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Title: Comparison of cytokines in the peritoneal fluid and conditioned medium of adolescents and adults with and without endometriosis

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Author Contributions

AB processed samples, performed statistical analysis, wrote the manuscript text, and prepared all tables. AH, MB, and CC developed protocols, assisted with sample processing, and provided significant editing of the manuscript. VS assisted with statistical analysis and provided significant editing of the manuscript. KI and ML performed all sample collections and provided significant editing of the manuscript. LG and SM contributed equally as senior authors and aided with protocol development, statistical analysis, and significant editing of the manuscript.

Abstract (Word Count: 233)

Problem: To compare inflammatory- and immune-associated peritoneal cytokines of adolescents and adults with and without endometriosis

Methods of Study: In a nested case-control study in multiple university-affiliated scientific centers, ten adolescents and thirteen adults with visually- and histologically-confirmed endometriosis (cases), thirteen adolescents with visually-suspected endometriosis but indeterminate (7 patients) or negative (6 patients) histology, and fifteen adults undergoing surgery for non-malignant gynecologic disease without endometriosis (controls) underwent laparoscopic aspiration of peritoneal fluid (PF), from which PF and conditioned medium (CM) cytokine levels were assayed.

Results: Compared to adults with endometriosis, MCP-3, IL-12p40, MIP-1β, and IL-15 were significantly higher among adolescents with endometriosis, while TNF-β and CTACK were

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lower among adolescents. These differences were similar comparing adolescents with endometriosis to adult controls except for MIP-1β, which was not statistically different. MIP-1β was, however, the only cytokine observed to differ between adult cases and controls. There were no significant differences in CM cytokines among the three groups. Results were similar when analyses were restricted to samples collected 1) during menstrual cycle days 1-10, 2) from patients unexposed to exogenous hormones, or 3) from all adolescents despite presence or absence of histologic endometriosis.

Conclusions: Biologically relevant and statistically significant differences in six PF cytokines were observed and suggest a more pro-invasion cytokine profile among adolescents with endometriosis. Adolescents with endometriosis have unique peritoneal cytokine profiles and molecular behavior when compared to adults with and without endometriosis.

Key words: endometriosis, adolescent, cytokine, peritoneal fluid, conditioned medium

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Introduction

Endometriosis is characterized by ectopic growth of tissue resembling the endometrium often associated with chronic pelvic pain and infertility. It affects ~10% of women of reproductive age [1] with onset of symptoms often occurring in the teenage years [2]. However, endometriosis is frequently unappreciated as a pediatric or adolescent disease [3] and typically remains undiagnosed and untreated for years [2] while young patients continue to experience severe pain and with great impact on quality of life and fertility [4,5]. This may be due in part to a reluctance to operate on a young population [6], yet surgery remains the only way to reliably diagnose endometriosis – particularly the superficial peritoneal lesions most common in adolescents [1].

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There is a 25-47% prevalence of endometriosis in adolescents undergoing surgery for pelvic pain [7-10], and approximately 75% of adolescents who do not respond to medical therapy for dysmenorrhea have the disease [10,11]. Despite these statistics, there is a paucity of data on adolescents with endometriosis [12]. We know this age group differs from adults with endometriosis in several ways. Symptom profiles vary between adults and adolescents [5], but this may reflect who undergoes a surgical evaluation for pain or infertility [10,11,13]. Superficial peritoneal lesion appearance also differs with red and clear lesions more frequently visualized in adolescents compared to adults, whereas adults more often present with the classically described blue/black "powder-burn" lesions [14-16]. As endometriosis is postulated to have multiple etiologies [1,17], adolescent endometriosis may represent a subset of disease with different natural history and mechanistic behaviors than endometriosis with onset later in life – independent of diagnostic delays.

Endometriosis has long been appreciated as a chronic inflammatory disease [18-21]. Animal models of endometriosis have demonstrated reduced lesion burden after inhibition of cytokines such as tumor necrosis factor-α, cyclooxygenase-2, peroxisome proliferator-activated receptor-γ, and c-Jun kinase [22-24]. One approach to molecular classification of adult endometriosis patients (hormone-treated or untreated) and controls into subgroups applied an unsupervised multivariate analysis to identify a "consensus signature" of 13 elevated peritoneal fluid immune network cytokines [25]. The signature was correlated with clinical severity of endometriosis with regard to infertility and partially with pain severity but did not correlate with the revised American Society for Reproductive Medicine (ASRM) classification of endometriosis, which is based on a weighted scoring index but has been noted to correlate poorly with infertility, pain severity, and prognosis [25-27]. This investigation suggested a critical role of macrophage activation in the pathophysiologic process and identified c-Jun kinase as a major regulator of cytokine production in cells cultured from patients; thus, such studies may indicate investigative targets for prediction of effective post-surgical symptom management.

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Our primary objective was to compare levels of inflammatory- and immune-associated cytokines in the peritoneal fluid of adolescents and adults with surgically visualized and histologically confirmed endometriosis and adults with non-malignant gynecologic pathology but no visual or histologic evidence of endometriosis. In addition, we evaluated molecular differences among these groups assayed from peritoneal fluid cell 24-hour conditioned medium to elucidate differences in cell growth and behavior *in vitro* as compared to *in vivo*. Prior to analysis, we hypothesized that the peritoneal fluid and 24-hour conditioned medium of adolescent patients with endometriosis would exhibit patterns of inflammatory- and immune-associated cytokines that differ from those in adults with endometriosis and adult controls.

