ORIGINAL ARTICLE: REPRODUCTIVE SCIENCE

Mycoplasma genitalium can modulate the local immune response in patients with endometriosis

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Objective: To detect Mollicutes in women with endometriosis and healthy peritoneal tissues and evaluate the participation of these bacteria in the immune response during endometriosis.
Design: Cross-sectional study.
Setting: University hospitals.
Patient (s): Women with endometriosis (n = 73) and without endometriosis (n = 31).
Intervention(s): Endocervical swabs, peritoneal fluid, and biopsied lesions of endometriosis of women with endometriosis (study group) and healthy peritoneal tissues (control group) were collected during surgery. Clinical characteristics were registered before surgery.
Main Outcome Measure(s): We determined the infectious agents with the use of quantitative polymerase chain reaction (PCR). The cytokine secretion profile was determined with the use of Luminex. The expression of immune response related genes was determined with the use of a PCR array kit.
Result(s): All target microorganisms were detected at least once in the swab samples analyzed. It was possible to observe higher diversity of microorganisms in the samples of swab and peritoneal fluid in the study group compared with the control. *Ureaplasma parvum* was associated with the severity of the symptom dyspareunia. *Mycoplasma genitalium* was associated with higher production of interferon-γ and interleukin-1β. Genes of inflammatory response activation and antigen presentation were up-regulated in biopsied tissue of women with endometriosis. In women with endometriosis, peritoneal fluid cells showed a down-regulation of genes associated with the inflammatory response. This down-regulation profile was higher in presence of *M. genitalium*.
Conclusion(s): *Mycoplasma genitalium* may play a key role in the immune tolerance process and, especially, the aggravation of this profile. More studies are needed to understand this immune tolerance profile of bacterial infections. (Fertil Steril® 2017;■:■–■. ©2017 by American Society for Reproductive Medicine.)
Key Words: Mollicutes, endometriosis, theory of contamination

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Endometriosis is an estrogen-dependent gynecologic disorder characterized by the implantation of endometrial tissue outside of the uterus (1, 2). The symptoms are variable which contributes to delayed diagnosis. Although some patients are asymptomatic, most women present dysmenorrhea, dyspareunia, intestinal and urinary alterations, pelvic pain, chronic fatigue, and infertility (3–6).

The pathogenesis and pathophysiology of endometriosis remain unclear (7). Among the most plausible explanations, the theory proposed by Sampson in 1927 (8) is based on the flowback of endometrial tissue. This happens through the fallopian tubes flowing into the peritoneal cavity with potential implantation and growth of endometrial tissue (7). Retrograde menstruation could explain the majority of events associated with the disease. Even so, only 10% of women with normal tubal patency develop the disease (8). Two other plausible hypotheses suggest that: abnormalities of the eutopic endometrium confer intrinsic resistance to elimination by the immune system; and the disease could be a consequence of the inability of macrophages and natural killer cells to eliminate endometrial implants (1).

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It is still difficult to identify a single mechanism that consistently explains the pathogenesis of endometriosis (9).

The exposure of the lower genital tract to microbes allows them to enter the upper genital tract (10). In this context, some studies investigated the participation of microorganisms in endometriosis (9,11–14). Recently, researchers demonstrated menstrual blood contamination by *Escherichia coli* in women with endometriosis and in control women without endometriosis. Furthermore, one study reported increased levels of bacterial endotoxin (lipopolysaccharide [LPS]) in menstrual fluid and peritoneal fluid of women with endometriosis compared with control women and an LPS/Toll-like receptor (TLR) 4 cascade in the growth of endometriosis (12). The same research group demonstrated that intra-uterine microbial colonization considered to be a subclinical infection was significantly higher in women with endometriosis than in the control. Therefore, the "theory of contamination" or "infectious theory" was proposed to explain these findings. During retrograde menstruation, it was suggested that endometrial tissue and microorganisms are carried to the ovary and peritoneal cavity (9).

Mollicutes are the smallest self-replicating free-living microorganisms (15). Mollicutes are found in genital disorders, but they are also found in healthy individuals. However, five decades ago, some Mollicutes were considered to be infectious agents of the human urogenital tract (16). This inconsistent history has made it challenging to clarify the role of these bacteria (17). *Mycoplasma hominis* is associated with nongonococcal urethritis (NGU), bacterial vaginosis, and post-birth fever (18). In turn, *M. genitalium* was identified as a possible etiologic agent of NGU and nonchlamydial urethritis (19). This species has been detected in cervical samples from patients with salpingitis and acute endometritis (20). Furthermore, *M. genitalium* has been strongly associated with cervicitis (21). In women, an increase of vaginal discharge may be reported as well as dysuria, but the infection may be asymptomatic (22). The Mollicutes mentioned above also present a complex relationship in the host immune response (23). Based on the above considerations, information about the participation of Mollicutes in the pathogenesis of endometriosis is lacking. There is a strong indication that microorganisms could be carried to the upper reproductive tract by means of retrograde menstruation. Therefore, the present study aimed to evaluate the influence of microorganisms with gynecologic importance in the peritoneal cavity and their association with the local immunologic response with endometriosis.

MATERIALS AND METHODS

Study Population

Subjects included in this study were women of reproductive age. Endocervical swabs were collected before laparoscopy, and peritoneal fluid and biopsy tissue samples were collected during laparoscopy, from 104 women with endometriosis ($n = 73$) and without endometriosis ($n = 31$). Patients were evaluated from August 2014 to August 2015. Women 15–51 years of age were recruited. Endometriosis was clinically suspected based on clinical symptoms of chronic pelvic pain, severe or incapacitating dysmenorrhea, deep dyspareunia, cyclic urinary abnormalities (pain and/or bleeding) or cyclic bowel abnormalities (pain and/or bleeding), and infertility. All patients were submitted to videolaparoscopy. According to the surgical findings, patients were divided into two groups: with and without endometriosis. Endometriosis was diagnosed when lesions were observed during surgery and subsequently confirmed by histology. The control group consisted of women without any evidence of endometriosis and who had been operated on for other reasons (Supplemental Table 1; Supplemental Tables 1–4 are available online at www.fertstert.org). The exclusion criteria for both groups were presence of any autoimmune or neoplastic disease and antibiotic therapy 1 month before collecting samples. The study complied with the Declaration of Helsinki standards and was approved by the Research Ethics Committees of the University of São Paulo, São Paulo, Brazil, and of the Hospital Sírio-Libanês, where the study was conducted (1170/CEPSH and AVAP/GBC260, respectively).

The patients in the endometriosis group were staged according to the 1996 classification established by the American Society for Reproductive Medicine (ASRM). Stages I and II were grouped as "initial stages" and stages III and IV as "advanced stages." Thirty-seven patients were classified as being in the initial stages of the disease and 35 in advanced stages. The severity of the disease in the affected organ was used to determine the most important site of the disease. Three categories were established: peritoneal disease, ovarian disease, and deep endometriosis. Among the deep lesions, retrocervical lesions (deep lesions >5 mm in depth), bowel lesions, and lesions of the urinary tract (in both cases affecting muscle layers) were considered. Sites affected by the disease were defined according to the following criteria. For the disease to be considered peritoneal, foci had to be exclusively peritoneal without presence of the deep disease (retrocervical, posterior vaginal wall and rectosigmoid regions, ureters, and bladder) or ovarian disease. Ovarian endometriosis was defined as the absence of deep disease regardless of peritoneal foci. And in the definition of deep endometriosis, no restrictions were made regarding the presence of peritoneal or ovarian disease. The phase of the menstrual cycle of the patient at the time of videolaparoscopy was evaluated (4).

