in sera and matched peritoneal fluids collected during the proliferative and secretory phases. **Table 1** details the descriptive statistics of the whole cohort of participants. Besides the stage and the progesterone levels, the characteristics of the study participants were similar across the groups as revealed by ANOVA and Fisher exact tests.

3.2. Quantification in the serum and peritoneal fluid

eCBs, N-acylethanolamines (AEA, LEA, OEA, PEA, SEA), SAG, PE, PC, and AA were detected in all samples. The levels of SAG, C18-20:4 PE, SEA, 2-AG, PEA, PGE2, and PGD2 were higher in PF than in the serum, whereas the levels of C20:4 PE, C18-20:4 PC, AEA, LEA, OEA, corticosterone, cortisol, and AA were higher in the serum (**Tables 2 and 3**). The values measured in PF and serum presented a strong positive correlation for C18-20:4 PE, C18-20:4 PC, C20:4 PE, corticosterone, and cortisol (**Table 4**). By contrast, the levels of SAG, SEA, 2-AG, AEA, LEA, PEA, OEA, AA, PGE2, or PGD2 in PF and serum did not correlate, suggesting an interplay with local metabolism involving different tissues. Some of the results presented in this paragraph should be viewed with caution. This is mainly the case for corticosterone, PGD2, and PGE2 because these compounds were barely detectable in PF (corticosterone) or in the serum (PGD2, PGE2, and corticosterone).

3.3. Targeted lipidomics in peritoneal fluid

An ANOVA analysis was performed to assess whether the lipids quantified in our study could be associated with menstrual cycle, endometriosis, or pain (dysmenorrhea, abdominal pain, or dyspareunia) (**Table 5**).

By taking into account these variables, we observed an association between the menstrual cycle and the lipids 2-AG, OEA, and PGE2 (**Table 5**) whose levels were higher in the proliferative phase than the secretory phase (**Table 2** and **Figs. 1A–C**). Endometriosis was associated with C18-20:4 PC (**Table 5**), in which the level was higher in the cases than in controls (**Table 2** and **Fig. 1D**). However, C18-20:4 PC did not correlate with rAFS (Fig. S1, available at http://links.lww.com/PAIN/B382). The levels of the other targeted lipids in PF were not significantly affected by endometriosis (**Table 5**).

Abdominal pain was significantly associated with elevated 2-AG and AA (**Table 5** and **Figs. 1E and F**). In addition to the ANOVA analysis, a positive linear correlation between abdominal pain score and 2-AG was found, although weak ($P = 0.049$) and significant only during the proliferative phase (Table S1, Fig. S2, available at http://links.lww.com/PAIN/B382). For dysmenorrhea, we reported the simple main effect alone because a significant interaction with the menstrual cycle was indeed observed for 2-AG ($P = 0.024$). Hence, dysmenorrhea was characterized by an increased 2-AG level occurring during the proliferative phase alone (**Fig. 1G**). Dysmenorrhea was not significantly linked to AEA (data not shown). However, the ratio AEA/2-AG was significantly lowered during the proliferative phase (**Fig. 1H**). In addition, the dysmenorrhea pain score correlated positively with 2-AG and negatively with AEA, during this phase (Table S1, Fig. S3, available at http://links.lww.com/PAIN/B382). Finally, we observed a negative association between dyspareunia and AEA (**Tables 5** and **Fig. 1I**), in which the level was lowered in women suffering from dyspareunia. In line, dyspareunia NRS and AEA levels correlated negatively (Fig. S4, available at http://links.lww.com/PAIN/B382). The levels of the other lipids measured in the PF were not significantly affected by pain (**Table 5**).

3.4. Targeted lipidomics in the serum

Considering the differences between the levels of the targeted lipids in PF and serum highlighted in **Tables 3 and 4**, a similar analytic approach was conducted with the sera to evaluate whether the values measured in the serum could serve as a marker of pain or endometriosis. The ANOVA showed an association between the menstrual cycle and ser AA, in which the level was lower during the secretory phase (**Table 5** and **Fig. 2A**). Significant differences in the serum that may account for endometriosis were found for LEA and OEA whose levels were lower in cases than in controls (**Table 5** and **Figs. 2B and C**). Lowest values for LEA and OEA were found in advanced endometriosis stages (Fig. S1, available at http://links.lww.com/PAIN/B382). In the serum, AA tended to be the best pain indicators (**Table 5**). In fact, ser AA was significantly elevated during the proliferative phase in women suffering from dysmenorrhea (**Fig. 2D**). Interestingly, 2 dysmenorrhea markers found in our study, ie, ser AA and PF. 2-AG, were strongly correlated (**Table 6**).

Contrasting with the results found in the PF and in line with the lack of correlation between serum and PF values, ser 2-AG, ser OEA, and ser PGE2 were not significantly affected by the menstrual phase, ser C18-20:4 PC was not significantly different in cases than in controls, and ser 2-AG and ser AEA/AEA did not associate with abdominal pain nor with dysmenorrhea (**Table 5**).

