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Total circulating microparticle levels are increased in patients with deep infiltrating endometriosis

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STUDY QUESTION: Are the levels of total circulating cell-derived microparticles (cMPs) and circulating tissue factor-containing microparticles (cMP-TF) increased in patients with endometriosis?

SUMMARY ANSWER: The levels of total cMP, but not cMP-TF, were higher in patients with endometriosis, and these were attributed to higher levels in patients with deep infiltrating endometriosis (DIE).

WHAT IS KNOWN ALREADY: Previous studies have reported elevated levels of total cMP in inflammatory conditions as well as higher levels of other inflammatory biomarkers in endometriosis. Increased expression of tissue factor (a transmembrane receptor for Factor VII/VIIa) in eutopic and ectopic endometrium from patients with endometriosis has been described. There is no previous data regarding total cMP and cMP-TF levels in patients with endometriosis.

STUDY DESIGN, SIZE, DURATION: A prospective case–control study including two groups of patients was carried out. The E group included 65 patients with surgically confirmed endometriosis (37 with DIE lesions) and the C group comprises 33 women without surgical findings of any form of endometriosis. Patients and controls were recruited during the same 10-month period. Controls were the next patient without endometriosis undergoing surgery, after including two patients with endometriosis.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Venous blood samples for total cMP and cMP-TF determinations were obtained at the time of surgery, before anesthesia at a tertiary care center. To assess total cMP, an ELISA functional assay was used and cMP-TF activity in plasma was measured using an ELISA kit.

MAIN RESULTS AND THE ROLE OF CHANCE: Total cMP levels in plasma were higher in the E group compared with the C group ($P < 0.0001$). The subanalysis of endometriosis patients with DIE or with ovarian endometriomas without DIE showed that total cMP levels were higher in the DIE group ($P = 0.001$). There were no statistically significant differences in cMP-TF levels among the groups analyzed.

LIMITATIONS, REASONS FOR CAUTION: This is a preliminary study in which the sample size was arbitrarily decided, albeit in keeping with previous studies analyzing cMP in other inflammatory diseases and other biomarkers in endometriosis. The control group included patients with other pathologies as well as healthy controls, and blood samples were taken at different phases of the cycle.

WIDER IMPLICATIONS OF THE FINDINGS: Elevated total cMP levels in DIE patients may reflect an inflammatory and/or procoagulant systemic status in these patients. Further studies are warranted to confirm our findings and to assess the role of cMP levels in the pathophysiology of DIE.

STUDY FUNDING/COMPETING INTEREST(S): This study was supported in part by a grant from FIS-PI11/01560 and FIS-PI11/00977 within the 'Plan Nacional de I + D + I' and co-funded by the 'ISCIII-Subdirección General de Evaluación' and 'Fondo Europeo de Desarrollo Regional (FEDER)' and by the grant 'Premi Fi de Residència Emili Letang 2015' from the Hospital Clínic of Barcelona. The authors have no competing interests to disclose.

Key words: endometriosis / deep infiltrating endometriosis / ovarian endometriomas / circulating cell-derived microparticles / total microparticles / tissue factor-containing microparticles / tissue factor / inflammation / coagulation

Introduction

In the last few years, there has been an increasing interest in the study of the so-called circulating 'microparticles' or 'microvesicles' (cMP). They are defined as 100–1000 nm cell-secreted phospholipid bilayer-bound structures shed from almost all cell membranes following cell activation or apoptosis (Piccin *et al.*, 2007; Buzas *et al.*, 2014). cMPs were initially considered to be inert cellular dust, but currently it is known that they display a wide range of activities in blood coagulation, inflammation, angiogenesis and cellular signaling (Buzas *et al.*, 2014). Furthermore, an intimate interaction between inflammation and coagulation has been reported in which cMP, platelets and the contact system (also named the plasma kallikrein–kinin system, composed of coagulation factors XI and XII, plasma prekallikrein and the nonenzymatic cofactor high-molecular-weight kininogen) may be involved (Wu, 2015). Higher levels of cMP have been described in systemic inflammatory diseases, thrombotic diseases and cancer (Barteneva *et al.*, 2013). A subset of cMP, which contain tissue factor (TF), called 'tissue factor-bearing cMP' (cMP-TF) has recently been described. In healthy individuals, low plasma levels of cMP-TF have been detected, but under pathological conditions, such as malignancy, sepsis and hypercoagulable states, it is thought that cMP-TF levels are increased (van Es *et al.*, 2015).