Collection of Clinical and Demographic Data

Before surgery, each woman included in the study self-completed validated questionnaires. Demographic data collected included age, ethnicity, level of education, marital status, weight and height, and familial history of endometriosis. Clinical data collected included previous use of hormones before surgery (which type and time since last use), previous use of an intrauterine device, average length of menstrual bleeding and average length of menstrual cycle, age at menarche, stage of endometriosis (ASRM, 1996), menstrual phase of the cycle, and severity of pain during menses, during intercourse, or at other times. Patients indicated on a visual analog scale (VAS) the intensity of five types of pain

(dysmenorrhea, dyspareunia, nonmenstrual pelvic pain, dyschezia, and urinary alterations). We considered severe pain when VAS was ≥ 7 on a scale of 0–10. None of the patients presented signs or symptoms of sexually transmitted infection before surgery.

Sample Collection

Before application of antiseptics to prepare the patient for the surgery, endocervical samples were collected from women with and without endometriosis with the use of a swab (n = 104). The flow chart of selected women with endometriosis and control women and study design is shown in Supplemental Figure 1 (Supplemental Figs. 1–4 are available online at www.fertstert.org). Samples were collected prior to the prophylactic use of antibiotics. To minimize the risk of contamination, swabs were inserted under visual control avoiding any contact with vaginal walls. After rubbing swabs in the endocervix, all samples were placed into a tube with 5 mL transport media (24) and kept at 4°C. Then the samples were homogenized, aliquoted to 1-mL samples, and stored at −20°C.

Laparoscopy was performed with the use of an umbilical puncture and pneumoperitoneum with no instillation of saline solution into the cavity. Following auxiliary puncture, the peritoneal fluid deposited in the anterior and posterior Douglas pouches was collected with the use of a puncture needle. The samples were aliquoted and stored in dry and sterile tubes placed in a nitrogen liquid container. Samples for quantitative polymerase chain reaction (qPCR) methodologies and for gene expression were centrifuged and stored at −20°C and −80°C, respectively. Samples destined for cytokine analysis were immediately stored at −80°C.

During laparoscopy, samples were collected from endometriotic lesions and healthy peritoneum from the retrocervical region from women with and without endometriosis, respectively. Biopsies from different anatomic sites were divided into three sections intracorporally during the surgery, placed into dry and sterile tubes, and kept in liquid nitrogen. These samples for PCR and gene expression analysis were stored with RNA Later (Ambion) at −80°C. For cytokine analysis, the samples were macerated with the use of the protease inhibitor buffer (20 mmol/L Tris-HCl, 150 μmol/L NaCl, 2 mmol/L EDTA, 1% Triton, 1 μg/mL pepstatin A, and 1 mmol/L PMSF at pH 7.5), centrifuged, and the supernate collected and stored at −80°C.

Real Time PCR

Genomic DNA samples of vaginal swabs were obtained according to the recommendations of Purelink Genomic DNA Mini Kit (Invitrogen). Initially, the extracted DNA was tested for the human β-globin gene to determine whether there were PCR inhibitors in the samples. Real-time PCR assays were performed on a StepOne Plus real-time PCR cycler (Life Technologies) in a 25-mL final volume with the use of TaqMan Real-Time PCR Master Mix (Thermo Fisher Scientific). Positive (DNA of microbial strains) control samples were included. In all experiments, PCR runs included a negative PCR control (without DNA) to detect any possible contaminating DNA in the reagents. Test and control samples were run in duplicate. Labeled probes in the TaqMan format were used to amplify the target gene of each microorganism. Nucleic acid extracts were tested against *M. hominis* (25), *M. genitalium* (26), *Ureaplasma urealyticum* (27), and *U. parvum* (27). For constructing DNA standards for absolute quantitation, the mycoplasmas were first cultured in 2 mL at 37°C and then expanded to 50 mL SP4 broth. In a logarithmic growth phase (based on colorimetric changes), the culture was centrifuged at 20,600*g* for 30 minutes at 25°C. The DNA was extracted with the use of a PureLink Genomic DNA Mini Kit. The genomic DNA copy number was then calculated by means of spectrophotometry (Denovix DS 11 Spectrophotometer; Uniscience). Tenfold serial dilutions (10^7–10 copies/μL) of the mycoplasma DNA standard were prepared and analyzed. For each assay, a novel standard curve was used and the following quality parameters were adopted: $r^2 \geq 0.950$, reaction efficiency 95%–105%, and slope ~3.32. The absolute quantification of microorganisms was done based on the standard curve.

Cytokine Dosage

Cytokine dosage was assessed in peritoneal fluid and biopsy tissue with the use of Luminex multiplex assay technology. This method allows for measuring several analytes at the same time in a single sample. Thus, these assays were based on the panel Procartaplex Immunoassay $T_H1/T_H2/T_H9/T_H17/T_H22$/Tfh/Treg (18 plex) (eBioscience-Affymetrix) to determine granulocyte-macrophage colony-stimulating factor, interferon (IFN) γ, interleukin (IL) 1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27, and tumor necrosis factor (TNF) α concentrations. To normalize the data obtained, we measured the protein concentration in each sample by with the use of the Pierce BCA Protein Assay kit (Thermo Fisher Scientific) following the manufacturer's instructions. For samples of peritoneal fluid, 50 μL of each sample were used, whereas for biopsy tissue 100 μL were used.

Gene Expression Analysis

Gene expression of the inflammasome pathway was verified by qPCR methodology. The cDNA obtained was subjected to analysis with the use of the Human Innate and Adaptive Immune Responses PCR Array kit (Qiagen-SABioscience) for the expression of 84 genes involved in the host response to bacterial infection. This array includes genes related to the host defense to bacteria that are represented in this array, including genes involved in the detection of bacteria and genes involved in the acute-phase response, complement activation, the inflammatory response, and the antibacterial humoral response. All procedures were performed according to the manufacturer's instructions.

Statistical Analysis

Clinical and demographic information was analyzed with the use of SPSS software (version 20.0). To verify association between variables, the Pearson chi-square test was

TABLE 1

Demographic and clinical features of women with endometriosis and the control group, n (%).