3.5. Modulation of inflammation by the endocannabinoid anandamide and 2-arachidonoylglycerol

Several inflammatory markers were quantified by ELISA (cytokines) or by HPLC (PGE2) in the peritoneal fluid to evaluate whether the eCBs AEA and 2-AG which were found to be associated with pain in our study could play a role in the inflammatory response and consequently in the inflammatory pain. The link between eCBs and inflammation was analyzed by linear regressions in the whole cohort independently of disease or pain (**Table 7**). We observed a positive correlation between 2-AG and both PGE2 and PGE2/PGD2. In addition, the ratio AEA/2-AG was significantly positively correlated with defensins. Although the effects of AEA or 2-AG taken independently were not significant, AEA and 2-AG seemed to exhibit opposite effects, AEA being positively correlated with defensins whereas 2-AG was negatively correlated. The other inflammation markers quantified in our study were not directly affected by the eCBs (or vice versa).

4. Discussion

Pain is one of the predominant clinical features of endometriosis, and understanding the biochemical mechanisms behind it is a prerequisite for new medical approaches. The ECS has emerged recently as a biological factor in endometriosis because of its potential modulatory role in pain, immune response, and proliferation.[4] Although others focused on eCBs in plasma or on eCB receptors levels in endometriosis affected women,[4,38] our study reports for the first time the levels of eCBs and other

Table 1

Characteristics of the study participants.

Characteristics of the study participants	Proliferative phase		Secretory phase		ANOVA (*P*)		Fisher exact test (*P*)	
	Controls (n = 6)	Cases (n = 12)	Controls (n = 13)	Cases (n = 11)	Menstrual phase	Disease	Menstrual phase	Disease
Age (y)								
Mean	33.1	32.5	36.8	33.4	0.295	0.250		
SEM	2.8	1.6	2.0	1.4				
BMI								
Mean	24.8	22.4	25.7	25.4	0.166	0.434		
SEM	1.1	1.2	1.2	1.8				
Volume of peritoneal fluid (mL)								
Mean	8.0	12.2	14.8	6.4	0.865	0.256		
SEM	1.8	1.8	3.9	0.9				
Stages (rAFS)								
I-II	—	7 (58.3%)	—	6 (54.5%)			>0.999	n.a.
III-IV	—	5 (41.7%)	—	3 (23.7%)				
Endometriosis subtypes								
Endometrioma	—	4 (33.3%)	—	5 (45.5%)				
Peritoneal	—	8 (66.7%)	—	5 (45.5%)				
DIE	—	2 (16.7%)	—	4 (36.4%)				
Concomitant procedures during laparoscopy								
Myomectomy	1 (16.7%)	0	4 (30.8%)	1 (9.1%)				
Hysterectomy	1 (16.7%)	0	6 (46.7%)	1 (9.1%)				
Salpingectomy	0	0	3 (23.1%)	1 (9.1%)				
Adnexectomy	1 (16.7%)	1 (8.3%)	0	0				
Ovarian cystectomy	0	2 (16.7%)	2 (15.4%)	0				
Dysmenorrhea (NRS 1-10)								
Mean	3.7	4.3	4.5	6.3	0.203	0.289		
SEM	1.9	1.1	1.1	1.0				
Patients with NRS Dysm. >5 (n, %)	2 (33.3%)	6 (50%)	7 (53.9%)	7 (63.6%)			0.533	0.757
Patients with NRS Dysm. <5 (n, %)	4 (66.7%)	6 (50%)	6 (46%)	4 (36.4%)				
NRS Dysm. >5 (& Dysp. <5 & Abdo. <5) (n, %)	1 (16.7%)	1 (8.3%)	5 (38.5%)	2 (18.2%)				
Abdominal pain (NRS 1-10)								
Mean	2.7	2.5	4.2	4.9	0.069	0722		
SEM	1.4	0.8	1.1	1.1				
Patients with NRS Abdo. >5 (n, %)	2 (33.3%)	3 (25%)	7 (53.8%)	5 (45.5%)			0.208	0531
Patients with NRS Abdo. <5 (n, %)	4 (66.7%)	9 (75%)	6 (46.2%)	6 (54.5%)				
NRS Abdo. >5 (& Dysm. <5 & Dysp <5) (n, %)	1 (16.7%)	1 (8.3%)	1 (7.7%)	0 (0.0%)				
Dyspareunia (NRS 1-10)								
Mean	1.0	1.7	1.5	2.6	0.351	0240		
SEM	0.8	0.7	0.7	0.9				
Patients with NRS Dysp. >5 (n, %)	1 (16.7%)	3 (25%)	2 (15.4%)	2 (18.2%)			0.698	0693
Patients with NRS Dysp. <5 (n, %)	5 (83.3%)	7 (58.3%)	11 (84.6%)	7 (63.6%)				
NRS Dysp. >5 (& Dysm. <5 & Abdo. <5) (n, %)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (9.1%)				
Missing data	0	2 (16.7%)	0	0				
Operation time (base 100)								
Mean	10.0	9.0	9.6	9.6	0.847	0.651		
SEM	0.6	0.9	0.6	0.8				
Missing data	1 (16.7%)	0	0	1 (9.10%)				
Serum progesterone (nmol/L)								
Mean	2.5	1.4	27.5	27.4	<0.001	0.938		
SEM	0.7	0.2	5.2	9.9				
Missing data	1 (16.7%)	0	0	1 (9.10%)				
Menstrual cycle								
Regular (n, %)	4 (66.7%)	9 (75.0%)	9 (69.2%)	6 (54.5%)			0.424	>0.999
Not regular (n, %)	1 (16.7%)	1 (8.3%)	3 (23.1%)	3 (27.3%)				
Missing data	1 (16.7%)	2 (16.7%)	1 (7.7%)	2 (18.2%)				

ANOVA, analysis of variance; BMI, Body Mass Index; DIE, Deep Infiltrating Endometriosis; NRS, Numeric Rating Scale; rAFS, revised American Fertility Society score.

