

Micro-RNA profile and proteins in peritoneal fluid from women with endometriosis: their relationship with sterility

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Objective: To define the microRNA (miRNA) profile and its relationship with cytokines content in peritoneal fluid (PF) from endometriosis patients.

Design: Case-control study.

Setting: University hospital, research institute.

Patient(s): One hundred twenty-six women with endometriosis (EPF) and 45 control women (CPF).

Main Outcomes Measure(s): MiRNA arrays were prepared from six EPF and six CPF. Quantitative reverse transcription-polymerase chain reaction validation of nine selected miRNAs (miR-29c-3p, -106b-3p, -130a-3p, -150-5p, -185-5p, -195-5p, -451a, -486-5p, and -1343-5p) was performed. Vascular endothelial growth factor-A (VEGF-A), thrombospondin-1 (TSP-1), urokinase plasminogen activator (uPA), plasminogen activator inhibitor-1 (PAI-1), matrix metalloproteinase-3 (MMP3), tissue inhibitor of metalloproteinases type 1 (TIMP-1), interleukin (IL)-6, IL-8, IL-17A, macrophage inflammatory protein 1 β (MIP1beta), platelet-derived growth factor α -polypeptide A, and regulated on activation, normal T cell expressed and secreted (RANTES) were quantified by ELISA and MILLIPIX.

Result(s): MiRNA arrays showed 126 miRNAs differentially expressed (fold change ± 1.2) (78 down-regulated, 48 up-regulated) in EPF. Validation showed higher levels of miR-106b-3p, -451a, -486-5p, IL-6, IL-8, uPA, and TIMP-1 in EPF. In menstrual phase, EPF presented up-regulation of miR-106b-3p, -130a-3p, -150-5p, -185-5p, -451a, -486-5p, VEGF-A, IL-8, MIF 1 β , uPA, and PAI-1 compared with other phases; however, CPF did not. MiRNA-486-5p was up-regulated in sterile EPF compared with sterile controls, and VEGF-A, IL-8, and TIMP-1 were increased in sterile and fertile EPF compared with fertile CPF.

Conclusion(s): MiRNAs seem to be involved in the peritoneal alterations in endometriosis, suggesting new mechanisms by which ectopic lesions could implant in endometriosis patients; and to serve as biomarkers for fertility outcome prediction. (Fertil Steril® 2017; ■:■-■. ©2017 by American Society for Reproductive Medicine.)

Key Words: Angiogenesis, endometriosis, inflammation, microRNA, peritoneal fluid

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Endometriosis is characterized by the presence of endometrial-like tissues in extrauterine locations,

mainly within the peritoneum and ovaries. The management of this disease ranges from medical therapies,

which are focused on symptomatology but limit the fertility status, to the surgical approach, which is addressed to

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eliminate ectopic lesions but implies invasive procedures (1–3). Endometriosis is still one of the most incapacitating diseases for women owing to the classic symptoms (dysmenorrhea, dyspareunia, dyschezia, dysuria) and is one of the most important etiologies of sterility and poor results of assisted reproductive techniques. Consequences of this disease deeply affect women's quality of life and imply an increasing economic and social burden. Simoens et al. (4) calculated the costs related to endometriosis care by means of a prospective, multicenter, questionnaire-based survey in ambulatory care in 12 centers in 10 countries enrolling 909 women, and they concluded that the average annual cost per woman was €9,579. This amount increased in parallel with severity of endometriosis and years of delayed diagnosis.

Despite its high incidence, prevalence, and its incapacitating symptoms, the etiopathogenic mechanisms of this benign gynecologic disease remain under study. Several theories have attempted to explain the biological events that could explain the presence of endometrial-like stroma and glands in the peritoneal cavity (1). Among them, the retrograde menstruation theory, described by Sampson in 1927 (5), is by far the most accepted proposal. According to this, in 90% of women during their menses (6), endometrial fragments migrate through the fallopian tubes and reach the peritoneum. Whereas women without endometriosis eliminate by physiologic mechanisms, patients with endometriosis are incapable of eliminating these fragments, which are able to implant, survive, and proliferate at different locations (3, 5, 6). Because of this, studying the peritoneal microenvironment of women with endometriosis becomes essential, to reveal the biological alterations responsible for the proliferation of endometriotic implants characteristic of patients with endometriosis.

Our research group has reported the regulatory potential of peritoneal fluid (PF) in patients with endometriosis (7–9). In these studies we described, for the first time, the alteration of the angiogenic state of stromal cells after being treated with endometriotic peritoneal fluid (EPF), enhancing the expression of the main angiogenic factors (7, 8) as well as the expression of microRNA (miRNA) regulators of angiogenesis (8, 9). This was performed by means of an *in vitro* model of endometriosis in which primary stromal cells from control women and patients were exposed to PF from controls (CPF) and patients (EPF).

MiRNAs are small noncoding RNAs (21–23 nt) able to inhibit the translation of their target genes by binding to their messenger RNA 3' untranslated region (3, 10). This posttranscriptional regulation occurs in both physiologic and pathologic conditions, including endometriosis (3, 11–15).