Variable	Group: Endometriosis (n = 73)[a]	Control (n = 31)	Total (n = 104)	OR	95% CI	*P* value
Age (y)						.424[b]
Mean	35.75	37.90	36.39			
Median (range)	36.00 (15–49)	39.00 (26–51)	36.50 (15–51)			
Ethnicity						.095[c]
White	55 (75.3)	19 (61.2)	74 (71.2)	1.00		
Black	2 (2.7)	6 (19.4)	8 (7.7)	0.74	0.24–2.24	
Asian	3 (4.1)	0 (0)	3 (2.9)	6.50	1.00–42.17	
Mulatto	13 (17.8)	6 (19.4)	19 (18.3)	ND	–	
Marital status[f]						.975[c]
Married	40 (70.2)	18 (81.8)	58 (73.4)	1.00		
Divorced	3 (5.3)	0 (0)	3 (3.8)	ND	–	
Single	12 (21.1)	4 (18.2)	16 (20.3)	1.000	–	
Widowed	2 (3.5)	0 (0)	2 (2.5)	ND	–	
BMI (kg/m^2)[f]						.351[c]
Underweight (<18.5)	1 (1.6)	2 (9.1)	3 (3.6)	0.96	0.35–2.65	
Healthy (18.5–25.0)	31 (50.0)	10 (45.5)	41 (48.8)	1.00		
Overweight (>25.0)	30 (48.4)	10 (45.5)	40 (47.6)	6.00		
Cycle phase[f]						.375[c]
Amenorrhea	22 (30.6)	5 (16.1)	27 (26.2)	1.00		
Follicular	24 (33.3)	17 (54.8)	41 (39.8)	1.96	0.64–5.99	
Luteal	26 (36.1)	9 (29.0)	35 (34.0)	1.08	0.32–3.59	
Endometriosis stage[g]						.652[d]
I	21 (29.1)		21 (29.1)			
II	16 (22.2)		16 (22.2)			
III	9 (12.5)		9 (12.5)			
IV	26 (36.1)		26 (36.1)			
Most severe site affected by disease						
No endometriosis		31 (100)	31 (30.1)			
Peritoneal	16 (22.2)		16 (15.5)			
Ovarian	3 (4.2)		3 (2.9)			
Deep	53 (73.6)		53 (51.5)			
Dysmenorrhea (VAS)[f]						<.001[d,e]
≥7	53 (80.3)	12 (42.9)	65 (69.1)	5.43	2.07–14.24	
<7	13 (19.7)	16 (57.1)	29 (30.9)			
Acyclic pelvic pain (VAS)[f]						.063[d]
≥7	30 (45.5)	7 (25.0)	37 (39.4)	2.50	0.93–6.68	
<7	36 (54.5)	21 (75.0)	57 (60.6)			
Dyspareunia (VAS)[f]						.009[d,e]
≥7	33 (50.8)	6 (21.4)	39 (41.9)	3.78	1.35–10.54	
<7	32 (49.2)	22 (78.6)	54 (58.1)			
Dyschezia (VAS)[f]						.145[d]
≥7	18 (26.9)	3 (10.7)	21 (22.1)	3.06	0.82–11.38	
<7	49 (73.1)	25 (89.3)	74 (77.9)			
Cyclic urinary alterations (VAS)[f]						.168[d]
≥7	11 (16.4)	1 (3.6)	12 (12.6)	5.30	0.65–43.22	
<7	56 (83.6)	27 (96.4)	83 (87.4)			
Infertility[f]						.014[d,e]
Yes	31 (47.0)	5 (19.2)	36 (39.1)	3.72	1.25–11.04	
No	35 (53.0)	21 (80.8)	56 (60.9)			
Smoking[f]						.779[d]
Ex-smoker or nonsmoker	9 (14.5)	2 (9.1)	11 (13.1)	1.69	0.33–8.54	
Smoker	53 (85.5)	20 (90.9)	73 (86.9)			

Note: BMI = body mass index; CI = confidence interval; ND = not determined; OR = odds ratio; VAS = visual analog scale of pain.
[a] Analysis includes data from all patients in the study independently from clinical samples obtained.
[b] Student *t* test.
[c] Likelihood ratio.
[d] Chi-squared test.
[e] $P < .05$.
[f] Loss of observation: marital status: loss of 24 (n = 79); body mass index: loss of 19 (n = 84); cycle phase and diagnosis/localization: loss of 1 (n = 103); dysmenorrhea: loss of 10 (n = 94); acyclic pelvic pain: loss of 10 (n = 94); dyspareunia: loss of 11 (n = 93); dyschezia: loss of 09 (n = 95); cyclic urinary alterations: loss of 9 (n = 95); infertility: loss of 12 (n = 92); smoking: loss of 11 (n = 92).
[g] According to the American Society of Reproductive Medicine (1996).

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applied. Statistical significance was considered to be at $P<.05$. The confidence level adopted for conclusions was 95%. To evaluate risk factors associated with infections, the odds ratio in univariate analysis was adopted.

The women's ages were described according to groups with the use of summary measures (mean, SD, median, minimum, and maximum). First, the normal distribution of data was checked with the use of a Shapiro-Wilk test. Finally, the groups were compared by means of Student *t* test. The use of hormone medications and the cycle phase were described as groups with the use of absolute and relative frequencies, and, to verify the existence of association, we used the chi-square test and the likelihood ratio, respectively.

To analyze the quantification of microorganisms and cytokine dosage statistical analysis was performed with the use of Graphpad Prism software (version 6.03). First, the Kolmogorov-Smirnov test was applied to evaluate the normal distribution of data. Finally, data were analyzed by means of nonparametric Mann-Whitney test when two groups (for quantification of microorganisms analysis) were evaluated and by one-way analysis of variance followed by Bonferroni post hoc analysis for comparison of infection/coinfection profiles between two groups. Values are presented as mean $\pm$ structural equation modeling (SEM) except for data of gene expression. Data were considered to be significant when $P<.05$.

RESULTS

At the time of surgery, the mean age of the women included in the study was 36.4 years (study group: 35.75 years; control group: 37.90 years). Among the women included in our study, 71.2% self-declared being white, 73.4% were married, and 48.8% had normal weight (body mass index (BMI) 18.5–24.9 kg/m^2). There were no significant differences in clinical characteristics between the women with endometriosis and control women without endometriosis when analyzing the variables listed above ($P>.05$). According to the ASRM 1996 classification, 38 patients had endometriosis in the initial stages (I and II) and 35 had advanced disease (stages III and IV). Of these patients, 33.3% were in the follicular phase of the menstrual cycle; 36.1% were in the luteal phase, and 30.6% were in amenorrhea due the hormone use. Among the clinical symptoms evaluated, the severity of pain in acyclic pelvic pain, dyschezia, and urinary disorders was not related to endometriosis in this study ($P>.05$). Nonetheless, the severity of pain reported for dysmenorrhea ($P<.001$) and dyspareunia ($P<.009$) were statistically higher in women with endometriosis. In addition, infertility was associated with the study group ($P=.014$). We did not find any significant difference between the groups regarding smoking habits ($P>.05$; Table 1).

The prevalence of each microorganism for type of sample in both groups, study and control, are presented in Table 2. All target microorganisms were detected at least once in the swab samples analyzed. The prevalences of *M. genitalium*, *M. hominis*, *U. urealyticum*, and *U. parvum* in the study group were 14.1%, 43.7%, 8.5%, and 5.9%, respectively, and in the control group they were 11.1%, 40.7%, 3.7%, and 3.7%. In the peritoneal fluid samples, three microorganisms were detected in the study group (*M. genitalium* [40.7%], *M. hominis* [27.8%], and *U. urealyticum* [3.7%]), and two were detected in the control group (*M. genitalium* [20.8%] and *M. hominis* [16.7%]). In the biopsied tissue samples, two microorganisms were detected in the study group (*M. genitalium* [13.2%] and

TABLE 2

Detection of Mollicutes (*Mycoplasma hominis*, *M. genitalium*, *Ureaplasma urealyticum*, and *U. parvum*) in endocervical swab sample, peritoneal fluid, and biopsied tissue obtained from women with and without endometriosis, n (%).

	Group					
	Endometriosis			Control		
qPCR	Swab[a] (n = 73)	Fluid[a] (n = 54)	Tissue[a] (n = 68)	Swab (n = 31)	Fluid (n = 24)	Tissue (n = 30)
M. genitalium						
Positive	10 (14.1)	22 (40.7)	9 (13.2)	3 (11.1)	5 (20.8)	2 (6.7)
Negative	61 (85.9)	32 (59.3)	59 (86.8)	24 (88.9)	19 (79.2)	28 (93.3)
M. hominis						
Positive	31 (43.7)	15 (27.8)	4 (5.1)	11 (40.7)	4 (16.7)	(0)
Negative	40 (56.3)	39 (72.2)	64 (94.1)	16 (59.3)	20 (83.3)	30 (100)
U. urealyticum						
Positive	6 (8.5)	2 (3.7)	0 (0)	1 (3.7)	0 (0)	0 (0)
Negative	65 (91.5)	52 (96.3)	68 (100)	29 (77.8)	24 (100)	30 (100)
U. parvum						
Positive	10 (14.1)	0 (0)	0 (0)	6 (22.2)	0 (0)	0 (0)
Negative	61 (85.9)	54 (100)	68 (100)	21 (77.8)	24 (100)	30 (100)
Mollicutes						
Yes	39 (54.9)	29 (53.7)	36 (52.9)	16 (59.3)	8 (33.3)	16 (53.3)
No	32 (45.1)	25 (46.3)	32 (47.1)	11 (40.7)	16 (66.7)	14 (46.7)

[a] No statistical difference among women with and without endometriosis ($P>.05$).