Table 2

Levels of eCBs and metabolites in the peritoneal fluid and serum (mean ± SEM).

	Peritoneal fluid				Serum				Peritoneal fluid/serum				PF	Serum	PF/serum
	Proliferative phase		Secretory phase		Proliferative phase		Secretory phase		Proliferative phase		Secretory phase		All cycles		
	Controls (n = 6)	Cases (n = 12)	Controls (n = 13)	Cases (n = 11)	Controls (n = 6)	Cases (n = 12)	Controls (n = 13)	Cases (n = 11)	Controls (n = 6)	Cases (n = 12)	Controls (n = 13)	Cases (n = 11)	Controls & cases (n = 42)		
SAG [ng/mL]															
Mean	59.27	65.24	40.93	54.32	9.53	7.12	11.41	10.01	6.99	11.12	6.00	7.32	54.00	9.55	7.95
SEM	8.64	9.76	3.84	5.94	1.66	0.76	2.31	1.81	1.27	2.49	1.51	1.32	3.80	0.92	0.96
C20:4 PE [ng/mL]															
Mean	85.46	138.16	123.90	115.59	324.45	446.04	391.83	376.41	0.27	0.33	0.33	0.31	120.30	393.65	0.32
SEM	7.43	16.77	11.51	10.41	16.71	59.34	50.41	30.39	0.03	0.02	0.03	0.02	6.94	24.53	0.01
C18-20:4 PC [ng/mL]															
Mean	2601.83	2984.42	2636.69	3185.18	3693.17	3630.67	3159.08	3814.64	0.73	0.85	0.84	0.87	2874.71	3541.81	0.83
SEM	214.70	155.53	193.20	156.22	409.59	309.34	159.14	327.67	0.06	0.04	0.05	0.06	94.79	145.35	0.03
C18-20:4 PE [ng/mL]															
Mean	1947.83	2174.00	1737.69	1711.09	1485.00	1479.42	1453.00	1244.27	1.45	1.56	1.27	1.42	1885.41	1410.45	1.42
SEM	213.69	180.82	125.69	213.89	269.91	147.96	136.34	164.76	0.17	0.15	0.10	0.12	92.78	81.29	0.07
SEA [ng/mL]															
Mean	12.16	12.20	12.80	11.63	7.91	7.71	8.03	6.72	1.57	1.65	1.70	1.83	12.23	7.58	1.70
SEM	0.72	0.71	0.40	0.82	0.54	0.43	0.54	0.48	0.13	0.14	0.15	0.18	0.33	0.26	0.08
2-AG [ng/mL]															
Mean	20.71	30.09	21.47	18.56	12.41	11.04	20.05	16.57	1.87	2.96	1.50	1.32	23.06	15.47	1.93
SEM	4.39	4.21	3.12	2.35	1.66	1.34	3.91	3.13	0.61	0.41	0.26	0.15	1.86	1.59	0.20
AEA [ng/mL]															
Mean	0.47	0.58	0.47	0.52	1.61	1.67	1.83	1.51	0.31	0.42	0.31	0.34	0.51	1.67	0.35
SEM	0.06	0.05	0.04	0.09	0.20	0.19	0.19	0.13	0.04	0.08	0.05	0.04	0.03	0.09	0.03
LEA [ng/mL]															
Mean	1.53	1.68	1.60	1.32	4.64	4.09	4.54	3.42	0.34	0.42	0.40	0.40	1.54	4.13	0.40
SEM	0.13	0.13	0.13	0.17	0.42	0.25	0.43	0.35	0.03	0.03	0.06	0.05	0.07	0.20	0.02
PEA [ng/mL]															
Mean	7.82	7.38	7.67	7.11	5.95	5.25	5.49	5.01	1.33	1.46	1.50	1.49	7.46	5.36	1.46
SEM	0.52	0.45	0.23	0.43	0.39	0.26	0.37	0.33	0.09	0.13	0.14	0.14	0.20	0.17	0.07
OEA [ng/mL]															
Mean	1.87	1.91	1.69	1.55	4.07	3.34	3.57	2.83	0.48	0.59	0.52	0.58	1.74	3.38	0.55
SEM	0.10	0.10	0.10	0.12	0.31	0.18	0.29	0.26	0.05	0.04	0.05	0.05	0.06	0.14	0.03
Corticosterone [ng/mL]															
Mean	1.03	1.19	1.66	1.58	3.67	4.41	3.32	3.56	0.74	0.76	0.77	1.05	1.41	3.74	0.83
SEM	0.13	0.15	0.51	0.41	1.11	1.07	0.57	0.84	0.34	0.22	0.18	0.34	0.19	0.43	0.13