Materials and Methods

This nested case:control study was conducted in collaboration between the Boston Center for Endometriosis (BCE) and the Center for Gynepathology Research (CGR). Aims and protocols were approved by Institutional Review Boards at Brigham and Women's Hospital, Boston Children's Hospital, and Newton-Wellesley Hospital; additionally, approval was obtained from the Massachusetts Institute of Technology Committee on the Use of Humans as Experimental Subjects. All methods were performed in accordance with the relevant guidelines and regulations of each Board, and informed consent was obtained from all participants in accordance with the high standards of these review boards. For minors, the parent provided consent, and the patient assented.

All adolescents and adults with endometriosis had symptoms consistent with endometriosis. To determine the cut point between adolescence and adulthood, time since menarche was calculated as a proxy for disease chronicity. Patients in the adolescent group were postmenarchal but ≤9.0 years since menarche at the time that they underwent laparoscopy, due to a suspicion for endometriosis based on history, physical exam, and/or imaging. Adults with endometriosis were

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premenopausal but ≥13 years since menarche at the time that they underwent laparoscopy, due to a suspicion for endometriosis based on history, physical exam, and/or imaging.

Like adult cases, adult controls were premenopausal but ≥13 years since menarche. However, this group underwent laparoscopy for non-malignant indications such as fibroids, adenomyosis, or unruptured ovarian cysts. This group's surgical and histological findings were negative for endometriosis. All study patients with a history that could be hypothesized to impact progression of endometriosis or affect immune and inflammatory cytokine levels were excluded, specifically, patients with prior bilateral tubal ligation, Crohn's disease, complex atypical endometrial hyperplasia, and tamoxifen use.

Adolescent patients were participants in the Women's Health Study: from Adolescence to Adulthood (A2A), a prospective cohort study conducted within the BCE that has been described in detail previously [5]. In brief, current and past symptoms, demographic and anthropometric characteristics, medical history, and lifestyle factors were collected via a modified (lengthened) version of the WERF EPHect standard clinical questionnaire [28] prior to surgery. Additional clinical details were abstracted from the participant's electronic medical record.

Surgical details for adolescents were collected via the WERF EPHect standard surgical form [29] and completed by the surgeon in the operating room. Peritoneal fluid collection and processing followed protocols established in prior work and recommended by the WERF EPHect standard fluids protocol [25,30]. All adolescent samples were aspirated from the posterior cul-de-sac immediately after laparoscopic entry and before peritoneal lavage and surgical manipulation. Two (2) milliliters of the peritoneal fluid were immediately aliquotted and transported in ice for processing. Samples were centrifuged within 15 minutes of aspiration for 5 minutes at 1000g and kept on wet ice as separate peritoneal fluid and peritoneal cell components. The supernate,

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used for subsequent cytokine analysis, was cryopreserved within 6 hours of collection by placement in a -20° Celsius freezer. The cell pellet was resuspended in phenol red-free Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% dextran-charcoal-stripped fetal bovine serum (FBS), 1% penicillin/streptomycin/amphotericin B, and 10% dimethyl sulfoxide and was cryopreserved within 6 hours of collection by placement in a -20° Celsius freezer.

For the adult participants, a member of the CGR at the Massachusetts Institute of Technology identified appropriate patients by medical record review. Once identified, patients answered questions on first day of last menstrual period, average cycle length, surgical history, presence or absence of pelvic pain, last exposure to hormones, gravidity, and parity via interview by one of the authors while awaiting laparoscopic surgery in the preoperative area at Newton-Wellesley Hospital. Data including history of diagnosed endometriosis, operative findings such as fibroids or endometriosis lesion distribution, and pathologic diagnoses from surgical specimens were abstracted from the surgical and clinical reports of the surgeon. All peritoneal fluid samples were collected and processed in the WERF EPHect-compliant manner described above for adolescents [25,30].

For all endometriosis patients, staging was performed visually according to the revised ASRM criteria [26]. Adult endometriosis patients included in this study were restricted to those with either stage I or II disease because no adolescent cases presented with stage III or IV disease at the index surgery. During all surgeries, a visualized "index" lesion believed by the surgeon to be endometriosis was sent to pathology for histologic confirmation based on the classic criteria of observed endometrial glands and stroma. All cases had visually-suspected endometriosis and were then further categorized as having histologically positive, histologically indeterminate, or histologically negative lesions. For the primary analysis, patients were only included in the adolescent and adult endometriosis categories if the lesions were histologically positive to meet

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the strictest diagnostic criteria for endometriosis [31]. An expanded adolescent population that required only visually confirmed endometriosis was included in one secondary analysis; these patients were categorized into histologically positive, histologically indeterminate, or histologically negative subsets.

The cryopreserved peritoneal cell samples from endometriosis cases (adolescents and adults) and controls were thawed and cultured on 96-well plates for 24 hours. All samples were plated at a concentration of $1x10^5$ viable cells/cm^2 (as measured by trypan blue exclusion) in 1 milliliter DMEM/F12 + 1% FBS + 1% penicillin/ streptomycin/ amphotericin B medium. Samples were plated in triplicate. After 24 hours in culture, 230 microliters of conditioned medium were collected by pipette into 2 milliliter tubes and centrifuged for 15 minutes in a 4°Celsius room. 210 microliters of supernatant were transferred by pipette into a 96-well V-bottom plate in three equal aliquots of 70 microliters each. This plate was sealed and frozen at -80°Celsius for subsequent cytokine analysis.