The proinflammatory state of EPF is well known, characterized by an enhanced number of peritoneal macrophages, T and B lymphocytes, and platelets (16–18). These cells, among others, are responsible for the elevated levels of such cytokines as vascular endothelial growth factor-A (VEGF-A), interleukin (IL)-6, IL-8, IL-10, IL-17A, and tumor necrosis factor- α (2, 6, 17, 19–21). Moreover, a higher presence of neutrophil extracellular traps (NETs) has been reported in EPF in comparison with CPF (22).

MiRNAs have been described to be present in all biofluids, including PF, because of their role as intercellular communicators in a paracrine way (23–26). They have even been assessed as predictors of peritoneal metastasis in patients with gastric cancer, owing to their important role as cancer dissemination signals (27).

In the present study we aimed to characterize, for the first time, the miRNA profile in PF from patients with endometriosis, its relationship with the altered proangiogenic and proinflammatory state of the peritoneal microenvironment, and its potential influence on fertility status.

MATERIALS AND METHODS

Ethics Statement

Written informed consent was obtained from all patients and control women, and the study was approved by the Ethics Committee of the Hospital Universitario y Politécnico La Fe, Valencia, Spain (#2008/0111) and Hospital General Universitario, Valencia, Spain, where patients and controls were recruited (#PBL00093).

Clinical Groups

Extended inclusion and exclusion criteria are provided in the [Supplemental Material](#), available online.

Endometriosis patients. A total of 126 Caucasian women with minimal–mild (stages I–II) or moderate–severe endometriosis (stages III–IV) (28) were studied. Sterility was observed in 54% ($n = 68$ of 126) of the endometriosis group.

Control nonendometriosis group. A total of 45 women without endometriosis were enrolled in the study. Sterility was observed in 49% ($n = 22$ of 45) of the non-endometriosis group, in which the indication for surgery was diagnostic laparoscopy to examine tubal patency. Control PF from fertile women ($n = 23$) was obtained while they were undergoing laparoscopic tubal sterilization.

The menstrual phase was identified according to sera hormonal analysis on the day of surgery and before the induction of anesthesia. Forty-seven percent of women with endometriosis were in the proliferative phase ($n = 59$), 44% in the secretory phase ($n = 55$), and 9% in the menstrual phase of the cycle ($n = 12$). Whereas 49% of the women without endometriosis were in the proliferative phase ($n = 22$), 42% were in the secretory phase ($n = 19$) and 9% in the menstrual phase of the cycle ($n = 4$).

Peritoneal Fluid Samples

Blood-contaminated PF samples were excluded. Peritoneal washings were not performed before collection of PFs, nor were anticoagulants used. The PF was immediately cleared of cells and cell debris by centrifugation at $1,500 \times g$ for 30 minutes at 4°C and stored at –80°C until processing.

A total of 126 PF samples from women with endometriosis (EPF) (mean age 32.4 years; range, 20–47 years) and 45 PF samples from women without endometriosis (CPF) (mean age 36.1 years; range, 28–47) were studied.

RNA Extraction

Total RNA was extracted from PF samples with the miRNeasy Mini Kit (Qiagen, #217004) according to the manufacturer's recommendations, with minor modifications. MS2 RNA (Roche, #10165948001) and cel-miR-39-3p mimic (Qiagen, #219610) were spiked-in as carrier and exogenous control, respectively. Quality of RNA for miRNA expression arrays was assessed using the Agilent Bioanalyzer 6000 (Agilent Technologies).

Analysis of miRNA Expression Profiles

MiRNA expression profiles were performed in six PF samples from women with endometriosis (two in each cycle phase) and 6 PF samples from women without endometriosis (two in each cycle phase) paired by age and day of the menstrual cycle. MiRNA expression profiles were performed using GeneChip miRNA 4.0 arrays of the Affymetrix platform according to the manufacturer's protocol. This array contains 2,578 probes for mature human miRNAs and 1,908 probes for their precursors. Arrays were prepared in the Array Service of our institution (IIS La Fe, Valencia, Spain). Data analysis was performed using Partek Genomic Suite software and normalized using the robust multiarray analysis algorithm. Statistical analysis of variance (ANOVA) allowed us to generate a list of differentially expressed miRNAs with ± 1.2 fold change (FC) and P values $< .05$.

Validation Phase

To corroborate results obtained from the array by real-time quantitative reverse transcription–polymerase chain reaction (qRT-PCR), a validation phase was focused on 9 miRNAs whose targets were implicated in angiogenesis, proteolysis, inflammation, or endometriosis on the basis of previous results from our group (miR-29c-3p, -106b-3p, -130a-3p, -150-5p, -185-5p, -195-5p, -451a, -486-5p, and -1343-5p). Cel-miR-39-3p served as exogenous normalizer. Quantitative RT-PCR was performed by the miRCURY LNA Universal RT microRNA PCR kit (Exiqon) using a LightCycler 480 II thermocycler (Roche Applied Science).

Protein Quantification

Levels of vascular endothelial growth factor-A (VEGF-A), thrombospondin-1 (TSP-1), urokinase plasminogen activator (uPA), plasminogen activator inhibitor-1 (PAI-1), matrix metalloproteinase-3 (MMP-3), and tissue inhibitor of metalloproteinases type 1 (TIMP-1) were quantified by commercial ELISAs. Levels of interleukins (IL-6, IL-8, IL-17A), macrophage inflammatory protein 1 β (MIP1 β), platelet-derived growth factor- α polypeptide A (PDGF-AA), and regulated on activation, normal T cell expressed and secreted (RANTES) were quantified using the MILLIPLEX MAP assay kit (Millipore Canada) in the Milliplex Service from our institution (IIS La Fe, Valencia, Spain). Both techniques were performed according to the manufacturer's instructions. Extended information about ELISA kits can be found in the [Supplemental Material](#).