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M. hominis [5.9%]) and one in the control group (*M. genitalium* [6.7%]). Positive samples in qPCR were sequenced for confirmation of results, and all sequenced PCR products showed similarity with the bacterial target. For some microorganisms (*M. hominis*, *U. urealyticum*, and *U. parvum*), it was possible to observe a decreasing trend of the prevalence in the order of swab > peritoneal fluid > biopsy tissue. However, the prevalence for *M. genitalium* did not follow the sample trend (Table 2).

The proportions of microorganisms in each sample for both groups are shown in Supplemental Figure 2. It is possible to observe higher diversity of microorganisms (infection and coinfection) in the samples of swab and peritoneal fluid in the study group compared with the control group ($P<.05$). The samples of biopsy tissue were more similar. However, there were statistical differences between study and control groups ($P<.05$).

With the use of a standard curve to determine the relative quantification of microorganisms, the burden of microorganisms was compared between study and control groups for each sample. There was no statistically significant difference between study and control groups when *M. genitalium* (study 48 $\pm$ 34 copies/mL, control 90 $\pm$ 87 copies/mL; $P=.551$), *M. hominis* (study $2.6 \times 10^4 \pm 1.4 \times 10^6$ copies/mL, control $2.5 \times 10^4 \pm 7.1 \times 10^4$ copies/mL; $P=.931$), *U. urealyticum* (study $4.7 \times 10^4 \pm 9.2 \times 10^4$ copies/mL, control $1.0 \times 10^4 \pm 0$ copies/mL; $P=.857$), and *U. parvum* (study $2.9 \times 10^4 \pm 3.1 \times 10^4$ copies/mL; control $6.7 \times 10^4 \pm 4.4 \times 10^4$ copies/mL; $P=.147$) were detected in the swab samples. Similarly, no statistically significant differences were observed between groups when analyzing the burden of *M. genitalium* and *M. hominis* in peritoneal fluid samples and of *M. genitalium* in biopsied samples (data not shown; $P>.05$). Moreover, no statistically significant differences were observed between women in different menstrual phases (data not shown; $P>.05$).

A positive association was observed between *U. parvum* detection in peritoneal fluid samples and higher severity of dyspareunia (VAS ≥ 7; $P=.019$) in women with endometriosis (Supplemental Table 2). However, when analyzing an association between other detected Mollicutes (*M. genitalium*, *M. hominis*, and *U. urealyticum*) in the same samples and even for *U. parvum* in swab and tissue samples, there was no statistically significant association with the severity of any reported symptoms ($P>.05$). Furthermore, no association was observed between Mollicutes detected, in all samples, and endometriosis, endometriosis staging (ASRM, 1996), and most severe sites affected by disease (Supplemental Tables 3 and 4).

IFN-γ levels in peritoneal fluid were found to be significantly higher in patients with endometriosis colonized by *M. genitalium* compared with the control group not colonized by *M. genitalium* ($P=.049$) and the study group not colonized by *M. genitalium* ($P=.012$). In the same manner, IL-1β levels were significantly higher in the study group colonized by *M. genitalium* compared with the same groups ($P<.05$). IL-6 levels, in turn, were statistically higher in the noncolonized study group and the study group colonized by *M. genitalium* compared with the control group ($P<.05$; Fig. 1). No statistically significant differences were found in the levels of IL-10, IL-12, IL-13, IL-18, and TNF-α ($P>.05$). Cytokines with levels under the standard curve were not represented. Moreover, no statistically significant differences were observed in cytokine levels between women in different menstrual phases (Supplemental Fig. 3).

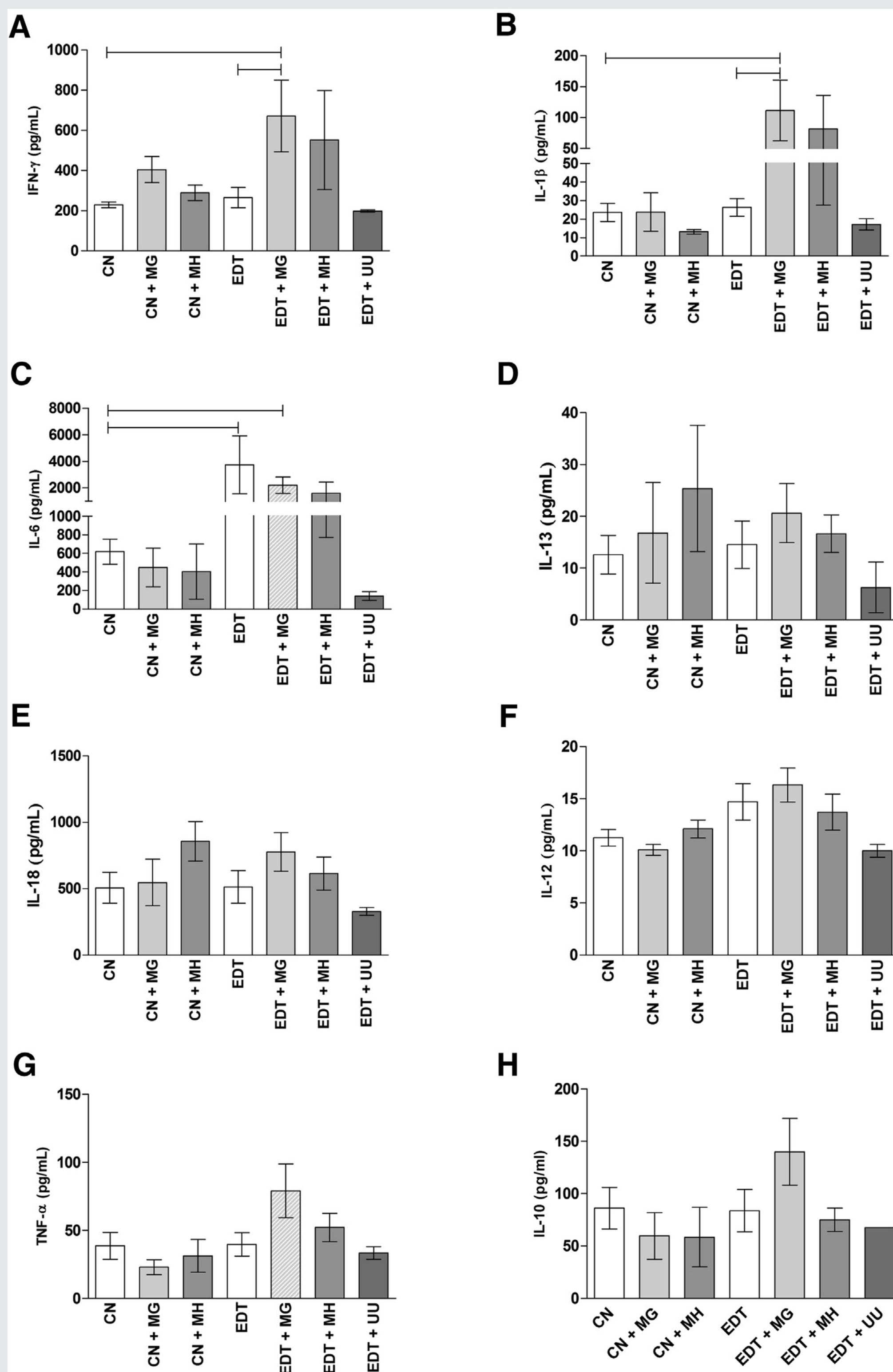
The expression of innate and adaptive immune responses genes were evaluated also in peritoneal fluid samples of both groups. Among 84 of the genes analyzed, 11 were up-regulated and two were down-regulated in endometriotic lesions compared with healthy peritoneum. There were statistical differences between groups for all of the up-regulated genes (*TLR8*, *TLR2*, *TLR1*, *IL1R1*, *IL1B*, *IFNG*, *IFNB1*, *CRP*, *CD86*, *CASP1*, and *APCS*), whereas for down-regulated genes, only one showed a statistical difference (*IFNAR1*; $P<.05$; Fig. 2A). Comparing gene expression in peritoneal fluid cells between the study group and the noninfected control group, 28 genes were down-regulated, five with statistical difference (*SLC11A1*, *NLRP3*, *NFKBIA*, *ITGAM*, and *CD80*; $P<.05$). There was no statistical difference in the two down-regulated genes ($P>.05$; Fig. 2B). Interestingly, comparing the gene expression in peritoneal samples of women with endometriosis colonized and noncolonized by *M. hominis*, 42 up-regulated genes were observed. Statistical difference was observed in four up-regulated genes (*STAT6*, *MAPK1*, *IRF3*, and *GATA3*) and one down-regulated (*CD4*; $P<.05$; Fig. 2C). Otherwise, comparing the gene expression of peritoneal fluid cells from women with endometriosis colonized by *M. genitalium* and noncolonized by any Mollicutes, we observed that down-regulation of genes prevailed (n = 30) compared with up-regulation (n = 1). Seven genes (*TBX21*, *MAPK8*, *IRAK1*, *IFNGR1*, *HLA-A*, and *CDA4*) presented statistically significant differences between groups ($P<.05$; Fig. 2D).