The cryopreserved peritoneal fluid samples were then thawed, and the concentrations of 48 cytokines, chemokines, and growth factors were measured using an immunobead assay protocol that operates similar to ELISA assays but enables multiplexing to increase the number of analytes. We have previously developed and validated protocols for sample preparation and quantitative analysis of the concentrations of cytokines, chemokines, growth factors, and proteases in human peritoneal fluid and cell culture samples, as well as ovarian fluid aspirate and other physiological fluids, using this approach[25,32-36]. For this study, both 21-plex and 27-plex human cytokine assays (Bio-Rad, Hercules, CA, USA) were run on each participant's peritoneal fluid and 24-hour conditioned medium in triplicate. All adult case and control samples and all adolescent case samples were assayed interspersed and in triplicate to minimize and identify intraassay artifacts; triplicate samples were also interspersed. Therefore, each plate contained samples from adult cases, adult controls, and adolescent cases with each patient's samples placed

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on different plates. Further, all samples that were compared to one another were run in a single batch to eliminate batch-to-batch variation. The resulting data were read by a FlexMap 3-D (Luminex, Austin, TX, USA) immediately after assay completion.

Coefficients of variation were calculated for log-transformed median fluorescence intensities from triplicates of each cytokine tested from both peritoneal fluid and conditioned medium. Means of the triplicates were calculated if the coefficient of variation was ≤10% (98.5% of triplicates); medians of the triplicate values were calculated if the coefficient of variation was >10% (1.5% of triplicates, which were unrelated to each other). Most cytokine sets (5,171) had triplicate values available for calculation; 201 sets had only two values available due to limitations in sample volume based on cell count, and therefore the mean was calculated; 4 samples had only one value available, therefore that value was used. Standard concentrations were run in each batch. There were three cytokine values out of 864 with a coefficient of variation >10% from the standard; none of these three values was found to be statistically significant in any analysis.

The primary analytic population included adolescents with both visually and histologically-confirmed endometriosis, adults with both visually and histologically-confirmed endometriosis, and adults with neither visually observed nor histologically identified endometriosis. Then, three sub-analyses were conducted. First, the primary analytic population was restricted to patients whose peritoneal fluid was collected during menstrual cycle day 1-10. Second, the primary analytic population was restricted to patients who had not been exposed to hormones for at least three months prior to the surgery during which the peritoneal fluid samples were collected. Third, the analytic population was expanded to include not only adolescents with both visually and histologically-confirmed endometriosis but also those without definitive histologic confirmation. This allowed for stratification of the adolescents by histologic criteria diagnostic for endometriosis, i.e. positive histology, indeterminate histology, or negative histology.

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In all analyses, study groups were compared by Kruskal-Wallis and ANOVA using SAS Version 9.3 (SAS Institute Inc., Cary, NC). All p-values were 2-sided. For cytokine level differences of p-value <0.05, the false discovery rate (FDR) was calculated post hoc using p-values for all cytokines to minimize α-error introduced by multiple comparisons (48 cytokine levels per patient). Results were discounted if an FDR ≥ 0.1 was found. For results with a FDR <0.1, pairwise comparisons between 2 of the 3 groups at a time were calculated by Mann-Whitney U tests using SPSS Version 21 (IBM, New York, USA).

Results

All patients who met criteria during the study period were included. The study population included ten adolescents with both visually and histologically-confirmed endometriosis, seven adolescents with lesions visually consistent with endometriosis but indeterminate histology, six adolescents with lesions visually consistent with endometriosis but negative histology, thirteen adults with both visually and histologically-confirmed endometriosis, and fifteen adults visually negative for endometriosis and also without evidence of endometriosis on any pathologic specimens. Several of the demographic differences noted among our study groups were expected based on the patient populations (Table 1). Adult patients were older and had a higher body mass index (BMI) and higher prevalence of fibroids and infertility than adolescents. No patients in this study underwent laparoscopic surgery for infertility alone. Pelvic pain was reported by all study patients except one adult control whose surgery was for menorrhagia attributed to fibroids. Of those patients included in the primary analysis, more adolescents (70%) were on hormones within three months of surgery than adults (23% of adults with endometriosis and none of the adult controls). All endometriosis patients in the study except for one adult were observed during surgery to have at least one superficial peritoneal lesion in the posterior cul-de-sac.

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Table 2 shows group-specific medians with interquartile (25^{th}-75^{th} percentile) ranges of the peritoneal fluid cytokines. Six cytokines were statistically significantly different in the three-group Kruskal Wallis comparison after FDR adjustment. All six cytokines were also significantly different in the comparison between the two groups with endometriosis: adults and adolescents (Mann-Whitney U). Compared to adults with endometriosis, cytokines that were observed to be statistically significantly higher in adolescents with endometriosis were MCP-3, IL-12p40, MIP-1β, and IL-15, while TNF-β and CTACK were lower among adolescents. These are the same findings comparing adolescents with endometriosis to adult controls (Table 2) except for MIP-1β, which was not statistically different. MIP-1β was, however, the only statistically different cytokine between the adult groups. There were no statistically significant or biologically-relevant differences in conditioned medium cytokine levels among the three study groups (Table 3).