Endometriosis is now considered a chronic systemic inflammatory condition (Bulun, 2009; Vercellini *et al.*, 2014). Although its etiopathogenesis is still under debate, many molecular and cellular alterations may be involved (Burney and Giudice, 2012). Three clinically different phenotypes have been described: endometriotic implants on the surface of the pelvic peritoneum, ovarian endometrioma (OE) and deep infiltrating endometriosis (DIE), the latter being the most severe form of the disease (Bulun, 2009; Vercellini *et al.*, 2014). Although the etiopathogenesis of different forms of endometriosis remains unclear (Bulun, 2009), the aggressiveness of DIE could be explained by an increased proliferation activity and more active invasive mechanisms, as well as an increased vasculogenesis, inflammation and neuroangiogenesis (Tosti *et al.*, 2015).

It has recently been postulated that women with endometriosis may be in an inflammatory and hypercoagulable state (Wu *et al.*, 2015). Previous studies analyzing endometriosis patients showed an increased expression of TF in eutopic and ectopic endometrium (Kikrun *et al.*, 2009; Lin *et al.*, 2012; Ding *et al.*, 2015). It has been hypothesized that cyclic bleeding in endometriotic lesions may activate and aggregate platelets in these sites, and may induce the production of cMP (Guo *et al.*, 2015).

This notwithstanding, to our knowledge, no previous studies have evaluated total cMP and cMP-TF levels in endometriosis patients. Therefore, this preliminary study was undertaken to investigate total functional cMP and cMP-TF levels in patients with endometriosis.

Materials and Methods

Study design and subjects

This is a prospective case–control study designed to evaluate the levels of total functional cMP and cMP-TF in plasma in surgically confirmed endometriosis patients (E group). Plasma level of soluble tissue factor (sTF) was also analyzed. A control group included patients without surgical findings of any form of endometriosis (C group). For the specific purposes of this study, 102 patients were prospectively asked to participate. Patients and controls were recruited during the same 10-month period. Controls comprise the next patient without endometriosis undergoing surgery after including two patients with endometriosis. The study was approved by the Ethics Committee of our hospital. All except two women provided written informed consent. The E group consisted of patients with clinical suspicion of endometriosis (DIE and/or OE) that underwent surgery due to painful symptoms. In all these patients, a preoperative work-up was performed including clinical examination and transvaginal ultrasound, and among those patients with suspicion of DIE magnetic resonance imaging was also performed. After surgery, two groups of endometriosis patients, with or without DIE, were identified for a subanalysis: the DIE group consisted of women with DIE and the OE group was composed of women who underwent surgery for suspicion of OE $\geq$ 3 cm without DIE lesions, which was confirmed during the surgical procedure. Endometriosis findings were always confirmed by histological study. The C group included patients who underwent laparoscopy due to mild benign adnexal pathology (cystectomy or adnexectomy) or a request for tubal sterilization with no presurgical suspicion of endometriosis and without endometriosis or signs of inflammatory pelvic condition during surgery.

The inclusion criteria were: women aged between 18 and 40 years with a BMI < 30.00 kg/m^2. The exclusion criteria were: history of past or present malignancy, endocrine, cardiovascular and systemic diseases, pregnancy or breastfeeding $\leq$ 6 months before sample collection, premature ovarian failure or menopausal status, endometrial hyperplasia or polyps, uterine leiomyomata, use of hormonal contraception or other hormonal treatments and intake of any medication including anti-inflammatory drugs $\leq$ 3 months before sample collection (except for oral acetaminophen as painkiller $\leq$ 72 hours before sample collection) or having had an inflammatory disease or infectious conditions $\leq$ 6 months before sample collection.

Before surgery, clinical and epidemiological data were collected from all the individuals participating in the study. Patients were asked about dysmenorrhea, dyspareunia, dyschezia, dysuria and chronic pelvic pain quantification according to the visual analog scale (Bourdel *et al.*, 2015). The severity of these symptoms was defined when patients referred a score $\geq$7 (Chapron *et al.*, 2012). Reporting of hematuria or rectal bleeding was also registered.