Statistical Analysis

All variables were checked for normal distribution by means of the Kolmogorov-Smirnov test. Differences in the studied variables between two groups were analyzed using the unpaired Student t test or Mann-Whitney U test, when appropriate. Differences in the studied variables among several groups were analyzed by a one-way ANOVA or Kruskal-Wallis test, when appropriate.

MiRNA data quantified by real-time qRT-PCR are presented as FC relative to women without endometriosis group (CPF = 1). Values are expressed as mean \pm SEM.

Levels of significance in correlations between variables were calculated by the bivariate Pearson correlation test. P values of $< .05$ (two-tailed) were considered significant. All these tests were performed using the statistical package SPSS release 20 for Windows (IBM).

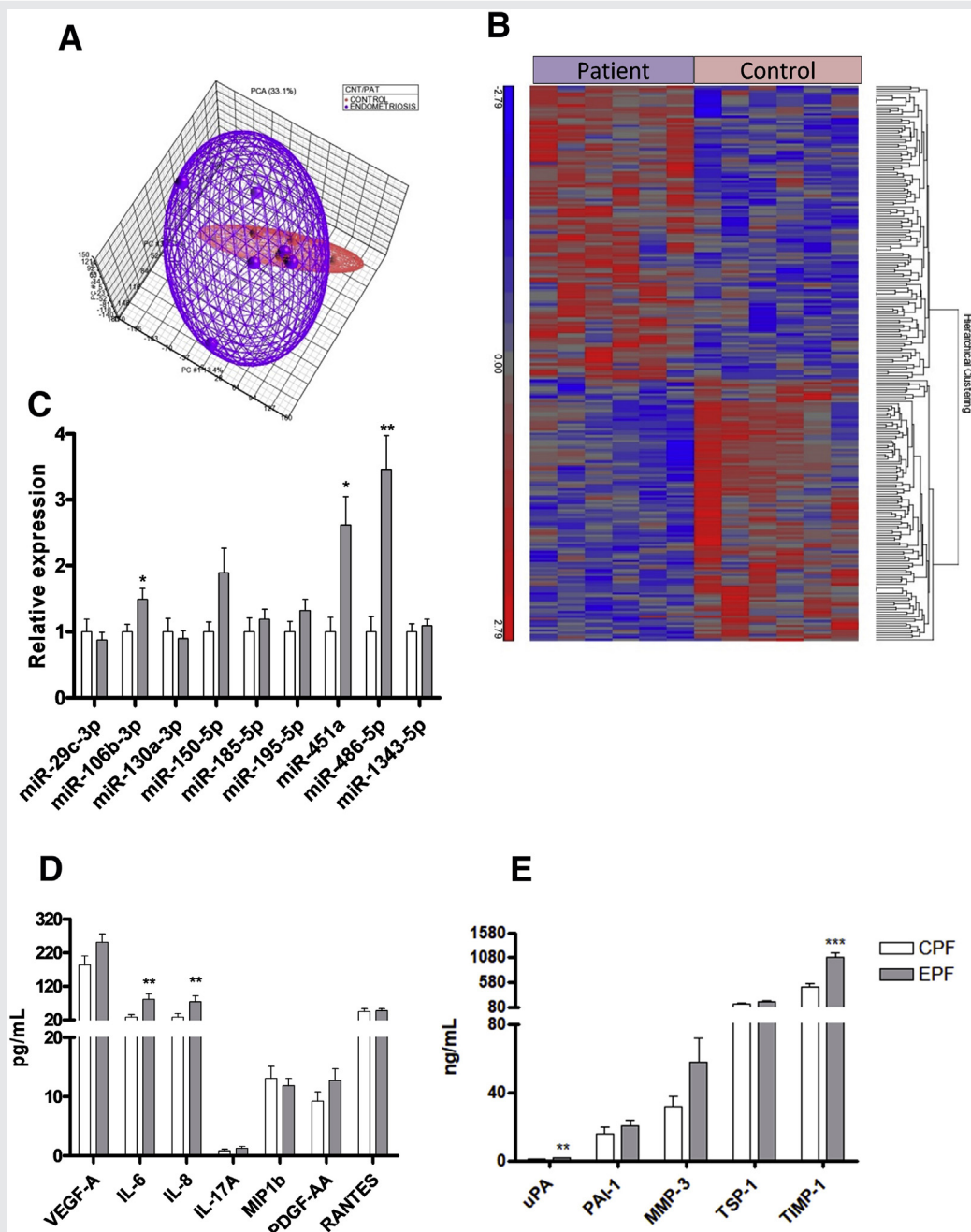
RESULTS

Analysis According to the Presence of Endometriosis

Characteristic miRNA profile in PF from patients with endometriosis. Profiling of miRNAs was completed in six PF samples from patients (two in each cycle phase) and in PF samples from six control women (two in each cycle phase). Principal component analysis graphically represents similarities and divergences among samples and, when factor "patient" was taken into account, the endometriosis group showed higher dispersion in terms of miRNA content ([Fig. 1A](#)). Data were analyzed by ANOVA according to disease status, by which 126 mature miRNAs were found significantly differentially expressed ($P < .05$; FC ± 1.2) (78 down-regulated and 48 up-regulated) between endometriosis patients and control women ([Supplemental Table 1](#)). The aforementioned 126 miRNAs were represented by a supervised hierarchical clustering demonstrating a different expression pattern between patients' and controls' PF ([Fig. 1B](#)). To better identify the magnitude of change, a volcano plot was performed representing the FC [$\log_2(\text{ratio endometriosis/control})$] vs. P value of every mature studied miRNA ([Supplemental Fig. 1A](#)).