DISCUSSION

Described for the first time more than 150 years ago, endometriosis remains an underdiagnosed disease (28). A definitive diagnosis of endometriosis may be delayed 3.8–7 years, which has significant impact on the women's quality of life, especially considering the prolonged pain they endure (29, 30). The nonspecificity of clinical manifestations and the necessity of surgical diagnosis is reflected in the late diagnosis (5, 28). Therefore, better understanding of the pathogenesis and pathophysiology of the disease remains essential to better diagnosis and treatment (28).

Association studies indicate a relationship between endometriosis and different factors, such as age, race, height, weight, socioeconomic status, among others (1, 31). In the present study, there was no association between factors such as age, ethnicity, marital status, BMI, and ASRM stage. In other aspects, women with endometriosis reported more severity in symptoms, such as dysmenorrhea and dyspareunia (VAS ≥ 7), in addition to infertility, which was consistent with the literature (32, 33). Therefore, diagnosis of endometriosis remains a matter of concern, and the most thorough and comprehensive assessment of the clinical

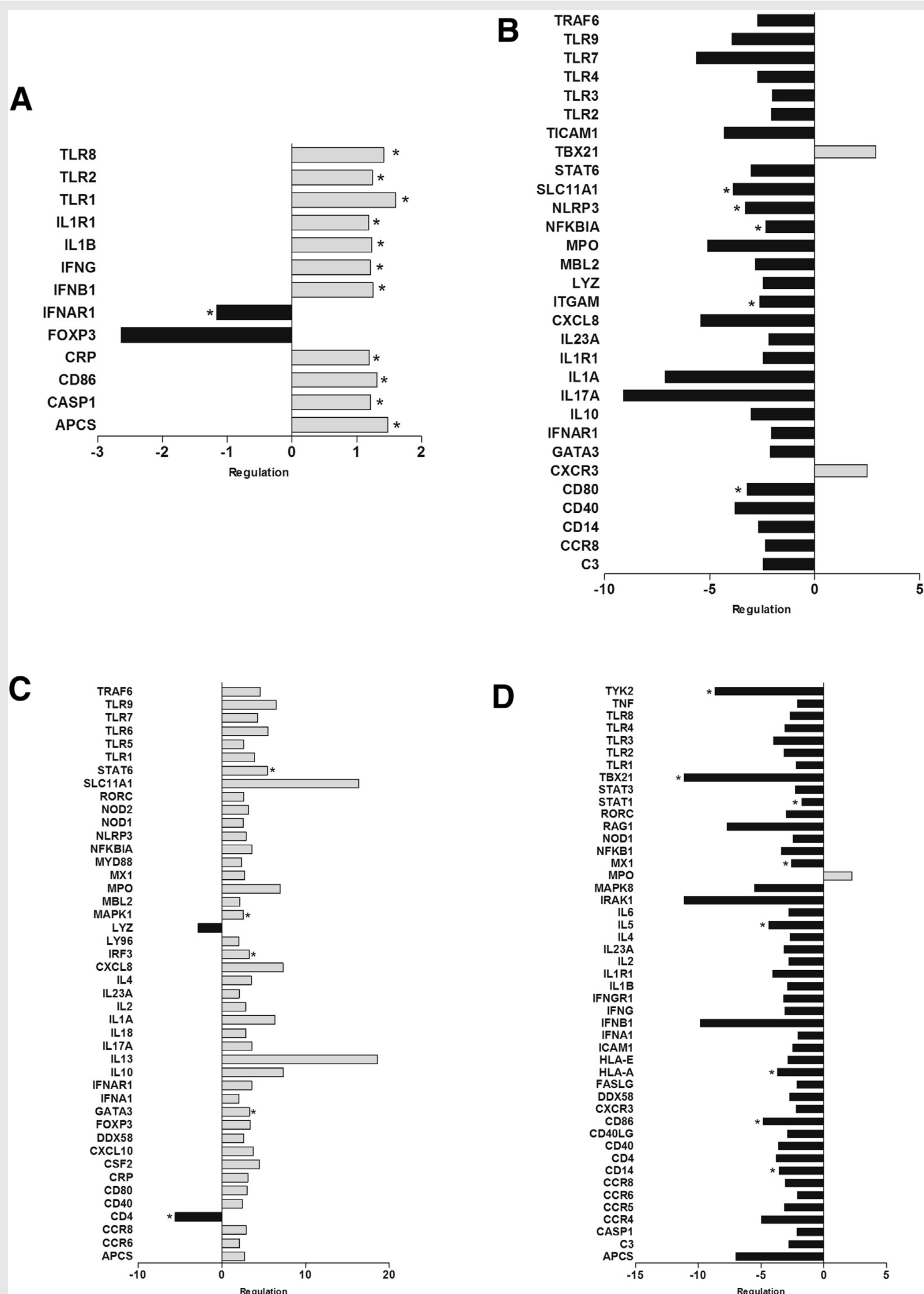
FIGURE 1



Cytokine levels in peritoneal fluid of women with (study group) and without (control group) endometriosis, colonized or not by Mollicutes. (**A**) IFN-γ levels in peritoneal fluid were found to be significantly higher in patients with endometriosis colonized by *Mycoplasma genitalium* compared with the noncolonized control (CN; P=.049) and study (EDT; P=.012) groups; (**B**) Similarly, IL-1β levels were significantly higher in the study group colonized by *M. genitalium* compared with the noncolonized control and study groups (P<.05). (**C**) Interleukin (IL) 6 levels were statistically higher in the noncolonized study group and the study group colonized by *M. genitalium* compared with the noncolonized control group (P<.05); There were no significant differences in the levels of (**D**) IL-13, (**E**) IL-18, (**F**) IL-12, (**G**) tumor necrosis factor (TNF) α, and (**H**) IL-10 (P>.05) MG = colonized by *M. genitalium*; MH = colonized by *M. hominis*; UU = colonized by *Ureaplasma urealyticum*. The results are expressed as mean + SD.