Operative laparoscopy was performed in all patients by insertion of a 12-mm umbilical trocar and two or three 5-mm trocars in the lower abdomen. An inspection of the pelvic organs and peritoneum was performed followed by the surgical procedure indicated in each case. All tissue excised was sent for pathology examination to confirm or exclude endometriosis. Patients were definitively assigned to one of the two groups of

patients (C or E) after undergoing laparoscopy and histological study. All patients were operated on by one of two experienced gynecological laparoscopic surgeons using the same technique and instruments. The surgical team and the operating room staff have lengthy experience with advanced gynecological laparoscopy and instrumentation. An expert gynecological pathologist performed all the histopathological analysis. Figure 1 shows the flow chart of inclusion and drop-out of the patients included in the study. Two women who did not give informed consent were excluded (Fig. 1).

Among 65 patients in the E group, there were 37 patients with DIE (DIE group) and 28 with OE without DIE (OE group). In the DIE group, 19 patients had concomitant OE: 5 cases of right OE, 10 cases of left OE and 4 cases of bilateral OE. The following DIE forms were recorded: vesical (n = 11), ureteral (n = 11), retrouterine (n = 21), retrocervical (n = 18), uterosacral ligaments (n = 25), sigmoid (n = 15), rectovaginal septum (n = 8), other intestinal location (n = 4) and vaginal (n = 3). All DIE implants were excised during surgery. Patients in the OE group underwent cystectomy due to right (n = 9), left (n = 12) and bilateral (n = 7) OE. Superficial peritoneal endometriotic (PE) lesions were found in 71% of patients in the DIE group and 46% in the OE group. All patients in the DIE group were classified as Stage IV of the revised American Fertility Society (rAFS) classification with a median rAFS score (25th; 75th percentiles) of 81 (58–107). The median rAFS score (25th; 75th percentiles) for patients of the OE group was 26 (22–36), all being classified as Stage III.

The C group was composed of 33 patients undergoing surgery for benign adnexal pathology (n = 23) or request for tubal sterilization (n = 10). Patients undergoing surgery for benign adnexal pathology included unilateral ovarian cystectomy due to dermoid cyst (n = 8), paraovarian cyst (n = 1), serous cystadenoma (n = 6), mucinous cystadenoma (n = 3); bilateral cystectomy due to bilateral serous cystadenomas (n = 1) and right dermoid cyst and left serous cystadenoma (n = 1); unilateral adnexectomy due to demoid cyst (n = 1) and ovarian fibroma (n = 1); and bilateral salpingectomy due to bilateral hydrosalpinx (n = 1).

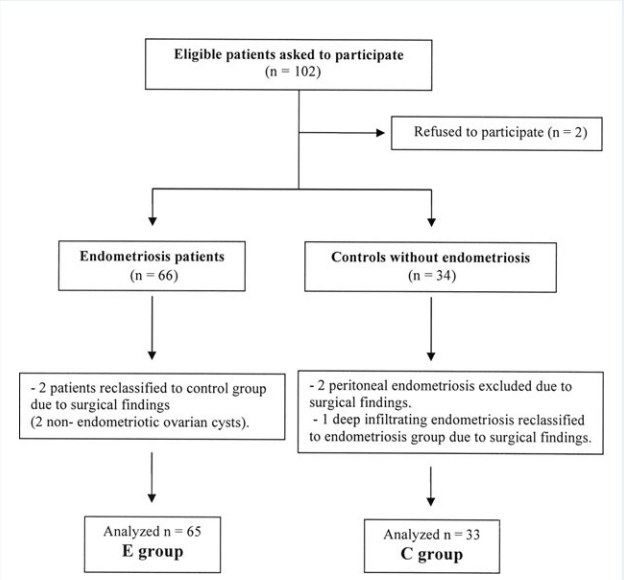

Figure 1 Flow chart of the inclusion and drop-out of patients in a study of circulating microparticles in endometriosis. E group = patients with surgically confirmed endometriosis; C group = women with benign adnexal pathology or request for tubal sterilization without surgical findings of any form of endometriosis.

Sample collection

Venous blood samples were all collected before preanesthesic medication intake and anesthesia induction by antecubital venous punction in tubes containing 3.8% trisodium citrate (1/9, vol/vol; Becton Dickinson, Rutherford, NJ, USA), and platelet-free plasma was immediately obtained by double centrifugation, first at 2000g for 10 min at 22°C and then at 5000g for 10 min at 4°C. Plasma was aliquoted, snap-frozen in a mixture of dry ice/ethanol (1/2, vol/vol) and stored at −80°C.