Table 4 shows the statistically significant peritoneal fluid to conditioned medium cytokine ratios in the primary analysis groups (Table 2 values: Table 3 values). Three cytokines had a significantly different ratio in the three-group Kruskal Wallis comparison: IL-12p40, TNF-β, and IL-15. All three ratios were statistically significantly different between the two groups with endometriosis: adults and adolescents. The same significant differences and directions of change were seen when adolescents with endometriosis were compared to controls. There were no significant differences in the peritoneal fluid to conditioned medium cytokine ratios observed when comparing adults with endometriosis to controls.

Restriction of the analyses to those whose surgery was performed on menstrual cycle days 1-10 (Supplementary Tables 1 and 2) or to those who were not exposed to hormones within three months of their surgery (Supplementary Tables 3 and 4) did not reveal any statistically or biologically significant differences in cytokine levels in either peritoneal fluid or conditioned medium nor in the peritoneal fluid to conditioned medium ratios. We also did not find any statistically or biologically significant differences in cytokine levels in peritoneal fluid,

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conditioned medium or in the peritoneal fluid to conditioned medium ratios when adolescent cases were further stratified based on their histologic diagnostic status (Supplementary Tables 5 and 6). Sample size for these sub-analyses was small.

Discussion

We hypothesized that the peritoneal fluid and 24-hour conditioned medium of adolescent patients with endometriosis would exhibit patterns of inflammatory- and immune-associated cytokines that differ from those in adults with endometriosis and adult controls. The results of this study support the hypothesis with regard to peritoneal fluid but not to conditioned medium.

This study showed significant differences in six peritoneal fluid cytokines (MCP-3, IL-12p40, TNFβ, CTACK, MIP-1β, IL-15) when comparing adolescents with endometriosis to adults with and without endometriosis. These differences suggest a more pro-invasion cytokine profile among the adolescents. MCP-3 promotes general cellular invasion while IL-15 is induced by progesterone and specifically promotes placental invasion *in utero*. Lower levels of CTACK likely lead to fewer T-cells in the peritoneal fluid thus leading to lower levels of TNF-β, which is produced by T cells; this shift favors angiogenesis in the peritoneal environment. The other two cytokines that are higher in adolescents than adults are IL-12p40 and MIP-1β; both are produced by and chemotactic for neutrophils, leading to a different inflammatory profile in adolescents. This profile could contribute to greater and/or earlier symptomatology, thus suggesting that adolescence is a distinct window in the natural history of endometriosis. Further, this pro-invasion cytokine profile is seen among adolescents despite a higher use of hormones for treatment compared to other groups, a finding that is supported by previously observed significant differences in peritoneal cytokine profiles in treated versus untreated endometriosis patients [25].

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We also noted significant differences in the peritoneal fluid to conditioned medium ratios of three cytokines: IL-12p40, TNF-β, and IL-15, all of which exhibited a lower magnitude of difference between *in vitro* and *in vivo* levels in adolescents as compared to both adult groups as evidenced by a ratio for adolescents that was closer to 1. The magnitudes of change in cytokine levels from *in vivo* to *in vitro* were not significantly different when the two adult groups were compared to each other. One explanation is that, in adolescents, cells outside of those within the peritoneal fluid have less effect on the peritoneal environment than in adults. For the other 45 cytokines that did not exhibit a significant peritoneal fluid to conditioned medium ratio, their regulation may not be by cells that exist in the peritoneal fluid but rather by cells that are intravascular or in the peritoneum itself, similar to the findings in adolescents. We did not identify significant or biologically-relevant differences in conditioned medium cytokines among the three groups. This could be due to a true lack of difference or due to the effects of sample processing or of the *in vitro* culture environment. Future studies that analyze the cellular composition within and between the groups that were used to generate the conditioned media may yield informative results.

Per clinical indication, more adolescents in this study were on hormones within three months of surgery than were the adult participants, which could be due to patient or provider preferences or due to greater symptom management efficacy of hormones in adolescent versus adult endometriosis. It may also suggest that the adolescents presented with more pain-intense symptoms that are responsive to hormonal treatment than did the adults. Even with greater use of hormones in the adolescent population, there were still significant cytokine differences reflective of a more pro-invasive profile in this age group. Analgesic and anti-inflammatory medication exposure was not quantified, and it is possible that these pharmaceuticals also impact cytokine levels similarly or differentially between adolescents and adults. However, inclusion of hormone-exposed participants leads to greater generalizability of these results and minimization of bias from anti-inflammatory effects of increased pain medication use by patients not on hormones.

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There are several strengths of this study. First, we were able to investigate agnostically a wide range of cytokines in peritoneal fluid versus conditioned medium in an adolescent population, whereas previous studies on adolescent peritoneal fluid have only focused on one or a few chosen cytokines, thus limiting the information gleaned. Second, only patients who met the strictest diagnostic criteria for endometriosis were included in the primary analyses. Additional strengths are that the same internationally ratified protocol was used for all adult and adolescent samples and that all laboratory methods to maximize quality control and minimize interassay variability were applied.