We next performed an "in silico" study to identify both the predicted and/or validated target genes of those miRNAs differentially expressed, to investigate which of these 126 miRNAs might regulate the expression of the most important factors involved in angiogenesis, proteolysis, and inflammation, and which of them could be implicated in endometriosis. This analysis led us to select nine miRNAs for validation by qRT-PCR in a larger cohort of samples. Among the selected miRNAs, three (miR-106b-3p, -451a, and -486-5p) were significantly up-regulated in EPF, and two miRNAs (miR-29c-3p and -1343-5p) were significantly down-regulated ([Supplemental Fig. 1A](#) and [Supplemental Table 1](#)). Although no significant differences were observed in miRNA arrays, miR-130a-3p, -150-5p, -185-5p, and -195-5p were also selected for qRT-PCR validation on the basis of previous results from our group (9), which demonstrated their down-regulation in stromal cells from endometrium from patients with endometriosis after being treated with EPF.

FIGURE 1



MicroRNA and protein levels in CPF and EPF. (A) Principal component analysis comparing miRNA patterns between patients with endometriosis and control women; (B) supervised hierarchical cluster; (C) miRNA levels in EPF and without CPF; (D, E) protein levels in CPF and EPF. Data are expressed as mean \pm SEM. MicroRNA expression is presented as FC relative to women without endometriosis (CPF = 1). * $P < .05$; ** $P < .01$ vs. CPF.

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Validation of selected miRNAs by qRT-PCR. We detected significantly higher levels of miR-106b-3p, miR-451a, and miR-486-5p in EPF compared with CPF (Fig. 1C). Increased levels of miR-150-5p were also detected, although differences did not reach statistical significance.

Protein levels in EPF compared with CPF. Aiming to explore the inflammatory and angiogenic status of the PF in endometriosis patients, the most relevant proinflammatory and angiogenic proteins were quantified in all samples. As shown in Figure 1D and 1E, IL-6, IL-8, uPA, and TIMP-1 levels were

Validation of miRNA levels in EPF and CPF according to the cycle phases. No significant differences were observed in CPF miRNA levels through any cycle phase. However, in the menstrual phase, EPF presented significantly higher levels of miR-106b-3p, -130a-3p, -150-5p, -451a, and -486-5p compared with the secretory and proliferative phases. Endometrial PF miR-29c-3p, -185-5p, and -195-5p levels were significantly up-regulated in the menstrual phase in comparison with the secretory phase. Additionally, EPF levels of miR-486-5p in the menstrual phase were significantly higher than levels in CPF (Fig. 2B).

Protein levels according to the cycle phases. Whereas VEGF-A, IL-8, MIP1 β , uPA, and PAI-1 were significantly up-regulated during the menstrual phase compared with the proliferative phase in EPF, CPF did not significantly modify their levels through these menstrual phases. Moreover, EPF VEGF-A, uPA, PAI-1, and MMP-3 levels in the menstrual phase were significantly higher in comparison with the secretory phase; in contrast, IL-17A was down-regulated. Finally, when EPF was compared with CPF, the cytokines IL-6, IL-8, and IL-17A were significantly different during the proliferative phase (Fig. 3).

Analysis According to the Endometriosis Severity Stages

Levels of miR-106b-3p, -451a, -486-5p, uPA, PAI-1, TIMP-1, IL-6, and IL-8 were significantly increased in PF from women with moderate-severe endometriosis (stages III-IV) compared with levels in CPF (Supplemental Tables 2 and 3). Moreover, TIMP-1 was also significantly increased in minimal-mild endometriosis (stages I-II) compared with CPF (Supplemental Table 3). Not only were peritoneal levels of IL-6 and IL-8 higher in patients than in controls (Fig. 1D), but their levels were also significantly increased as the disease advanced (Supplemental Table 3). Although it did not reach statistical significance, an important trend to up-regulation with severity of disease was observed for miR-106b-3p and -451a, whose levels increased mainly in the most advanced stage (Supplemental Table 2).

Analysis According to Fertility Status

All the parameters quantified in PF were also evaluated according to sterility status (Fig. 4, Supplemental Fig. 2).

MicroRNAs levels according to fertility status. Levels of miR-106b-3p and miR-486-5p were significantly higher in EPF from sterile patients compared with CPF from sterile control women (Fig. 4A). Moreover, miR-486-5p was significantly higher in EPF from fertile patients compared with CPF from fertile control women.