In the E group, 57% of patients were sampled in the follicular phase and 43% in the luteal phase, while corresponding figures in the C group were 61 and 39%, respectively.

Sample analysis

Assays were performed by a single experienced operator. Samples were tested in duplicate. The mean absorbance values for each set of duplicate samples were calculated. When discordant results were obtained (sample values >20% of the mean), the samples were re-tested. There were no missing data or outliers. The operator was blind to the group allocation.

Total cMP analysis

To assess total cMP, we used a readily available commercial functional assay (Zymuphen MP-Activity, Hyphen BioMed, Neuville, France: Catalog number: 521096) as previously reported (Martínez-Zamora *et al.*, 2016a,b). It is a prothrombinase assay based on the property of annexin-V, immobilized onto plastic wells, to bind phosphatidylserine (PS). Calibrators with known amounts of PS are used to obtain a standard curve, and the results are expressed as nanomolar PS equivalents. The calibration is validated when the quality controls provided by the kit are measured within their acceptance range, indicated for each lot on the flyer provided in the kit. The detection threshold of the assay is 0.05 nM. For cMP, the intra-assay coefficient of variation (CV) was 5% and the inter-assay CV was 8%. In this study, we used assay batch number 130207C.

Analysis of cMP-TF

cMP-TF activity in plasma was measured using an ELISA commercial kit according to the manufacturer's instructions (Zymuphen MP-TF, Hyphen Biomed, Neuville, France). In a first step, the samples are introduced into the wells of the microplate coated with a murine monoclonal antibody specific for human TF extracellular domain, which does not interfere with TF activity. cMP-TF present in the sample bind to the solid phase through an epitope localized in the extracellular domain of TF. Following overnight incubation and a washing step, wash solution is immediately introduced into the wells. Then, Factor VIIa and Factor X are added. The TF–FVIIa complex forms and subsequently activates Factor X into activated Factor X (FXa) on the surface of the anionic phospholipids present in the cMP and in the presence of Ca^{2+}. After that, a specific substrate for FXa is added and reacts with FXa producing a yellow color. The absorbance is recorded at 405 nm on a spectrophotometer and is directly proportional to the amount of cMP-TF present in the sample. A calibration curve is constructed. The calibration is validated when the quality controls are measured within their acceptance range, indicated for each lot on the flyer provided in the kit. The results were expressed as pg/ml. The detection threshold is ≤1 pg/ml. For cMP-TF, the intra-assay CV was 7% and the inter-assay CV was 10% (catalog number: 521196; batch number: 110315A).

Analysis of sTF in plasma

sTF was determined by quantitative sandwich ELISA (Quantikine Human Coagulation Factor III/Tissue Factor Immunoassay, R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. The

results were expressed as pg/ml. Intra- and inter-assay CVs were 3 and 6%, respectively.

Sample size and statistical analysis

As this is a preliminary study to investigate the levels of total cMP and cMP-TF in patients with endometriosis, the sample size was decided arbitrarily, albeit in keeping with previous studies analyzing cMP in other inflammatory diseases (Giuducci *et al.*, 2008; Leonetti *et al.*, 2013) and biomarkers in endometriosis (Fassbender *et al.*, 2013).

Statistical analysis was performed with the Statistical Package for the Social Sciences software, Release 20.0 for Windows (SPSS, Chicago, IL, USA). Continuous variables were compared using the nonparametric Mann–Whitney *U* test or Kruskal–Wallis test using the post hoc Dunn's multiple comparison test, when appropriate, and presented as median with interquartile range (25th; 75th percentiles). Categorical variables were compared using the Chi-square test and presented as total count and relative percentages (%). Statistical significance was defined as a P-value < 0.05.

Results

Clinical characteristics of the subjects

The baseline clinical characteristics of the two groups of patients included in the study are presented in Table I. The median age, BMI and tobacco use were similar in the two groups analyzed. As expected, there were more patients in the E group with severe dysmenorrhea and dyschezia compared with the C group.