There are some weaknesses of the study. Since adolescent patients only undergo surgery for a strong indication, there was no adolescent control population ethically available without disease or inflammation (e.g. those undergoing bariatric surgery or appendectomy). Also, the adult controls were not healthy women but rather all had non-endometriosis pathology - again due to ethical determination of surgical indication. We attempted to minimize this bias by excluding controls who presented with symptoms or conditions known to be highly inflammatory. Comparing adolescents with endometriosis directly to an adolescent population if that control group was available might demonstrate more cytokine differences than we saw in our study.

A second weakness was the potential for differential patient recruitment and sample collection methods between the adolescent and adult groups, since they were collected at two sites; however, the same protocols were applied and the geographic catchment area was similar. Also, moderate interassay and intraassay variability are known weaknesses of immunoassays including bead-based multiplex immunoassays. We mitigated intraassay variability in our study by running triplicates for each patient, using either means or medians of the triplicate values based on the coefficient of variation, interspersing samples on the assay plate as detailed above, and running all samples in the same batch along with calibrations. Future research that expands upon these multiplex findings for analytes outside the range of the employed assay should consider

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marker-specific ELISA and western blot approaches. All samples that were compared to one another in the analyses were run in a single batch to eliminate interassay variability.

Further, adolescent endometriosis is especially difficult to diagnose histologically based on the currently utilized criteria of finding both glands and stroma, in part because the adolescent lesions are smaller and more delicate than adult samples. Thus, histologic diagnostic criteria may not be as informative for diagnostic confirmation in adolescents.

Conclusions

Improving our characterization of endometriosis, especially in the adolescent population for whom data are sparse, is paramount in understanding this disease and, ultimately, diagnosing and treating this complex pathology more successfully. As part of this pursuit, it is critical to describe the physiologic environment in which endometriosis exists as the peritoneal fluid inflammatory milieu in adolescence may impact fertility in adulthood. Our study suggests that adolescents with endometriosis have a unique cytokine profile and demonstrate different peritoneal fluid molecular behavior when compared to both adults with endometriosis and adults with nonmalignant gynecologic pathology but no endometriosis. This pro-invasive cytokine profile in adolescents may impact future childbearing and need for assisted reproductive technology to conceive. Further, as only one-third of endometriosis patients have infertility, identifying predictors of who is at risk and why is a critical step in preserving future fertility. There are numerous future research avenues in this area, including identification of cytokine profiles that contribute to unique clinically informative endometriosis subtypes, investigation into how nonsteroidal anti-inflammatory drug and narcotic use affect cytokine levels, investigation into secondary analyses undertaken here with more patients included in each group, and correlation of pre-treatment and post-treatment cytokine levels with symptom severity and disease progression.

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Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Tables

Table 1. Demographic Characteristics of Study Groups

	Adolescents with Visual and Histologic Endometriosis	Adults with Visual and Histologic Endometriosis	Adults without Endometriosis (Control)	Adolescents with Visual Endometriosis but Indeterminate Histology	Adolescents with Visual Endometriosis but Negative Histology
Number of Patients	10	13	15	7	6
Medians (25th-75th percentile)					
Age (years)	17 (16-18)	36 (32-40)	40 (36-44)	14 (13-16)	16 (14-16)
BMI (kg/m²)	21.3 (19.3-22.4)	27 (22.3-31)	27 (22.9-30.6)	21 (20.1-21.8)	22 (19.5-22.3)
Cycle Day at Surgery†	12 (8-14)	14 (8-17)	11 (6.5-17)	19.5 (7.5-20.6)	13 (5-14.5)
Patients with any Prior Abdominal Surgery	1 (0-1)	1 (0-2)	1 (0-1)	0.5 (0-1.25)	0 (0)
Gravidity	0 (0)	0 (0-2)	1 (0.5-2.5)	0 (0)	0 (0)
Parity	0 (0)	0 (0-1)	1 (0-2)	0 (0)	0 (0)
Peritoneal Fluid Volume Collected (mL)	9.5 (5.6-14)	11 (7.5-18.8)	7 (3.3-15.0)	12 (9.3-13.0)	7 (6.1-8.8)
N (%)					
BMI Category:					
Underweight (<18.5 kg/m²)	1 (10)	0	0	0	0
Lean (18.5-24.9 kg/m2)	8 (80)	5 (38)	6 (40)	6 (86)	5 (83)
Overweight (25-29.9 kg/m2)	0	4 (31)	4 (27)	1 (14)	1 (17)

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	Adolescents with Visual and Histologic Endometriosis	Adults with Visual and Histologic Endometriosis	Adults without Endometriosis (Control)	Adolescents with Visual Endometriosis but Indeterminate Histology	Adolescents with Visual Endometriosis but Negative Histology
Obese (>30 kg/m2)	1 (10)	4 (31)	4 (27)	0	0
Cycle Phase at Surgery[‡]:					
Follicular	2 (20)	4 (31)	8 (53)	1 (14)	0
Mid-Cycle	0	2 (15)	4 (27)	0	1 (17)
Luteal	1 (10)	4 (31)	3 (20)	2 (29)	0
Unexposed to Hormones	3 (30)	10 (77)	15 (100)	3 (43)	1 (17)
Continuous or Cyclic Hormones	7 (70)	3 (23)	0	4 (57)	5 (83)
Presence of Pelvic Pain[§]	10 (100)	13 (100)	14 (93)	7 (100)	6 (100)
Presence of Fibroids	0	7 (54)	7 (47)	0	0
Infertility History	N/A	2 (15)	2 (13)	N/A	N/A
Anatomic Lesion Distribution[¶]:					
Anterior cul-de-sac/ Bladder	5 (50)	4 (31)	0	3 (43)	2 (33)
Pelvic sidewall/ Ovarian fossa/ Broad and Round ligaments	5 (50)	6 (46)	0	5 (71)	4 (67)
Posterior cul-de-sac/ Uterosacral ligaments	10 (100)	12 (92)	0	7 (100)	6 (100)