Whereas some of the studied miRNAs (miR-106b-3p, -150-5p, -451a, and -486-5p) did not differ within the control group by their fertility status, some others, namely miR-185-5p and -195-5p, trended to be up-regulated in PF from both sterile groups (control and endometriosis) compared with fertile groups, although without reaching statistical significance; a similar result was observed for miR-150-5p levels in sterile patients (Fig. 4A).

Protein levels according to fertility status. VEGF-A, IL-8, and TIMP-1 levels were significantly increased in both EPF groups (sterile and fertile) compared with CPF from fertile control women. In addition, VEGF-A was also significantly increased in sterile versus the fertile CPF groups (Fig. 4B). When sterile groups were compared, IL-6, IL-8, and TIMP-1 showed significantly higher levels in EPF than in CPF. Interestingly, although PAI-1 was overexpressed in EPF independently of fertility status, only the fertile group was significantly different between EPF and CPF (Fig. 4B).

Correlations Between Evaluated Parameters

No significant correlations were observed between age of women and any of the studied parameters in either the endometriosis or the control group.

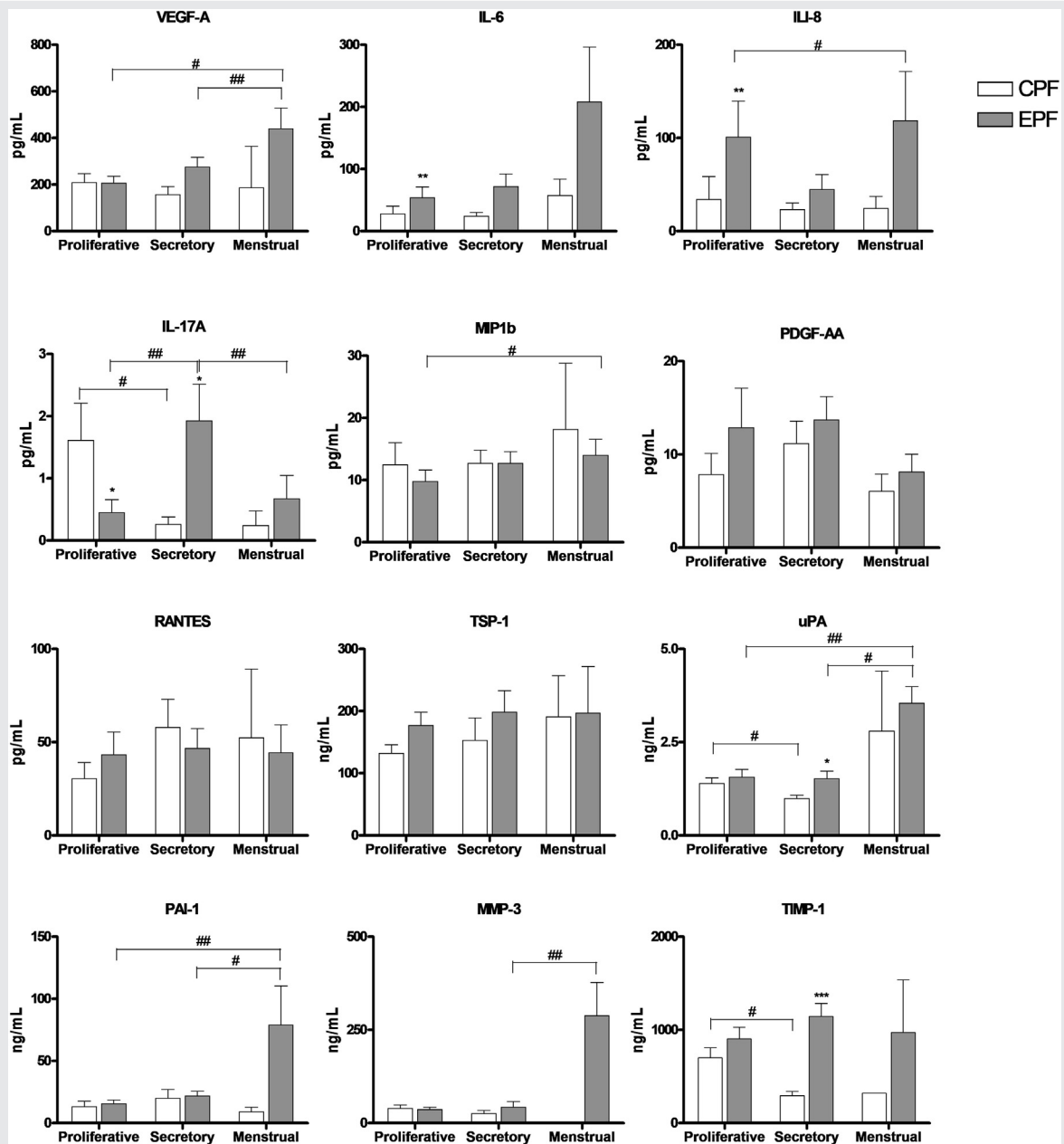
The biggest differences between EPF and CPF were observed in levels of miR-451a and -486-5p; moreover, these miRNAs correlated significantly with RANTES levels (CPF: $r = 0.688$, $P < .001$ and $r = 0.581$, $P = .001$; EPF: $r = 0.281$, $P = .019$ and $r = 0.282$, $P = .022$, respectively). However, miR-451a and -486-5p levels only correlated significantly with MMP-3 in EPF ($r = 0.440$, $P = .009$ and $r = 0.550$, $P = .001$, respectively). Finally, miR-486-5p levels were significantly correlated with uPA in EPF but not in CPF ($r = 0.374$, $P = .015$ and $r = -0.034$, $P = .874$).