Total cMP levels

Total cMP levels were significantly higher in the E group compared with the C group ([median nM PS eq (25th; 75th percentiles)]: E group: 28.50 (17.4; 41.20); C group: 15.15 (8.85; 26.45); $P < 0.0001$) (Fig. 2). The subanalysis of endometriosis patients with or without DIE showed that total cMP levels were statistically higher in the DIE group compared with the C group ($P = 0.001$), and no differences were found between the OE group and the C group ([median nM PS eq (25th; 75th percentiles)]: DIE group: 33.10 (20.28; 40.30); OE group: 24.80 (12.45; 41.80) and C group: 15.15 (8.85; 26.45)) (Fig. 3). The presence or absence of PE in the DIE and OE group did not alter these results (data not shown).

The subanalysis between the women with and without pelvic pain, regardless of endometriotic lesions did not show significant differences ([median nM PS eq (25th; 75th percentiles)]: no pelvic pain: 23.25 (13.55; 37.93); pelvic pain: 20.80 (10.64; 37.35)).

Among all patients with OE ($n = 47$), 27 patients (57.4%) had associated PE. Total cMP levels were statistically higher in patients with OE compared with endometriosis-free patients with ovarian cysts ($n = 22$) ([median nM PS eq (25th; 75th percentiles)]: patients with OE: 27.6 (16.50; 38.90); endometriosis-free patients with ovarian cysts: 13.7 (10.70; 29.2); $P = 0.03$), irrespective of whether OE was associated with PE or not (data not shown). The comparison of total cMP levels between OE patients and healthy women (patients who underwent tubal ligation) showed similar results (data not shown).

The comparison of follicular versus luteal phase did not show statistical differences in cMP levels ([median nM PS eq (25th; 75th percentiles)]: cMP follicular phase: 27.60 (11.33; 34.65); cMP luteal phase: 24.10 (16.60; 29.30)). The subanalysis of cMP levels among the groups analyzed showed no differences between the follicular and luteal phase (data not shown).

cMP-TF and sTF levels

There were no statistically significant differences in cMP-TF levels between the two groups analyzed [median pg/ml (25th; 75th percentiles)]: E group: 0.91 (0.73; 1.23); C group: 0.92 (0.69; 1.21) (Fig. 2). The subanalysis of endometriosis patients with or without DIE showed no statistically significant differences [median pg/ml (25th; 75th percentiles)]: DIE group: 0.91 (0.74; 1.26); OE group: 0.90 (0.69; 1.16); C group: 0.92 (0.69; 1.21) (Fig. 3).

No differences in the cMP-TF levels were found between patients with and without pelvic pain, regardless of endometriotic lesions ([median pg/ml (25th; 75th percentiles)]: no pelvic pain: 0.91 (0.71; 1.22); pelvic pain: 1.00 (0.74; 1.25)).

The comparison of the cMP-TF levels between patients with OE ($n = 47$) compared with endometriosis-free patients with ovarian

Table I Clinical characteristics of the patients included in a study of circulating microparticles in endometriosis.

	E group ($n = 65$)	C group ($n = 33$)	*P* value
Age (years)	35 (31; 36)	32 (27; 38)	NS
BMI (kg/m^2)	21.68 (19.99; 24.30)	22.72 (20.70; 25.39)	NS
Tobacco use	25 (38.5%)	12 (36.4%)	NS
Dysmenorrhea VAS ≥ 7	44 (67.7%)	7 (21.2%)	<0.0001
Dyspareunia VAS ≥ 7	15 (23.1%)	3 (9.1%)	NS
Dyschezia VAS ≥ 7	14 (21.5%)	1 (3%)	0.017
Dysuria VAS ≥ 7	6 (9.2%)	1 (3%)	NS
Chronic pelvic pain VAS ≥ 7	14 (21.5%)	2 (6.1%)	NS
Hematuria	5 (7.7%)	0	NS
Rectal bleeding	5 (7.7%)	0	NS

The values are median (25th; 75th percentiles) or *n* (%). NS, not significant; VAS, visual analog scale. E group = women with surgically confirmed DIE and/or OEs; C group = women with benign adnexal pathology or request for tubal sterilization without surgical findings of any form of endometriosis. Continuous variables were compared using the nonparametric Mann–Whitney *U* test. Categorical variables were compared using the Chi-square test.