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	Adolescents with Visual and Histologic Endometriosis	Adults with Visual and Histologic Endometriosis	Adults without Endometriosis (Control)	Adolescents with Visual Endometriosis but Indeterminate Histology	Adolescents with Visual Endometriosis but Negative Histology
Peritoneal window	0	0	0	0	1 (17)

[†]Cycle day 1 is the first day of full menstrual flow

[‡]For those patients unexposed to hormones

[§]At any point of the cycle and occurring within the three months prior to surgery

[¶]Lesion distributions are not mutually exclusive

BMI = body mass index

Table 2. Peritoneal Fluid Cytokines by Group

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	Adolescents with Visual and Histologic Endometriosis (n=10)	Adults with Visual and Histologic Endometriosis (n=13)	Adults without Endometriosis (Control) (n=15)	Kruskal-Wallis p-values (All 3 groups)	FDR (All 3 groups)	Adolescent Endometriosis vs Adult Endometriosis Mann-Whitney U p-values	Adolescent Endometriosis vs Adult Control Mann-Whitney U p-values	Adult Endometriosis vs Adult Control Mann-Whitney U p-values
Interleukin-2ra (IL-2ra)	611 (398-666)	292 (244-522)	472 (341-666)	0.31				
Monokine induced by interferon-γ (MIG)	2690 (2164-3445)	2225 (1947-3968)	1879 (1509-3992)	0.64				
Interferon-α2 (IFN- α2)	3901 (2837-4927)	3521 (2304-5999)	3337 (2424-4512)	0.87				
Stromal cell-derived factor 1α (SDF-1α)	156 (145-200)	168 (129-182)	160 (123-259)	0.97				
Monocyte chemotactic protein-3 (MCP-3)	145 (110-194)	86 (66-92)	76 (66-106)	0.006	0.06	0.008	0.002	0.68
Interleukin-16 (IL-16)	565 (467-658)	622 (359-1209)	545 (339-661)	0.85				
Interleukin-12p40 (IL-12p40)	750 (631-952)	330 (289-389)	289 (227-373)	0.001	0.02	0.002	<0.001	0.75
Leukemia inhibitory factor (LIF)	122 (106-138)	126 (85-135)	104 (84-125)	0.59				
Tumor necrosis factor-β (TNF- β)	646 (583-674)	1607 (1395-3815)	1693 (1215-5119)	<0.001	<0.001	<0.001	<0.001	0.96
Macrophage migration inhibitory factor (MIF)	965 (901-1127)	1523 (630-2328)	998 (673-1405)	0.78				
Interleukin-18 (IL-18)	322 (234-444)	545 (250-720)	456 (193-842)	0.45				
β-nerve growth factor (β-NGF)	381 (329-417)	420 (378-522)	547 (421-577)	0.04	>0.10			
Growth-regulated oncogene-α (GRO- α)	78 (70-97)	80 (71-85)	84 (69-106)	0.71				
Hepatocyte growth factor (HGF)	569 (314-906)	1136 (574-1626)	1292 (854-1912)	0.03	>0.10			
Interleukin-1α (IL-1 α)	50 (47-53)	50 (46-52)	49 (47-51)	0.67				

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	Adolescents with Visual and Histologic Endometriosis (n=10)	Adults with Visual and Histologic Endometriosis (n=13)	Adults without Endometriosis (Control) (n=15)	Kruskal-Wallis p-values (All 3 groups)	FDR (All 3 groups)	Adolescent Endometriosis vs Adult Endometriosis Mann-Whitney U p-values	Adolescent Endometriosis vs Adult Control Mann-Whitney U p-values	Adult Endometriosis vs Adult Control Mann-Whitney U p-values
Interleukin-3 (IL-3)	319 (309-361)	313 (251-433)	336 (263-453)	0.92				
Stem cell factor (SCF)	3822 (3059-4798)	4905 (4044-6173)	5129 (3645-7038)	0.28				
Tumor necrosis factor-related apoptosis inducing ligand (TRAIL)	212 (129-382)	152 (115-295)	114 (89-205)	0.29				
Monocyte colony stimulating factor (M-CSF)	80 (71-86)	95 (75-101)	87 (82-119)	0.22				
Cutaneous T-cell-attracting chemokine (CTACK)	396 (313-609)	712 (590-844)	680 (610-1149)	0.01	0.08	0.02	0.004	0.50
Stem cell growth factor-β (SCGF- β)	1891 (1534-2210)	2753 (1938-5024)	3172 (1840-4862)	0.06				
Macrophage inflammatory protein-1β (MIP-1 β)	274 (164-489)	121 (107-135)	155 (141-339)	0.001	0.02	<0.001	0.37	0.002
Interleukin-6 (IL-6)	1455 (697-2337)	977 (538-3478)	1160 (750-3562)	0.87				
Interferon-γ (IFN- γ)	110 (91-185)	101 (89-122)	125 (88-149)	0.68				
Interleukin-1ra (IL-1ra)	87 (72-107)	79 (68-96)	75 (65-94)	0.80				
Interleukin-5 (IL-5)	64 (58-70)	56 (51-57)	55 (52-59)	0.04	>0.10			
Granulocyte-monocyte colony stimulating factor (GM-CSF)	84 (69-95)	78 (76-93)	77 (71-84)	0.92				
Tumor necrosis factor-α (TNF- α)	62 (53-76)	60 (50-78)	62 (55-75)	0.88				
Regulated upon activation normal T-cell expressed, and	48 (45-52)	53 (50-234)	52 (50-55)	0.37				