(*continued on next page*)

Table 2 (*continued*)

	Peritoneal fluid				Serum				Peritoneal fluid/serum				PF	Serum	PF/serum
	Proliferative phase		Secretory phase		Proliferative phase		Secretory phase		Proliferative phase		Secretory phase			All cycles	
	Controls (n = 6)	Cases (n = 12)	Controls (n = 13)	Cases (n = 11)	Controls (n = 6)	Cases (n = 12)	Controls (n = 13)	Cases (n = 11)	Controls (n = 6)	Cases (n = 12)	Controls (n = 13)	Cases (n = 11)	Controls & cases (n = 42)		
Cortisol [ng/mL]															
Mean	17.44	30.68	27.62	33.05	55.40	71.59	61.59	82.42	0.34	0.44	0.49	0.39	28.46	69.02	0.43
SEM	4.23	6.06	4.22	6.13	13.29	11.44	6.87	7.75	0.07	0.04	0.08	0.05	2.78	4.85	0.03
PGE2 [ng/mL]															
Mean	0.82	1.19	0.74	0.73	0.19	0.20	0.32	0.27	4.97	7.77	3.64	4.19	0.88	0.25	5.15
SEM	0.20	0.20	0.15	0.08	0.04	0.03	0.08	0.08	0.91	1.87	0.70	0.78	0.08	0.03	0.66
PGD2 [ng/mL]															
Mean	1.50	1.24	1.32	1.34	0.19	0.16	0.25	0.15	10.30	10.58	9.90	10.13	1.33	0.19	10.21
SEM	0.13	0.13	0.08	0.14	0.04	0.03	0.08	0.03	2.41	1.78	1.59	1.21	0.06	0.03	0.82
AA [ng/mL]															
Mean	1284.33	1589.08	1171.54	1345.73	4101.00	3669.33	3100.85	2841.36	0.33	0.48	0.41	0.51	1352.57	3338.19	0.44
SEM	59.00	172.51	134.34	216.38	413.82	390.20	286.35	263.92	0.04	0.06	0.05	0.10	87.30	176.99	0.04

The number of patients (n) is indicated for each group.
2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; AEA, anandamide; eCBs, endocannabinoids; LEA, N-linoleyl-ethanolamine; OEA, N-oleoyl-ethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEA, palmitoylethanolamide; PF, peritoneal fluid; PGD2, prostaglandin D2; PGE2, prostaglandin E2; SAG, stearoyl-arachidonoylglycerol; SEA, N-stearoyl-ethanolamines.

Table 3

Comparison between peritoneal fluid and matched serum values for each metabolite (paired *t* test).

Paired samples *t* test	t	*df*	*P*
Peritoneal fluid vs serum			
SAG	11,046	41	<0.001
C20:4 PE	−13,521	41	<0.001
C18-20:4 PC	−5856	41	<0.001
C18-20:4 PE	6515	41	<0.001
SEA	11,598	41	<0.001
2-AG	3388	41	0.002
AEA	−12,436	41	<0.001
LEA	−13,629	41	<0.001
PEA	7829	41	<0.001
OEA	−11,711	41	<0.001
Corticosterone	−5696	41	<0.001
Cortisol	−11,245	41	<0.001
PGE2	7044	41	<0.001
PGD2	16,346	41	<0.001
AA	−11,133	41	<0.001

Student *t* test.
2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; AEA, anandamide; LEA, N-linoleyl-ethanolamine; OEA, N-oleoyl-ethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEA, palmitoylethanolamide; PGD2, prostaglandin D2; PGE2, prostaglandin E2; SAG, stearoyl-arachidonoylglycerol; SEA, N-stearoyl-ethanolamines.

related lipids in peritoneal fluids in relation to pain, inflammation, and endometriosis.

AEA and 2-AG as well as the structurally related lipids (SAG, SEA, OEA, PEA, LEA, C20:4 PE, C18-20:4 PC, and C18-20:4 PE) were all present in the peritoneal fluid. The 2-AG level was higher

Table 4

Linear correlation between peritoneal fluid and matched serum values for each metabolite.

Pearson correlations	r	*P*
Peritoneal fluid vs serum		
SAG	−0.128	0.419
C20:4 PE	0.708***	<0.001
C18-20:4 PC	0.622***	<0.001
C18-20:4 PE	0.656***	<0.001
SEA	0.086	0.588
2-AG	0.161	0.308
AEA	0.082	0.608
LEA	0.244	0.119
PEA	−0.052	0.745
OEA	0.234	0.135
Corticosterone	0.345*	0.025
Cortisol	0.675***	<0.001
PGE2	0.058	0.715
PGD2	−0.130	0.412
AA	0.231	0.141

*P<0.05, ***P< 0.001.
2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; AEA, anandamide; LEA, N-linoleyl-ethanolamine; OEA, N-oleoyl-ethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEA, palmitoylethanolamide; PGD2, prostaglandin D2; PGE2, prostaglandin E2; SAG, stearoyl-arachidonoylglycerol; SEA, N-stearoyl-ethanolamines.