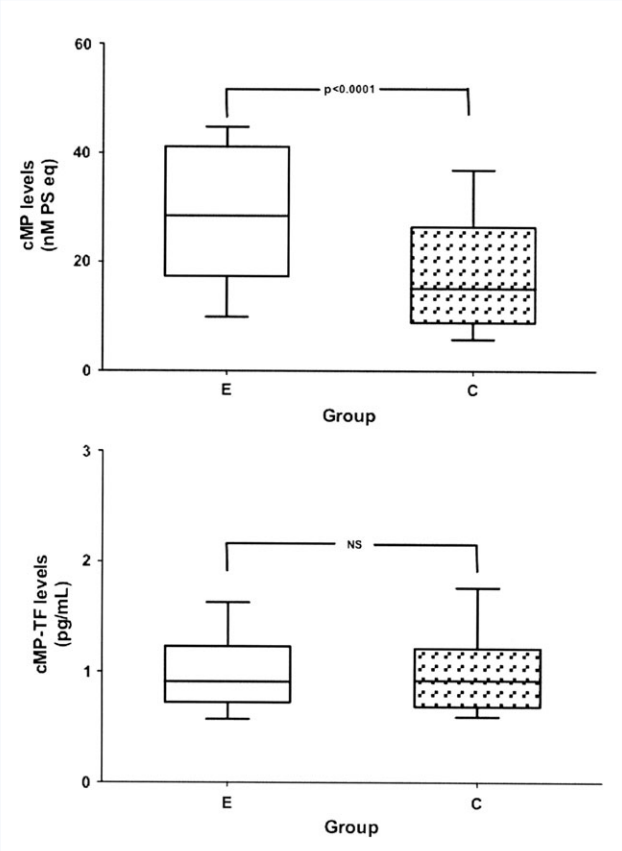

Figure 2 Box plot showing total circulating cMP levels and circulating cMP-TF levels in patients with surgically confirmed endometriosis (E group, $n = 65$) and patients with benign adnexal pathology or request for tubal sterilization without surgical findings of any form of endometriosis (C group, $n = 33$). Each box represents the middle 50% of the data (25th–75th percentiles range). The central horizontal line represents the median. Vertical lines represent the 10th–90th percentiles range of the data, as indicated by the small horizontal lines. Data from the E and C groups were compared using the nonparametric Mann–Whitney *U* test. Statistical comparisons between the groups are indicated. cMP, cell-derived microparticle; cMP-TF, tissue factor-containing microparticle; NS, not significant; nM PS eq, nM phosphatidylserine equivalent.

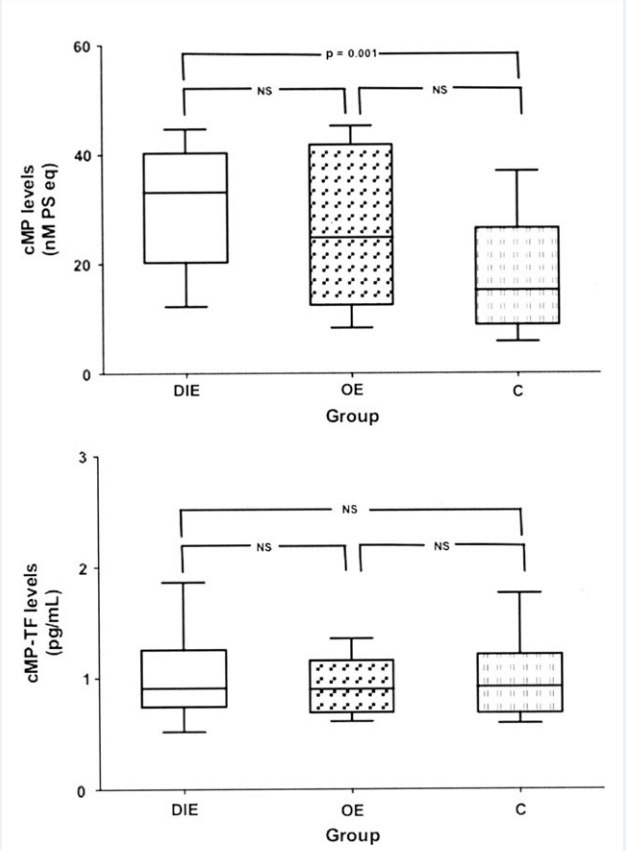

Figure 3 Box plot showing total cMP levels and cMP-TF levels in patients with DIE (DIE group, $n = 37$), patients with OEs but without DIE (OE group, $n = 28$) and patients with benign adnexal pathology or request for tubal sterilization and without endometriosis (C group, $n = 33$). Each box represents the middle 50% of the data (25th–75th percentiles range). The central horizontal line represents the median. Vertical lines represent the 10th–90th percentiles range of the data, as indicated by the small horizontal lines. Data from the three groups were compared using the nonparametric Kruskal–Wallis test and the post hoc Dunn's multiple comparison test. Statistical comparisons between groups are indicated. DIE, deep infiltrating endometriosis; OE, ovarian endometrioma.