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	Adolescents with Visual and Histologic Endometriosis (n=10)	Adults with Visual and Histologic Endometriosis (n=13)	Adults without Endometriosis (Control) (n=15)	Kruskal-Wallis p-values (All 3 groups)	FDR (All 3 groups)	Adolescent Endometriosis vs Adult Endometriosis Mann-Whitney U p-values	Adolescent Endometriosis vs Adult Control Mann-Whitney U p-values	Adult Endometriosis vs Adult Control Mann-Whitney U p-values
presumably secreted (RANTES)								
Interleukin-2 (IL-2)	83 (68-116)	73 (65-81)	69 (63-75)	0.33				
Interleukin-1β (IL-1β)	76 (60-86)	87 (73-104)	69 (59-87)	0.37				
Eotaxin	636 (352-1758)	579 (459-774)	640 (307-1118)	0.89				
Basic fibroblast growth factor (Basic FGF)	78 (70-86)	74 (69-81)	68 (67-72)	0.29				
Vascular endothelial growth factor (VEGF)	709 (474-1001)	440 (342-1018)	315 (286-505)	0.12				
Platelet-derived growth factor-BB (PDGF-BB)	82 (74-87)	78 (76-85)	75 (71-82)	0.63				
Interferon-γ-induced protein 10 (IP-10)	10883 (5889-13030)	3612 (3110-6311)	6911 (2664-9210)	0.08				
Interleukin-13 (IL-13)	93 (75-108)	82 (74-105)	89 (79-127)	0.86				
Interleukin-4 (IL-4)	79 (71-82)	75 (65-95)	83 (72-96)	0.55				
Monocyte chemoattractant protein-1 (MCP-1)	1865 (884-5455)	2033 (1340-5766)	2408 (881-5325)	0.96				
Interleukin-8 (IL-8)	444 (377-523)	546 (399-1067)	472 (337-829)	0.53				
Macrophage inflammatory protein-1α (MIP-1α)	77 (74-84)	71 (69-78)	69 (67-75)	0.26				
Interleukin-10 (IL-10)	246 (192-314)	177 (121-199)	149 (96-212)	0.08				
Granulocyte colony stimulating factor (G-CSF)	105 (89-135)	117 (95-133)	102 (88-117)	0.56				
Interleukin-15 (IL-15)	913 (694-1430)	628 (332-787)	444 (320-510)	0.003	0.04	0.04	<0.001	0.17

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	Adolescents with Visual and Histologic Endometriosis (n=10)	Adults with Visual and Histologic Endometriosis (n=13)	Adults without Endometriosis (Control) (n=15)	Kruskal-Wallis p-values (All 3 groups)	FDR (All 3 groups)	Adolescent Endometriosis vs Adult Endometriosis Mann-Whitney U p-values	Adolescent Endometriosis vs Adult Control Mann-Whitney U p-values	Adult Endometriosis vs Adult Control Mann-Whitney U p-values
Interleukin-7 (IL-7)	132 (74-163)	112 (86-158)	139 (56-171)	0.91				
Interleukin-12p70 (IL-12p70)	209 (195-241)	179 (134-269)	160 (123-194)	0.19				
Interleukin-17a (IL-17a)	71 (70-77)	69 (66-70)	68 (66-73)	0.10				
Interleukin-9 (IL-9)	191 (165-199)	171 (149-203)	153 (128-178)	0.09				

All medians presented with 25th-75th percentile range; False discovery rate (FDR) <0.1 denotes significance

Table 3. Conditioned Medium Cytokines by Group

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	Adolescents with Visual and Histologic Endometriosis (n=10)	Adults with Visual and Histologic Endometriosis (n=13)	Adults without Endometriosis (Control) (n=15)	Kruskal-Wallis p-values (All 3 groups)	FDR (All 3 groups)
IL-2ra	116 (83-129)	105 (102-117)	127 (115-141)	0.06	
MIG	2181 (314-4280)	331 (184-3979)	3631 (699-9027)	0.29	
IFN-α2	137 (121-141)	125 (115-137)	131 (119-144)	0.59	
SDF-1α	58 (56-60)	58 (55-60)	62 (57-65)	0.049	0.38
MCP-3	174 (158-185)	168 (151-185)	203 (181-253)	0.06	
IL-16	183 (164-286)	203 (188-223)	176 (145-191)	0.38	
IL-12p40	1607 (1491-1739)	1505 (1318-1706)	1604 (1482-1653)	0.24	
LIF	207 (194-218)	195 (188-207)	207 (196-232)	0.35	
TNF-β	297 (273-310)	266 (261-285)	286 (271-329)	0.21	
MIF	1624 (758-1831)	1147 (736-2440)	710 (422-1030)	0.06	
IL-18	152 (135-163)	157 (138-166)	164 (146-236)	0.28	
β-NGF	74 (71-77)	70 (68-74)	73 (68-82)	0.49	
GRO-α	1912 (1189-5136)	4063 (2226-5743)	6509 (2918-12170)	0.06	
HGF	123 (97-144)	130 (103-142)	131 (112-167)	0.72	
IL-1α	661 (584-778)	605 (538-696)	582 (550-626)	0.33	
IL-3	387 (363-400)	357 (326-406)	378 (346-431)	0.44	
SCF	243 (211-293)	220 (201-274)	279 (229-385)	0.27	
TRAIL	165 (151-174)	160 (138-169)	166 (150-195)	0.50	
M-CSF	2636 (1771-4357)	2754 (1946-4027)	4095 (2190-5380)	0.25	
CTACK	114 (105-125)	113 (105-119)	117 (114-134)	0.38	
SCGF-β	281 (264-309)	317 (293-348)	364 (284-392)	0.11	