Campos. Mycoplasma genitalium *in endometriosis. Fertil Steril 2017.*

FIGURE 2



Gene expression analysis: up-regulated (*gray*) and Down-regulated genes (*black*). (**A**) Biopsied tissue of women with endometriosis (study group) compared with biopsied tissue of women without endometriosis (control group). There were statistical differences ($P<.05$) between groups in all 11 up-regulated genes (*TLR8*, *TLR2*, *TLR1*, *IL1R1*, *IL1B*, *IFNG*, *IFNB1*, *CRP*, *CD86*, *CASP1*, and *APCS*) but in only one down-regulated gene (*IFNAR1*). (**B**) Peritoneal fluid cells between the study group and the noninfected control group. Twenty-eight genes were down-regulated, five with statistical differences between groups (*SLC11A1*, *NLRP3*, *NFKBIA*, *ITGAM*, and *CD80*; $P<.05$). There was no statistical difference in the two down-regulated genes ($P>.05$) (**C**) Peritoneal fluid cells of women with endometriosis colonized and not colonized by *Mycoplasma hominis*. Forty-two genes were up-regulated. Statistical significance ($P<.05$) was seen for four up-regulated genes (*STAT6*, *MAPK1*, *IRF3* and *GATA3*) and one down-regulated gene (*CD4*). (**D**) Peritoneal fluid cells from women with endometriosis colonized by *M. genitalium* and noncolonized for any Mollicutes. We observed down-regulation of 30 genes (*TBX21*, *MAPK8*, *IRAK1*, *IFNGR1*, *HLA-A*, and *CDA4*; $P<.05$) and up-regulation of one. *Statistical significance ($P<.05$) (nonparametric Mann-Whitney analysis, one-tailed test; Graphpad Prism 1, version 6.01).

Campos. Mycoplasma genitalium *in endometriosis. Fertil Steril 2017.*

findings of each patient is recommended for diagnosis and early management (33).

After the first sexual intercourse, various microorganisms that were not part of the microbiota can be found in the urogenital tract of women. Mollicutes species, in turn, can be isolated from the lower genital tract of healthy individuals and most commonly from individuals with infectious etiology disorders (34). The species *M. hominis*, *M. genitalium*, *U. urealyticum*, and *U. parvum* have been most closely associated with disorders, such as pelvic inflammatory disease, infertility, and NGU (35). Few clinical-epidemiologic studies have connected microorganisms to endometriosis until now. In addition, there are no data in the literature regarding the difference of microorganisms between women with and without endometriosis. Therefore, this study is a pioneer in investigating and demonstrating the presence of Mollicutes in peritoneal fluid and biopsied tissue obtained from the peritoneal cavity of women with and without endometriosis. The prevalence data indicate a decreasing trend in the majority of Mollicutes (*M. hominis*, *U. urealyticum*, and *U. parvum*) in the order of samples: endocervical swab > peritoneal fluid > biopsied tissue. The presence of Mollicutes in the peritoneal cavity follows the hypothesis proposed by Khan et al. (11), that retrograde menstruation could deliver microorganisms throughout fallopian tubes up to the ovaries. Thus, we suggest that, as in the Sampson theory, the tissue reaching the ovaries could pass through to the peritoneal cavity carrying microorganisms attached to it. This hypothesis is supported by evidence of different viable microorganisms in the uterine cavity (9, 12) and in the cystic fluid derived from women with ovarian endometrioma (11). Cicinelli et al. (36) reports that the diagnosis of chronic endometritis was more frequent in patients with endometriosis than in the nonendometriosis group ($P < .001$). The authors could not establish a causal relationship between endometriosis and chronic endometritis, but speculated that in women who have endometriosis, common symptoms such as pain or abnormal uterine bleeding may actually stem at least in part from chronic endometritis. Another study observed that the incidence of endometriosis was higher in patients with genital tract infection than in control subjects (37). These findings suggest that the uterine cavity and anatomic sites where endometriosis lesions are commonly found could be colonized frequently by means of the process proposed by Sampson. In the present study, a molecular strategy was used to search for microorganisms because of their fastidious characteristics (these bacteria grow only in specially fortified artificial culture media under specific culture conditions, which makes bacterial isolation difficult). The qPCR methodology is more sensitive than culture (the technique usually used in the clinical routine). However, qPCR measures bacterial DNA, not bacteria. Nevertheless, further studies using a culture approach are needed to better understand the relation of these microorganisms and endometriosis.

We propose two explanations for the decrease in microorganism prevalence in the samples in this order: cervical samples > peritoneal fluid > biopsied tissue: 1) During endometrial tissue migration through the fallopian tubes, the microbial load would decrease along the route; and 2) the peritoneal fluid extravasated through the peritoneum travels down from different regions and accumulates in anatomic sites, such as the Douglas cul de sac, thus explaining the higher prevalence of *M. hominis*, *U. urealyticum*, and *U. parvum* in the peritoneal fluid than in the biopsied tissue. Intriguingly, the findings of *M. genitalium* did not present the same trend. Baczynska et al. (38) described the ability of *M. genitalium* to adhere to the epithelium of the oviduct and to infect the upper reproductive tract. Similarly, Ashshi et al. (39) reported *M. genitalium* prevalence (14.1%) in fallopian tubes of women who experienced ectopic pregnancy. Grzesko et al. (40), in turn, reported *M. genitalium* prevalence (8.4%) in peritoneal fluid samples, demonstrating the presence of mycoplasma in the peritoneal cavity. Moreover, according to that author, *M. genitalium* is able to promote asymptomatic infections that are consequently untreated. Under these circumstances, *M. genitalium* could persist in infecting the upper reproductive tract, allowing it to travel to the peritoneal cavity, independently from infecting the cervix or uterine cavity. Thus, these aspects could contribute to differences in the prevalence in each sample analyzed.

In women with endometriosis, functional alterations in immune system cells (macrophages, natural killer cells, cytotoxic lymphocytes, and maybe neutrophils) render the peritoneal microenvironment immunotolerant and allow for endometriosis establishment after retrograde menstruation. Furthermore, cytokines and chemokines produced by different cells could lead to endometriosis progression rather than prevention (41). In the peritoneal fluid, we found IL-6 levels to be significantly higher in patients with endometriosis compared with the control group, which was consistent with findings by Bersinger et al. (42). Similarly, women with endometriosis colonized by *M. genitalium* presented the same results as the control group. McGowin et al. (43) observed that *M. genitalium* might induce higher production of IL-6 by A2EN and ShEN101 human cells during the acute phase of infection (48 hours). Nonetheless, in persistent infection (36 days), insignificant production of cytokines was observed. Campos et al. (44) reported similar results where IL-6 presented higher levels in healthy women compared with those not infected by *M. genitalium*. Nevertheless, it was suggested to use IL-6 levels as a biomarker in peritoneal fluid and serum owing to its positive association with endometriosis stage (45).

Interleukin-1β levels were significantly higher in patients with endometriosis colonized by *M. genitalium* compared with the control and study groups not colonized by any Mollicutes. There was no difference between the study and noncolonized control group, suggesting that *M. genitalium* was decisive for the increased production of this cytokine. Some authors have reported increased production of IL-1β in women with endometriosis (46–48); however, others did not observe differences between women with and without endometriosis (45, 49). Faced with these discordant results, it is plausible to suggest that the presence or absence of *M. genitalium* could be the reason for these discrepant results. Likewise, IFN-γ levels were increased in the study group

colonized by *M. genitalium*, reinforcing the hypothesis that the microorganism was the trigger for the increased cytokine production in women with endometriosis. Mollicutes have a large number of lipoproteins, called lipid-associated membrane proteins (LAMPs). The recognition of LAMPs by the innate immune system may induce the production of proinflammatory cytokines (50–52).