Although without reaching statistical significance, miR-150-5p showed an important up-regulation in EPF in comparison with CPF. Moreover, as occurred with miR-451a and -486-5p, miR-150-5p levels significantly correlated with RANTES both in CPF and EPF (CPF: $r = 0.354$, $P = .004$ and $r = 0.550$, $P = .001$; EPF: $r = 0.440$, $P = .009$ and $r = 0.550$, $P = .001$).

DISCUSSION

In the present study we have described, for the first time, the characteristic miRNA content in PF from women with endometriosis as well as its relationship with the main proangiogenic and proinflammatory factors. Endometrial PF presented 126 mature miRNAs with significantly different levels compared with CPF (78 down-regulated and 48 up-regulated). For the validation phase in a larger cohort of PFs, we selected five miRNAs whose target genes were related to angiogenesis or inflammation (miR-29c-3p, -106b-3p, -150-5p, -451a, -486-5p, and -1343-5p) (9, 15, 29). Moreover, miR-130a-3p, -185-5p, and -195-5p were also selected for the validation phase, because in a previous study by our group (9) we detected that cultured stromal cells from endometrium from patients significantly reduced the expression of these miRNAs in response to EPF. The analysis of PF showed a significantly higher level of miR-106b-3p, -486-5p, -451a, TIMP-1, IL-6, IL-8, and uPA in EPF compared with CPF. Levels of miR-150-5p were also increased, although differences did not reach statistical significance (Fig. 1C). Regarding miR-451a, Graham's team has recently published two studies showing increased levels of this miRNA in ovarian endometriomas that correlate with lesion size and with its serum levels (30, 31). Provided that PF is mainly composed

FIGURE 3



Protein levels in EPF and CPF, according to cycle phases. Data are expressed as mean \pm SEM. * $P < .05$; ** $P < .01$; *** $P < .001$ vs. CPF same cycle phase. # $P < .05$; ## $P < .01$; ### $P < .001$ vs. any cycle phase.

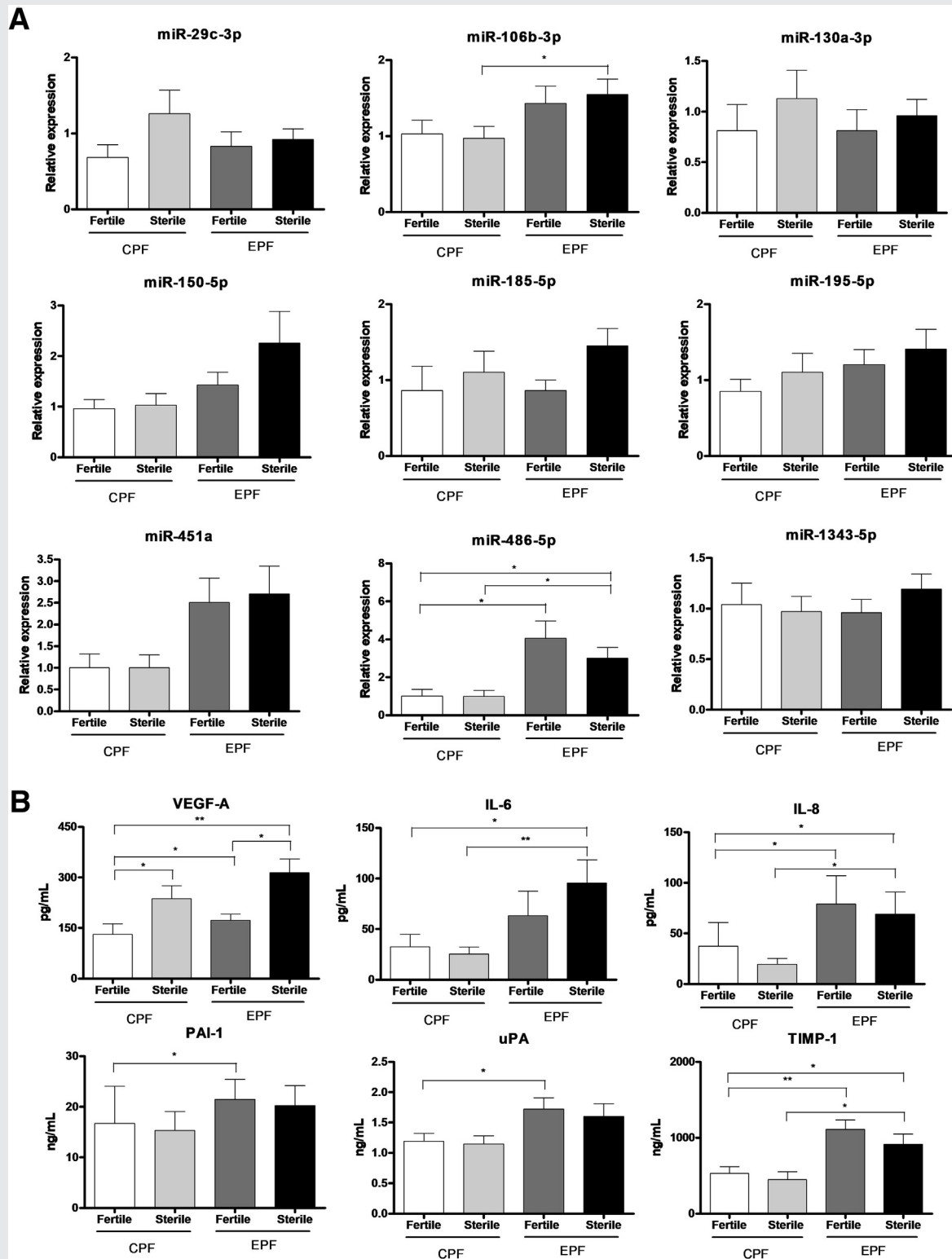
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of blood components (32), and usually up-regulated proteins in serum are proportionally up-regulated in PF (33), our results are in accordance with Graham's, suggesting an important role for miR-451a as a mediator of inflammation in the peritoneal microenvironment. Furthermore, miR-451a has been characterized as the most abundant miRNA in plasma and the main miRNA within erythrocytes (34, 35). In concordance with the retrograde menstruation theory (5), blood and endometrial fragments migrate during