cysts ($n = 22$) showed no significant differences ([median pg/ml (25th; 75th percentiles)]: patients with OE: 0.90 (0.72; 1.17); endometriosis-free patients with ovarian cysts: 0.87 (0.63; 1.13)).

The cMP-TF levels were statistically similar in the follicular and luteal phase ([median pg/ml (25th; 75th percentiles)]: cMP-TF follicular phase: 0.85 (0.74; 1.07); cMP-TF luteal phase: 0.92 (0.76; 1.01)) and comparison among groups according the follicular or luteal phase showed no differences (data not shown).

There were no statistically significant differences in sTF levels between the two groups analyzed ([median pg/ml (25th; 75th percentiles)]: E group: 26.20 (21.60; 30.80); C group: 27.80 (23.90; 33.00)). The subanalysis of the DIE and OE groups showed no statistically significant differences ([median pg/ml (25th; 75th percentiles)]: OE group: 24.25 (19.50; 30.80); DIE group: 27.80 (22.60; 31.49); C group: 27.80 (23.90; 33.00)).

Discussion

The current investigation is a preliminary report to study functional cMP and cMP-TF levels in patients with endometriosis. Our study has shown increased total cMP but not cMP-TF levels in endometriosis patients. This notwithstanding, it should be stressed that these increased total cMP levels in endometriosis patients seem to be attributed to higher levels in DIE patients but not in patients with OE without DIE.

In the last two decades, the interest in cell-derived cMP has risen. Indeed, cMPs have been described to be involved in the processes of coagulation, inflammation, angiogenesis, cell remodeling and proliferation (Barteneva *et al.*, 2013) and have been implicated in several diseases associated with hypercoagulability, such as atherosclerosis, venous thromboembolism, sepsis and cancer (Piccin *et al.*, 2007). Nonetheless, elevated levels of cMP have also been reported in

inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, systemic sclerosis, multiple sclerosis and Crohn's disease (Barteneva *et al.*, 2013; Buzas *et al.*, 2014).

In the last few years, there has been increasing evidence about the intimate interplay between inflammation and coagulation, which seem to interact by many mechanisms including cMP (Wu, 2015). It has been reported that platelets are activated and aggregated in endometriotic lesions contributing to angiogenesis, inflammation and evolution to fibrosis (Ding *et al.*, 2015). Moreover, it has recently been suggested that endometriosis patients may be in a hypercoagulable state (Wu *et al.*, 2015; Guo *et al.*, 2015). Furthermore, it has been hypothesized that cyclic bleeding that takes place in endometriotic lesions may activate and aggregate platelets in these sites, inducing the production of cMP (Guo *et al.*, 2015), which would contribute to the development of endometriosis by several mechanisms. The results of our preliminary study provide data showing higher levels of total cMP in endometriosis patients with DIE compared with endometriosis patients with OE without DIE and patients without endometriosis, reflecting the chronic inflammatory and/or procoagulant systemic status in these patients. The higher cMP levels found in the DIE patients in the subanalysis suggest that there may be a more intense grade of inflammation and angiogenesis in this more aggressive form of endometriosis. It remains unknown whether these cMP levels are causative, a consequence or a coincidence in DIE patients, as our research is preliminary. Nevertheless, our results suggest that cMP levels may have a role in the pathophysiological mechanisms of DIE, although the relationship between the severity and extension of DIE lesions and cMP levels cannot be ascertained from our preliminary study, and these issues remain to be established in larger further studies.