Comparing gene expression in tissue samples of study and control groups without infection, a high expression of genes related to activation of the inflammatory response (i.e., *TLR1*, *TLR2*, *TLR8*, *CASP1*, *IL1R1*, *IL1B*, and *CRP*), activation of macrophages (*IFNG*), activation of the complement system (*APCS*), and antigen presentation (*CD86*) was observed. These findings clearly indicate the inflammatory process in the lesions of endometriosis analyzed. Ahn et al. (53) also noted the inflammatory profile in the lesions and its importance to the process of proliferation, survival, and differentiation of these cells. Luo et al. (54) suggest that Toll-like receptors may play an important role in the origin and development of endometriosis by promoting inflammation. Inducing IL-8 secretion (autocrine and paracrine) allows the invasion and proliferation of tissue in the peritoneal cavity via focal adhesion kinase signaling. Although no gene expression evaluation has been undertaken to compare study and control groups, both colonized by Mollicutes, it is worth noting that *TLR2* was up-regulated. This molecule is recognized as the main inflammatory response activating component to Mollicutes (15). Other authors associated *TLR1* and *TLR2* activation with *M. genitalium* recognition (55, 56), but another study reported that the *M. genitalium*–mediated inflammatory process could also be dependent on TLR6 (57).

The down-regulation profile of genes observed in the peritoneal fluid cells reveals the inhibition of the cells recruited to the site. Genes associated with activation of inflammatory response (*NLRP3*), presentation of antigen and integrins (*ITGAM* and *CD80*), and activation of macrophages (*SLC11A1*) were down-regulated. Owing to the low expression of essential receptors for recognition functions, presentation, and cell activation, unresponsive macrophages and activated T cells are recruited (41). Interestingly, the down-regulation profile described was accentuated by the presence of *M. genitalium* in the same cells. Genes associated with the activation of an inflammatory response (*TYK2*, *STAT1*, *CD14*), T_H1 response activation (*TBX21*), T_H2 response activation (*IL5*), and antigen presentation (*HLA-A*, *CD86*) were down-regulated. Comparing the study group colonized with any Mollicutes and the control group without infection, genes involved in similar processes were down-regulated (*TBX21*, *MAPK8*, *IRAK1* [IL-1 receptor], *IFNGR1* [INFG receptor], and *HLA-A* and *CDA4*–recognizing major histocompatibility complex). It is still unclear how microorganisms are able to induce immune tolerance. However, the ability of *M. genitalium* to persist in the host by using immune modulation mechanisms is well known (15). In contrast, comparing the control group not colonized by any Mollicutes with the study group colonized by *M. hominis*, an inverse profile of gene expression with up-regulation predominance was observed. However, in the same manner, the immune-modulating role of *M. hominis* remains unclear (58). Thus, the complex relationship between microorganisms and immune response in women with endometriosis requires further studies to clarify these issues.

CONCLUSION

To the best of our knowledge, no other similar study has been published in the literature considering Mollicutes in the context of endometriosis. *M. genitalium*, in the peritoneal fluid, may play a key role in the immune tolerance process and, especially, the aggravation of this profile, although more studies are needed to understand this immune tolerance aspect of bacterial infections. It is suggested that endometriosis, regardless of the presence of *Mollicutes*, is able to induce the secretion of inflammatory cytokines in peritoneal fluid, leading to a proinflammatory environment. The inflammatory condition generated by microorganisms associated with endometriosis could contribute to immune cell recruitment, even if they are not functional, which leads to tolerance of the implanted tissue (Supplemental Fig. 4). Therefore, the results of this study may contribute to understanding the physiopathology of endometriosis and may be helpful to better understanding microbial contribution in the development or maintenance of the disease. However, more studies are needed to understand if these organisms are the cause or the effect.

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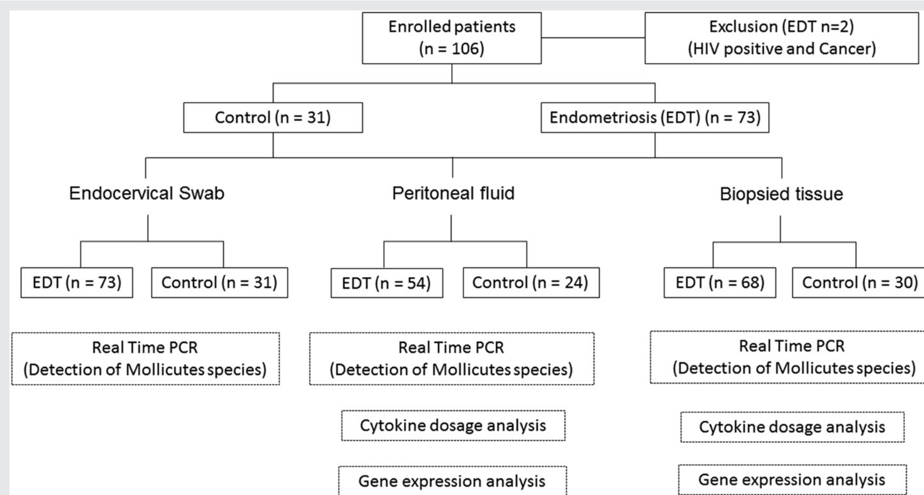
SUPPLEMENTAL FIGURE 1



Flow chart of study design. PCR = polymerase chain reaction.