menstruation and are deficiently eliminated by the peritoneal microenvironment in patients with endometriosis, remaining and proliferating; it could be an explanation of the observed enhancement of an erythrocyte-specific miRNA in EPF.

The second most abundant miRNA in plasma is miR-486-5p (35), similar to our findings in EPF (data not shown). In 2013 Rekker et al. (35) reported that this miRNA did not modify its levels through the menstrual cycle in plasma

FIGURE 4



MicroRNA levels in EPF and CPF, according to fertility status. Data are expressed as mean \pm SEM. MicroRNA expression is presented as FC relative to women without endometriosis (CPF = 1). * P <.05; ** P <.01 between groups.

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from healthy women. Similarly, we have not found differences in miR-486-5p levels in CPF across the menstrual cycle (35). However, our results indicate that miR-486-5p not only is up-regulated in EPF in all three phases compared with CPF, but it also presented different levels in each menstrual phase (Fig. 2). Moreover, miR-486-5p has been defined as an exosomal miRNA with a crucial role in intercellular communication in colorectal cancer (36), which, combined with other miRNAs, could be used as biomarker of disease. Taking these studies into account, our results suggest an interesting role for miR-486-5p as a potential intercellular communicator in endometriosis, which should be closely evaluated in future studies.

In our study, with the exception of only miR-1343-5p, all eight studied miRNAs presented increased levels in EPF in the menstrual phase compared with other phases of the menstrual cycle. Similar findings were observed regarding VEGF-A, IL-8, MIP1 β , uPA, PAI-1, and MMP-3 proteins. These findings are in accordance with Sampson's theory (5), because migrated endometrial fragments in the peritoneal cavity require an increased capacity of implantation and vascularization.

Regarding the revised American Society for Reproductive Medicine stages of the disease, most of the miRNAs (miR-106b-3p, -451a, and -486-5p) and proteins (VEGF-A, uPA, PAI-1, IL-6, IL-8, IL-17A, MIP1 β , PDGF-AA, and RANTES) presented higher levels the more advanced the disease, suggesting that the PF might be a potential source of biomarkers (37, 38).

Finally, and taking into consideration the fertility status, whereas the enhancement of miR-106b-3p and -451a levels seemed to be due to endometriosis, miR-185-5p trended to be up-regulated in both sterile groups, suggesting its potential role within endometriosis-related sterility, although additional validations are required. Interestingly, we found VEGF-A levels significantly higher in both sterile groups (EPF and CPF) compared with their corresponding fertile groups. Moreover, EPF from fertile women presented significantly higher levels than CPF from fertile control women. These findings suggest new role of peritoneal VEGF-A not only in endometriosis pathogenesis but also in pregnancy outcome.