Table 5

ANOVA in the peritoneal fluid and serum (*P*-values).

	Peritoneal fluid					Serum				
	Menst. cycle	Endometriosis	Dysmenorrhea	Abdo. pain	Dyspareunia	Menst. cycle	Endometriosis	Dysmenorrhea	Abdo. pain	Dyspareunia
SAG	0.056	0.127	0.781	0.619	0.133	0.265	0.218	0.093	0.661	0.571
C20:4 PE	0.922	0.278	0.571	0.281	0.344	0.649	0.435	0.885	0.262	0.182
C18-20:4 PC	0.662	0.008**	0.427	0.167	0.651	0.626	0.166	0.515	0.454	0.311
C18-20:4 PE	0.064	0.668	0.958	0.611	0.894	0.293	0.290	0.079	0.858	0.333
SEA	0.892	0.301	0.477	0.746	0.846	0.319	0.109	0.540	0.858	0.852
2-AG	0.009**	0.487	0.077	0.024*	0.149	0.110	0.453	0.560	0.609	0.348
AEA	0.362	0.081	0.115	0.058	0.032*	0.979	0.270	0.308	0.506	0.370
LEA	0.153	0.498	0.436	0.814	0.102	0.298	0.026*	0.477	0.603	0.762
PEA	0.631	0204	0.622	0.691	0.974	0.329	0.108	0.474	0.883	0.925
OEA	0.022*	0.561	0.818	0.737	0.606	0.068	0.010*	0.436	0.982	0.852
Corticosterone	0.220	0.986	0.893	0.665	0.960	0.304	0.454	0.708	0.165	0.171
Cortisol	0.498	0.113	0.851	0.162	0.584	0.588	0.062	0.548	0.279	0.656
PGE2	0.033*	0.233	0.615	0.102	0.051	0.274	0.746	0.883	0784	0.693
PGD2	0.755	0.471	0.514	0.853	0.360	0.863	0.274	0.318	0.204	0.569
AA	0.098	0.127	0.734	0.044*	0.166	0.005**	0.276	0.070	0.735	0.527
AEA/2-AG	0.285	0.760	0.768	0.057	0.224	0.198	0.841	0.670	0.274	0.249
PGE2/PGD2	0.056	0.094	0.304	0.115	0.121	0.269	0.237	0.160	0.342	0.439

*$P < 0.05$, **$P < 0.01$.

2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; AEA, anandamide; LEA, N-linoleyl-ethanolamine; OEA, N-oleoyl-ethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEA, palmitoylethanolamide; PGD2, prostaglandin D2; PGE2, prostaglandin E2; SAG, stearoyl-arachidonoylglycerol; SEA, N-stearoyl-ethanolamines.

than that of AEA which is in line with the ratio previously found in the plasma[11,19,21,24,38] and brain.[5,8] It should be noted that the levels of all the lipids quantified in the peritoneal fluid were significantly different from those found in the serum. This demonstrates the value of quantifying these molecules locally in addition to measuring them in the circulation. We also observed that circulating and peritoneal levels of eCBs did not correlate. This finding supports the concept of a local production, or of a local metabolism, within the peritoneal cavity happening either by immune cells or the reproductive tract. This is in line with current knowledge on circulating eCBs which can be produced by multiple organs and tissues, including brain, muscle, adipose tissue, and circulating cells.[21] In mice and humans, AEA, 2-AG, and their regulatory enzymes are produced in the endometrium, and their levels fluctuate significantly over the estrus cycle.[7,17,40,46] Interestingly, endocannabinoid hydrolysis especially increases during the midsecretory phase.[40] We also found that the levels of 2-AG, OEA, and PGE2 apparently fluctuate during the menstrual phase in the peritoneal fluid; their levels being lower during the secretory phase. An increase in MAGL expression during the secretory phase as reported previously[40] could explain the level of 2-AG in our study. However, further studies are needed to validate this finding, especially in the case of 2-AG which is in addition strongly affected by pain in a cycle-specific manner.

Endometriosis was associated with a higher level of C18-20:4 PC measured in the peritoneal cavity. C20:4 PE, C18-20:4 PE, and C18-20:4 PC are phosphatidylethanolamine or phosphatidylcholine lipids which function as reservoir precursors for the polyunsaturated fatty acid, arachidonic acid. PC species are also precursors of *N*-acylethanolamines (AEA, LEA, OEA, PEA, and SEA) generated by *N*-acetyltransferase and phospholipase D. This is the reason why they have been measured in our study. Interestingly, we found a higher level of C18-20:4 PC in patients with endometriosis. This finding is in line with previous studies showing an alteration of phosphatidylcholine metabolites lipid profiles in plasma,[44] peritoneal fluid,[45] and eutopic endometrium[27] of endometriosis women. In mice, artificial induction of endometriosis led to a global increase of circulating PCs, independently of inflammation.[10] Interestingly, plasma and serum levels of C18-20:4 PC in our study correlated strongly and a similar trend was observed for ser C18-20:4 PC. However, the area under the curve was low (0.698 and 0.580 for PF and serum, receiver operating characteristic analysis, data not shown), suggesting that the value of this lipid alone does not qualify as an endometriosis marker. Moreover, our results have to be interpreted with care because of the possible degradation of C18-20:4 PC during the sample storage (to be evaluated). PC and PE lipids were not associated with pain in our study.