cMP-TFs are described to be involved in the coagulation activation process and higher plasma levels have been detected in cases of hypercoagulable states (van Es *et al.*, 2015). Apart from their role in hemostasis, cMP-TFs have been described to be involved in inflammation, tumor progression and angiogenesis (Lin *et al.*, 2012). It has been reported that women with endometriosis have an increased expression of TF in eutopic and ectopic endometrium-derived cells (Kikrun *et al.*, 2009; Lin *et al.*, 2012; Ding *et al.*, 2015). However, according to our results, this fact does not correlate with plasma cMP-TF levels, since we found no differences in cMP-TF levels among the groups analyzed. Although no increase in cMP-TF levels was found in our series, these results do not rule out the hypothesis of a possible role of TF in endometriosis at a tissue/local level (Kikrun *et al.*, 2009; Lin *et al.*, 2012; Ding *et al.*, 2015). In our study, we determined plasma cMP-TF levels in peripheral blood, as sTF plasma levels may not necessarily reflect the amount or function of TF located at the site of endometriosis lesions. Therefore, total cMP but not cMP-TF levels might be implied in the pathophysiology of DIE. Another possible explanation, as previously described by other authors (Key and Mackman, 2010; Hellum *et al.*, 2012), is that the measurement of cMP-TF functional activity may be technically difficult in plasma with low levels of cMP-TF. Nevertheless, this hypothesis is less probable since we additionally determined sTF levels by ELISA obtaining concordant results with those of cMP-TF, with very low sTF levels in patients and no differences among groups.

Our study has several strengths. First is that all blood samples were obtained just before surgery in order to assess the basal plasma total cMP and cMP-TF levels of these patients. Second, all patients underwent surgery and were definitively classified into the study or control groups according to the surgical findings and histology and not only according to the presurgical work-up.

On the other hand, the present study also has some limitations. First, the sample size was small and arbitrarily decided according to previous studies analyzing cMP in other inflammatory diseases (Giuducci *et al.*, 2008; Leonetti *et al.*, 2013) and biomarkers in endometriosis (Fassbender *et al.*, 2013). Therefore, our research should be considered a preliminary study since there are no previous studies exploring the involvement of cMP in endometriosis patients. Second, we did not include a group of patients with isolated peritoneal lesions and therefore, since it has been reported that early endometriosis with red peritoneal lesions may induce a higher inflammatory response in the pelvic cavity than advanced endometriosis (Khan *et al.*, 2004), this research cannot evaluate if DIE is associated with a higher degree of inflammation than peritoneal endometriosis. Another drawback of the current investigation is that the C group was composed of controls without endometriosis who underwent surgery for benign adnexal pathology or tubal sterilization to verify the absence of endometriosis lesions and therefore not all of them were healthy controls. The fact that we included patients in the C group without presurgical suspicion of endometriosis and not patients with endometriosis-like symptoms after laparoscopic and histological examinations showing no endometriosis may lead to an overestimation of diagnostic characteristics, and, therefore, our results may have more pathophysiological interest than diagnostic implications. Moreover, it should be noted that blood samples were obtained at different phases of the menstrual cycle, which could constitute a limitation owing to the potential for cMP concentrations to change over the menstrual cycle and the subanalysis of our data could be biased because of the small sample size. Finally, another limitation of our study is that, apart from the subset of cMP-TF, no other subtypes of cell-derived cMP were investigated, and we did not use other methodological approaches to determine cMP levels.

In conclusion, our preliminary study shows that DIE patients have increased functional cMP levels. These high total cMP levels in DIE patients were not found in either OE patients without DIE or in controls without endometriosis. The presence of elevated functional cMP levels in women with DIE may reflect an inflammatory and/or procoagulant systemic status in these patients. Nonetheless, further studies are warranted to confirm our findings and to assess the role of total cMP in the pathophysiological mechanisms of this disease. Further research should focus on analyzing cMP levels and other cMP subtypes in different types of endometriosis including peritoneal endometriosis and in different sites and extension of DIE lesions.

Authors' roles

J.M. has contributed to the study design, recruitment of patients, sample extraction and analysis, results analysis and wrote the manuscript. M.A.M.-Z. contributed to the study design, recruitment of patients, results analysis, manuscript preparation and critical discussion. D.T. contributed to the study design, sample analysis, results analysis, manuscript preparation and critical discussion. J.L.C. contributed to the recruitment of patients, sample extraction and results analysis. M.A.T., J.C.R., F.C. and J.B. contributed to the study design, results analysis, manuscript preparation and critical discussion.

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Conflict of interest

None declared.

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