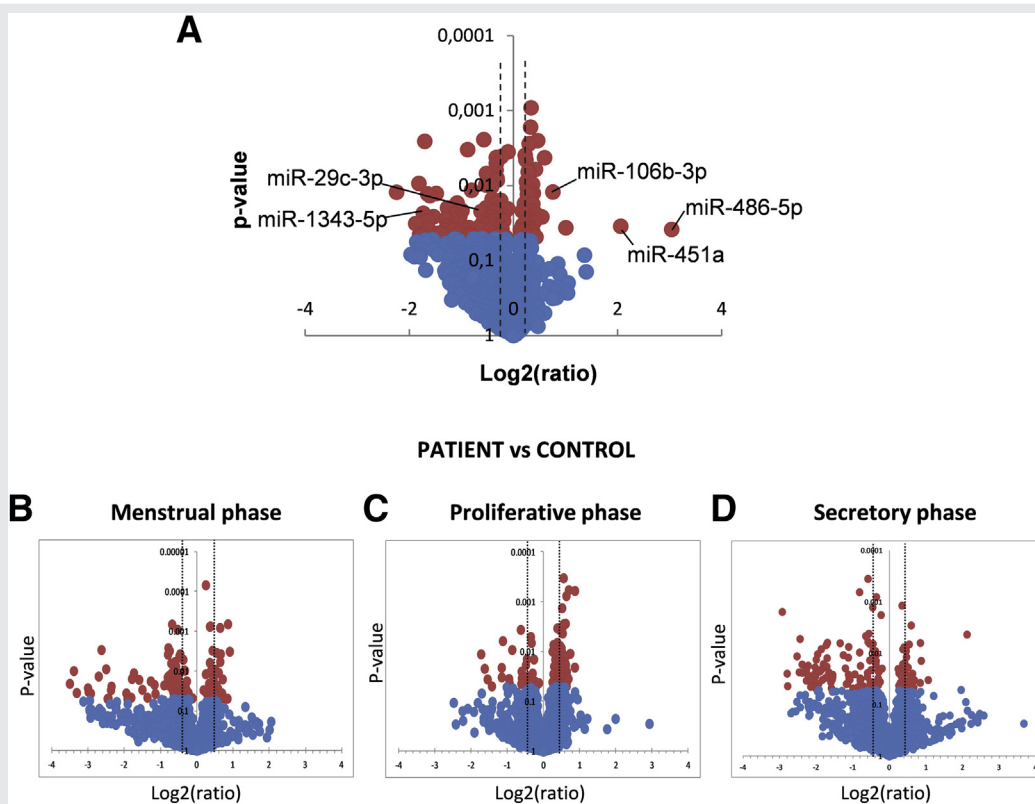
In conclusion, we have described, for the first time, a characteristic miRNA profile in PF from patients with endometriosis. Moreover, the validation phase confirmed a significant increase of miR-106b-3p, -451a, and -486-5p in comparison with CPF, independently of fertility status. Interestingly, these miRNAs presented their highest levels in the most advanced stages of the disease, as well as in the menstrual phase in comparison with other phases of the menstrual cycle. In addition, VEGF-A levels, although significantly higher in EPF from sterile women, were significantly up-regulated in both sterile groups (EPF and CPF), suggesting its role as a biomarker for fertility outcome. Summarizing, because of the role of miRNAs as intercellular communicators, these findings may be useful to better understand the peritoneal mechanisms that allow the implantation and survival of endometriotic lesions, although additional experiments are required to validate this hypothesis.

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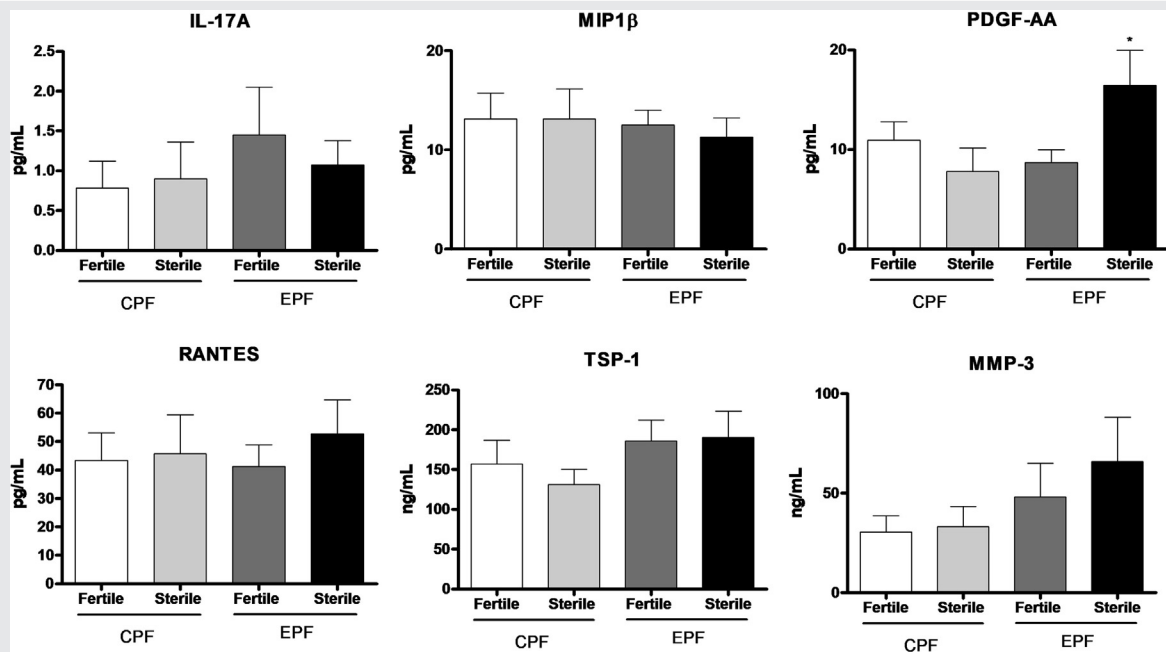
SUPPLEMENTAL FIGURE 1



Vulcano plots from miRNA arrays performed in EPF and CPF. (A) Vulcano plot representing different expression levels between controls and patients. (B–D) Vulcano plots representing miRNA differently expressed between EPF and CPF in each menstrual phase. Red dots: $P < .05$; blue dots: $P > .05$. Broken lines represent $FC \pm 1.2$.

Mari-Alexandre. Peritoneal fluid miRNAs in endometriosis. *Fertil Steril* 2017.

SUPPLEMENTAL FIGURE 2



Protein levels in EPF and CPF, according to fertility status. Data are expressed as mean \pm SEM. * $P < .05$; ** $P < .01$ between groups.

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