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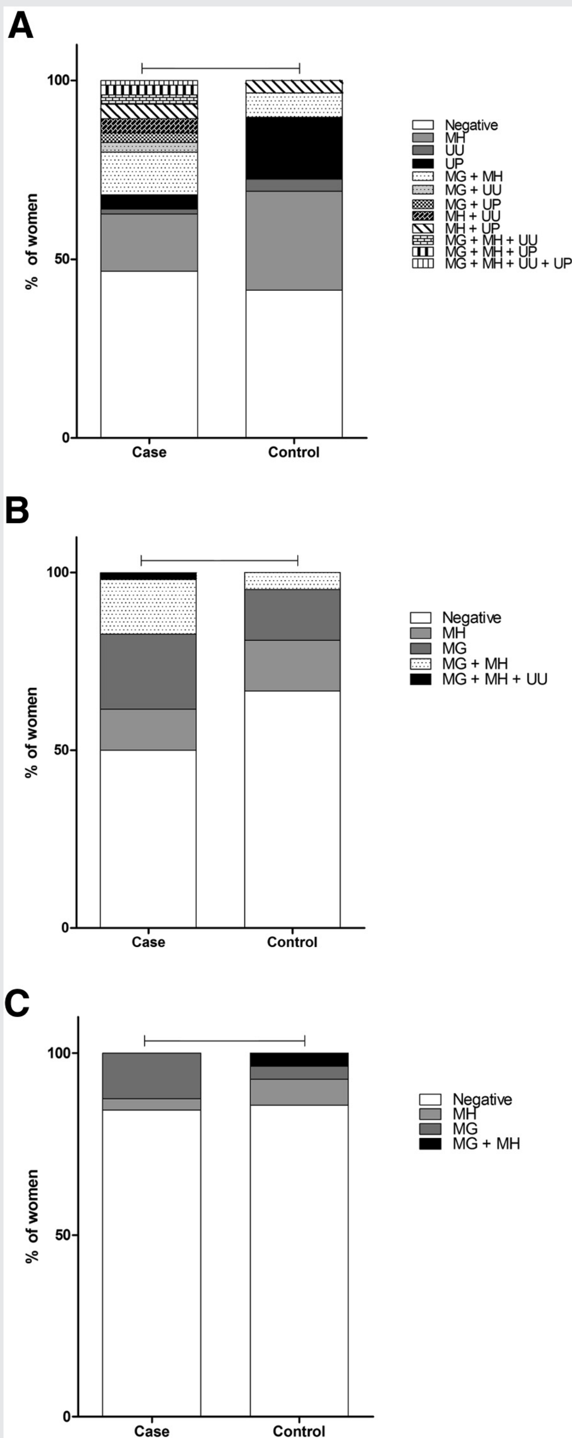
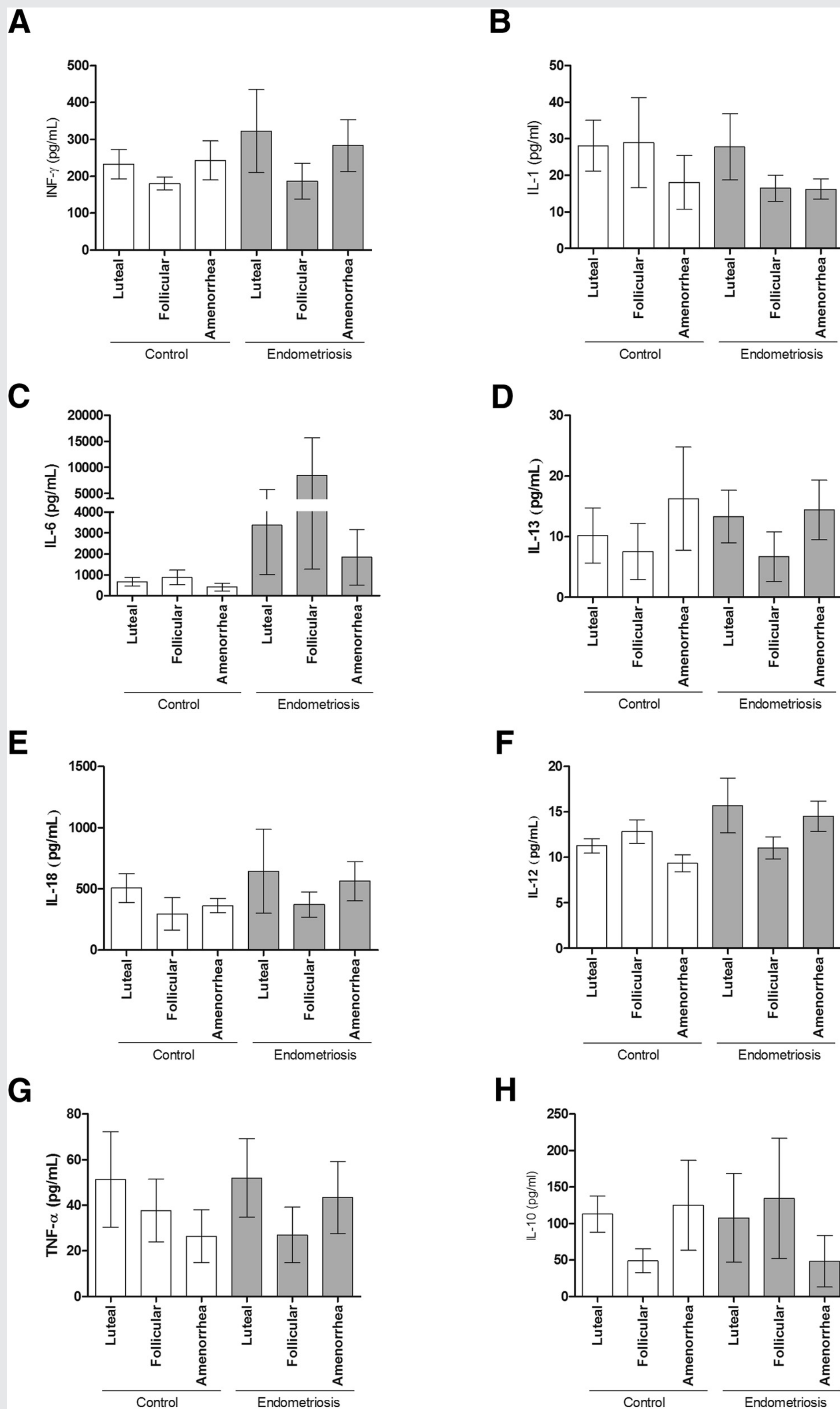
SUPPLEMENTAL FIGURE 2



Mollicutes proportions among women with endometriosis (case) and without endometriosis (control) in (**A**) endocervical swab samples, (**B**) peritoneal fluid samples, and (**C**) biopsied tissue samples. MG = *Mycoplasma genitalium*; MH = *M. hominis*; UU = *Ureaplasma urealyticum*; UP = *U. parvum*. Comparison between proportions (two-way ANOVA and Bonferroni post test) was considered to be significant when $P<.05$.

Campos. Mycoplasma genitalium *in endometriosis. Fertil Steril 2017.*

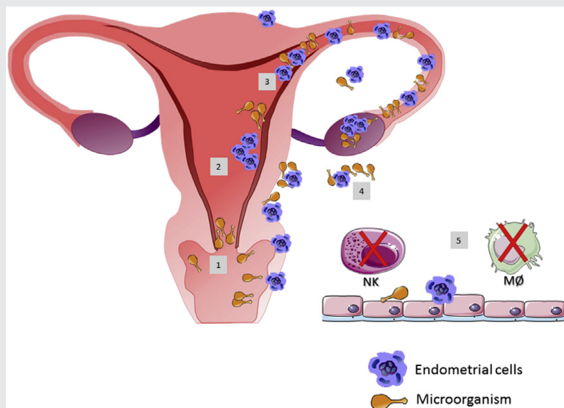
SUPPLEMENTAL FIGURE 3



Cytokine levels in peritoneal fluid of women with and without (control) endometriosis, not colonized by Mollicutes in different menstrual phases. There were no significant differences in the levels of (**A**) interferon (IFN) γ (P=.6835), (**B**) interleukin (IL) 1β (P=.8110), (**C**) IL-6 (P=.6136), (**D**) IL-13 (P=.7456), (**E**) IL-18 (P=.8309), (**F**) IL-12 (P=.1589), (**G**) tumor necrosis factor (TNF) α(P=.7451), and (**H**) IL-10 (P=.6333). The results are expressed as mean + SD.

Campos. Mycoplasma genitalium in endometriosis. Fertil Steril 2017.

SUPPLEMENTAL FIGURE 4



The scheme shows events occurring in the pelvis with retrograde menstruation and microorganism influence on the immune response. 1) Microorganisms could colonize, infect, and persist in the lower genital tract, leading to genital disorders or asymptomatic infections. 2) Microorganisms may ascend to the upper reproductive tract and infect the uterine cavity. Pathogen-associated molecular patterns are recognized by Toll-like receptors involved in the initial inflammatory response and innate immunity. 3) Retrograde menstruation could carry endometrial cells and microorganisms to the fallopian tubes, ovaries, and peritoneal cavity. Species such as *Mycoplasma genitalium* are able to persistently and asymptomatically infect the fallopian tubes and be carried even when they do not colonize or infect the lower tract. 4) Reaching the peritoneal cavity, endometrial cells can attach to peritoneal surfaces, establish a blood supply, and invade nearby structures. Lesions and microorganisms activate macrophages (MØ), which are abundant in the peritoneal fluid in women with endometriosis, and can promote the secretion of proinflammatory cytokines (interleukin IL 1β, IL-8, IL-6, and tumor necrosis factor α). 5) Besides the recruitment of natural killers cells (NK) and MØ, these cells are not responsive, probably owing to the repression of several genes related to their activation and responsiveness locally. Thus, microorganisms and endometrial cells could cooperate to persist in the peritoneal cavity by means of negative regulation of the immune system.

Campos. Mycoplasma genitalium *in endometriosis. Fertil Steril 2017.*