Lower levels of circulating LEA and OEA were found in women with endometriosis. This is contrasting with a recent study that reported an increase of AEA, 2-AG, and OEA levels in the plasma of women with endometriosis during the secretory phase.[38] This apparent discrepancy between both studies is puzzling but could be attributed to differences in the methodology used. First, in the study by Sanchez et al., the pain was not characterized in the women without endometriosis, and therefore, it remains to be elucidated whether the reported association was disease or symptom dependent. Second, the data were expressed as mg of total extracted lipids which makes a direct comparison difficult. Third, eCBs were quantified in mononuclear cell layers (Peripheral Blood Mononuclear Cells, PBMCs) containing plasma, which is not strictly plasma. In our study, the lipids were quantified in the serum. This difference may suggest a dysregulation of the ECS at the level of the PBMCs which is relevant considering the key role of the ECS to modulate migration and activation state of these cells[42] and the

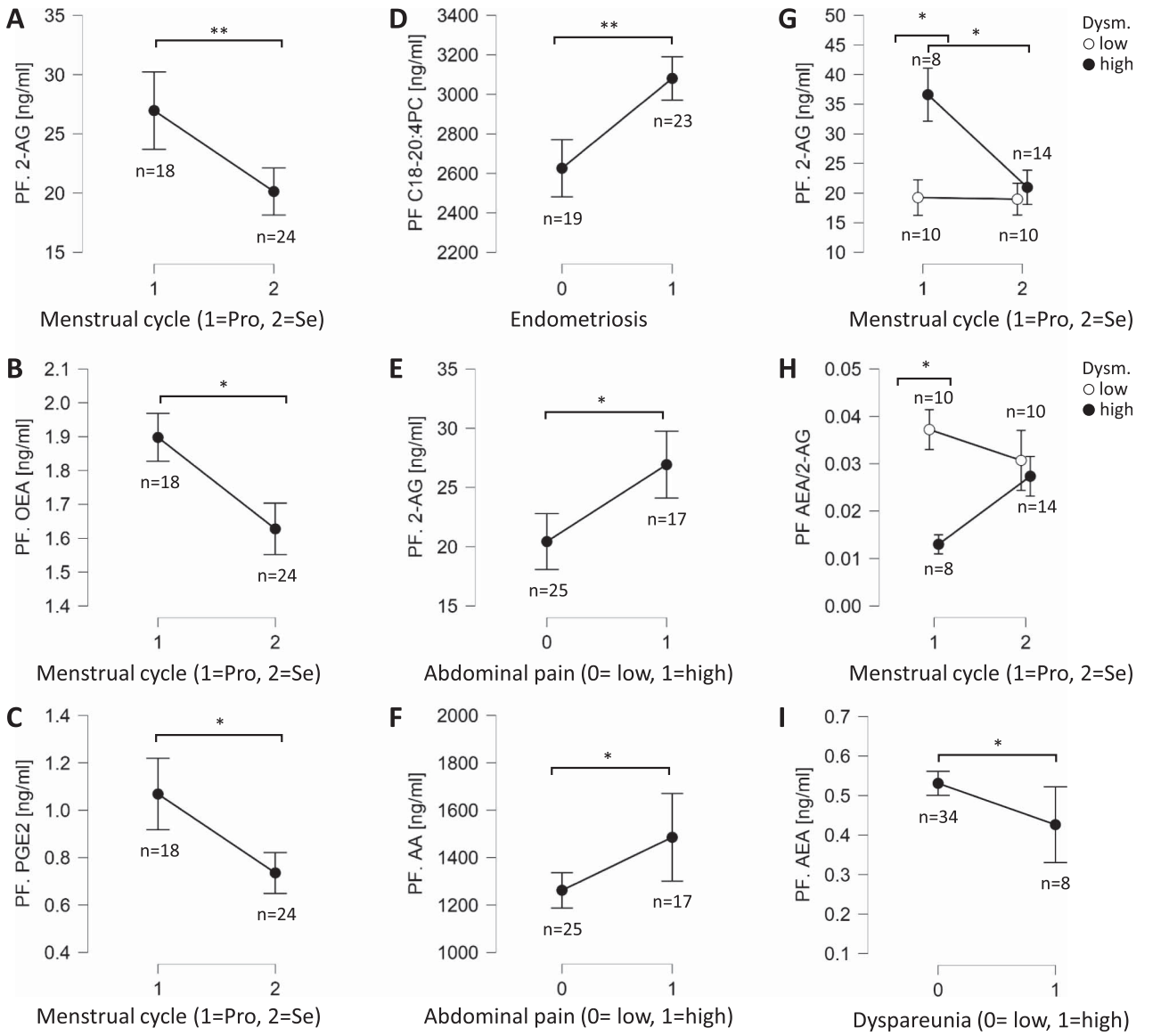

Figure 1. Targeted lipidomics in PF. Descriptive plots for 2-AG (A), OEA (B), and PGE2 (C) in the proliferative ("1 = Pro") and secretory ("2 = Se") phases. Descriptive plots for C18-20:4 PC (D) in patients without ("0") and with endometriosis ("1"). Descriptive plots for 2-AG (E) and AA (F) in patients with low ("0") and high ("1") abdominal pain. Descriptive plots for 2-AG (G) and AEA/2-AG (H) across the menstrual cycle in patients with low (white circles) and high (dark circles) dysmenorrhea. Descriptive plot for AEA (I) in patients with low ("0") and high ("1") dyspareunia. The number of patients is indicated on the figure. The values represent mean ± SEM. ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$ (A–F, I). Two-way ANOVA, Tukey post hoc test, $^{*}P < 0.05$ (G and H). ANOVA, analysis of variance.

transcriptomic modulation previously observed in PBMCs of patients with endometriosis.[12]