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	Adolescents with Visual and Histologic Endometriosis (n=10)	Adults with Visual and Histologic Endometriosis (n=13)	Adults without Endometriosis (Control) (n=15)	Kruskal-Wallis p-values (All 3 groups)	FDR (All 3 groups)
MIP-1β	30623 (10586-67798)	39836 (35703-50283)	72611 (51982-91568)	0.06	
IL-6	3058 (834-5167)	1644 (872-4783)	3982 (2180-9864)	0.21	
IFN-γ	189 (165-203)	180 (175-189)	189 (172-222)	0.62	
IL-1ra	367 (281-497)	619 (562-834)	745 (528-1020)	0.009	0.38
IL-5	59 (56-62)	61 (60-63)	63 (56-67)	0.86	
GM-CSF	5473 (4328-6075)	6102 (5756-6311)	6106 (5886-6418)	0.10	
TNF-α	603 (224-803)	258 (204-379)	379 (302-501)	0.57	
RANTES	459 (312-853)	359 (319-522)	415 (332-640)	0.88	
IL-2	686 (522-838)	557 (542-611)	644 (594-758)	0.36	
IL-1β	1065 (238-1775)	400 (305-975)	997 (521-1648)	0.57	
Eotaxin	172 (162-185)	167 (151-189)	168 (151-188)	0.82	
Basic FGF	662 (591-705)	671 (615-698)	670 (646-715)	0.85	
VEGF	1637 (1249-1944)	1662 (1583-1735)	1770 (1497-1920)	0.50	
PDGF-BB	318 (289-337)	314 (290-352)	314 (290-343)	0.95	
IP-10	1797 (125-9877)	279 (133-13218)	7962 (1006-30021)	0.13	
IL-13	122 (105-145)	115 (105-118)	110 (104-120)	0.54	
IL-4	205 (170-240)	194 (189-231)	227 (193-263)	0.25	
MCP-1	26290 (4112-34454)	29362 (14159-48775)	59796 (23576-68071)	0.08	
IL-8	86291 (83626-89728)	92031 (89448-95422)	89131 (87017-93407)	0.07	
MIP-1α	9196 (1353-26344)	2732 (1051-25608)	14185 (4560-25331)	0.67	
IL-10	403 (333-888)	381 (329-565)	491 (395-580)	0.66	

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	Adolescents with Visual and Histologic Endometriosis (n=10)	Adults with Visual and Histologic Endometriosis (n=13)	Adults without Endometriosis (Control) (n=15)	Kruskal-Wallis p-values (All 3 groups)	FDR (All 3 groups)
G-CSF	242 (203-264)	236 (219-252)	248 (225-308)	0.48	
IL-15	3342 (2965-3768)	3620 (3325-4294)	4129 (3746-4878)	0.03	0.38
IL-7	83 (81-85)	83 (80-89)	82 (76-87)	0.51	
IL-12p70	379 (314-441)	345 (316-379)	343 (326-400)	0.80	
IL-17a	817 (680-888)	777 (746-787)	873 (758-915)	0.29	
IL-9	1103 (866-1564)	1198 (1131-1375)	1556 (1271-1653)	0.16	

All medians presented with 25th-75th percentile range; False discovery rate (FDR) <0.1 denotes significance

Table 4. Peritoneal Fluid to Conditioned Medium Cytokine Ratios with Significant Differences

	Adolescents with Visual and Histologic Endometriosis (n=10)	Adults with Visual and Histologic Endometriosis (n=13)	Adults without Endometriosis (Control) (n=15)	Kruskal-Wallis p-values (All 3 groups)	FDR (All 3 groups)	Adolescent Endometriosis vs Adult Endometriosis Mann-Whitney U p-values	Adolescent Endometriosis vs Adult Control Mann-Whitney U p-values	Adult Endometriosis vs Adult Control Mann-Whitney U p-values

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IL-12p40	0.47 (0.31-0.65)	0.22 (0.19-0.27)	0.18 (0.13-0.25)	0.003	0.07	0.009	0.001	0.45
TNF-β	2.18 (1.47-2.73)	6.04 (4.81-10.82)	5.92 (4.54-17.42)	<0.001	<0.001	0.001	<0.001	0.91
IL-15	0.27 (0.18-0.46)	0.17 (0.09-0.21)	0.11 (0.08-0.13)	<0.001	0.05	0.03	<0.001	0.06

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