In the peritoneal fluid, 2-AG and AEA were both associated with pain. ECs and related lipids are involved in nociception, neuropathic pain, and inflammatory pain by acting on cannabinoid and noncannabinoid receptors, including CB1, CB2, TRPV1, PPARs, GPR55, GPR18, and $GABA_A$, among others.[15,35,41] By comparing the levels of eCBs and other lipids in the peritoneal fluid with the pain scale, we identified 2-AG and AEA as dysregulated signaling molecules in pain. However, it is unclear yet whether this effect is endometriosis dependent as the number of samples were not high enough to perform subgroup analysis. Abdominal pain and dysmenorrhea were characterized both by a high level of 2-AG in the peritoneal fluid. However, our study revealed differences in the timing when this dysregulation occurs. Hence, the dysregulation was found essentially the proliferative phase for dysmenorrhea while it was seen throughout the cycle for abdominal pain. Strengthening our finding, 2-AG correlated positively with dysmenorrhea and abdominal pain scores. The other particularity of dysmenorrhea is its low level of AEA which is negatively correlated with the pain score during the proliferative phase. Hence, our data suggest that 2-AG concentrations in PF represent a possible marker of pain. The mechanism explaining the change in 2-AG level and AEA/2-AG ratio in abdominal pain and dysmenorrhea, respectively, deserves further investigations. Because of the positive correlation observed between PF 2-AG and ser AA, we propose that the high level of 2-AG observed in dysmenorrhea may be consecutive to a systemic increase of arachidonic acid.

In the peritoneal fluid, eCBs are moderately associated with inflammation. Endometriosis-associated pain has in part been attributed to inflammatory pain and the establishment of a chronic local inflammatory environment. Hence, interleukin-6, tumor necrosis factor alpha, and RANTES are increased in endometriosis-associated dysmenorrhea.[39,43] Among the various inflammatory modulators analyzed in the peritoneal fluid in

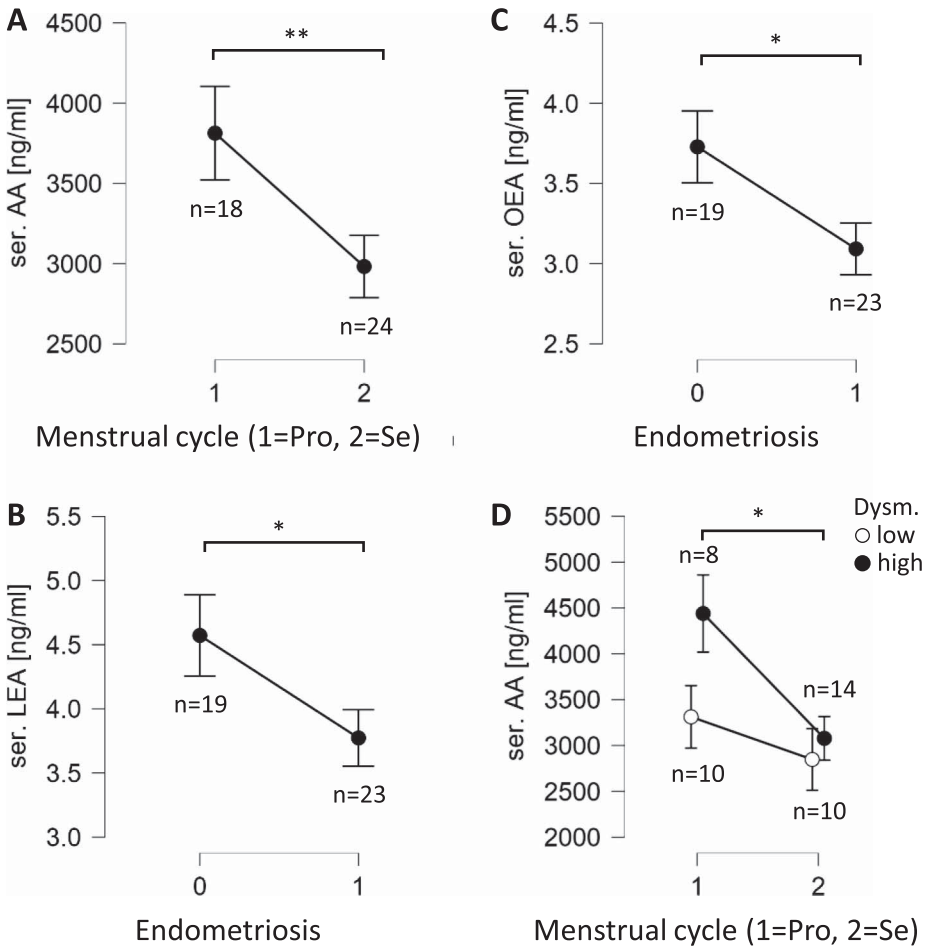

Figure 2. Targeted lipidomics in the serum. Descriptive plots for AA (A) in the proliferative ("1 = Pro") and secretory ("2 = Se") phases. Descriptive plots for LEA (B) and OEA (C) in patients without ("0") and with endometriosis ("1"). Descriptive plots for AA (D) across the menstrual cycle in patients with low (white circles) and high (dark circles) dysmenorrhea. The number of patients is indicated on the figure. The values represent mean ± SEM. ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$ (A–C). Two-way ANOVA, Tukey post hoc test, $^{*}P < 0.05$ (D). ANOVA, analysis of variance.

our study, only PGE2 and defensins were directly positively correlated with eCBs. Hence, our data suggest that both 2-AG and PGE2 could participate to the modulation of abdominal pain, whereas AEA/2-AG and defensins may modulate dysmenorrhea. As reviewed by Hillard,[21] eCBs have the potential to increase or decrease the perception of pain, depending on the mechanism of action. Although the administration of the eCB-like molecule PEA showed to efficiently reduce pain in patients with endometriosis,[4,23] other studies showed that 2-AG, its COX-2 metabolite PGE2-glycerol ester, and PGE2 reduce the pain threshold and exacerbate pain and neurogenic inflammation by modulating

Table 6

Linear correlation between ser AA and PF. 2-AG, PF.

Pearson correlations	All patients (n = 42)	
	r	*P*
ser. AA		
PF. 2-AG	0.510*	<0.001
PF. AEA	0.152	0.335
PF. AEA/2-AG	−0.109	0.494
PF. AA	0.231	0.141

AEA, PF. AEA/2-AG, and PF. AA in the whole cohort (n = 42).
*** $P < 0.001$.
2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; AEA, anandamide; PF, peritoneal fluid.

Table 7

Linear correlation between the endocannabinoids PF.

Pearson correlations	All conditions and cycles	
	All patients (n = 42)	
	r	*P*
PF. AEA		
PF. PGE2	0.187	0.235
PF. PGD2	0.122	0.443
PF. PGE2/PGD2	0.142	0.370
PF. defensins	0.222	0.174
PF. 2-AG		
PF. PGE2	0.354*	0.021
PF. PGD2	−0.155	0.326
PF. PGE2/PGD2	0.412**	0.007
PF. defensins	−0.278	0.086
PF. AEA/2-AG		
PF. PGE2	−0.157	0.322
PF. PGD2	0.030	0.849
PF. PGE2/PGD2	−0.166	0.293
PF. defensins	0.423**	0.007

AEA, PF. 2-AG, PF. AEA/2-AG, and the inflammation markers PGE2, PGD2, and defensins in the whole patient cohort.
* $P < 0.05$, ** $P < 0.01$.
2-AG, 2-arachidonoylglycerol; AEA, anandamide PGD2, prostaglandin D2; PF, peritoneal fluid; PGE2, prostaglandin E2.

TRPV1 sensitivity.[8,22,25,28] Thus, elevated 2-AG and PGE2 levels, as found in our study, may mediate pain sensation.

Our patient samples have been collected under standardized protocols, but over several months, we are aware that many factors such as storage and circadian rhythm can affect the level of eCBs.[16,18] In addition, we would like to highlight the fact that the data related to ser PGD2, ser PGE2, ser corticosterone, and PF corticosterone should be taken with caution because of the reported sensitivity issue. Another limitation to our study is the relatively small number of patients which did not allow us to perform a complete subgroup analysis. Nevertheless, the fact that eCBs present in the peritoneal fluid fluctuate with the sensation of pain mainly dysmenorrhea and abdominal pain shows, that it would now be justified and worthwhile to perform a more in-depth study with a larger cohort.

Endometriosis-associated pain has a strong impact on the quality of life. Unfortunately, current treatment strategies are not fully satisfactory[33] so that novel treatments with better effectiveness and tolerability are urgently needed. Our study demonstrated a link between eCB especially 2-AG, selected inflammation indicators, and pain in women undergoing laparoscopy, suggesting that the ECS may be involved in endometriosis-associated pain. Further investigation will be needed to elucidate these links and to evaluate whether ECS could provide a target model for new therapeutic intervention.

Conflict of interest statement

A. Chicca and J. Gertsch are cofounders of Synendos Therapeutics, a spin-off company of the University of Bern that develops first-in-class eCB modulators. The remaining authors have no conflicts of interest to declare.

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Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/B382.

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