Protein oxidative stress markers in peritoneal fluids of women with deep infiltrating endometriosis are increased

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STUDY QUESTION: Are protein oxidative stress markers [thiols, advanced oxidation protein products (AOPP), protein carbonyls and nitrates/nitrites] in perioperative peritoneal fluid higher in women with histologically proven endometriosis when compared with endometriosis-free controls?

SUMMARY ANSWER: Protein oxidative stress markers are significantly increased in peritoneal fluids from women with deep infiltrating endometriosis with intestinal involvement when compared with endometriosis-free controls.

WHAT IS KNOWN ALREADY: Endometriosis is a common gynaecologic condition characterized by an important inflammatory process. Various sources of evidence support the role of oxidative stress in the development of endometriosis.

STUDY DESIGN, SIZE, DURATION: We conducted a prospective laboratory study in a tertiary-care university hospital between January 2011 and December 2012, and included 235 non-pregnant women, younger than 42 year old, undergoing surgery for a benign gynaecological condition.

PARTICIPANTS/MATERIALS, SETTING, METHODS: After complete surgical exploration of the abdomino-pelvic cavity, 150 women with histologically proven endometriosis and 85 endometriosis-free controls women were enrolled. Women with endometriosis were staged according to a surgical classification in three different phenotypes of endometriosis: superficial peritoneal endometriosis (SUP), ovarian endometrioma (OMA) and deeply infiltrating endometriosis (DIE). Perioperative peritoneal fluids samples were obtained from all study participants. Thiols, AOPP, protein carbonyls and nitrates/nitrites were assayed in all peritoneal samples.

MAIN RESULTS AND THE ROLE OF CHANCE: Concentrations of peritoneal AOPP were significantly higher in endometriosis patients than in the control group (median, 128.9 µmol/l; range, 0.3–1180.1 versus median, 77.8 µmol/l; range, 0.8–616.1; P < 0.001). In a similar manner concentrations of peritoneal nitrates/nitrites were higher in endometriosis patients than in the control group (median, 24.8 µmol/l; range, 1.6–681.6 versus median, 18.5 µmol/l; range, 1.6–184.5; P < 0.05). According to the surgical classification, peritoneal fluids protein AOPP and nitrates/nitrites were significantly increased only in DIE samples when compared with controls (P < 0.001 and P < 0.05; respectively), whereas the others forms of endometriosis (SUP and OMA) showed non-statistically significant increases. We found positive correlations between peritoneal fluids AOPP concentrations, nitrates/nitrites levels and the total number of intestinal DIE lesions (r = 0.464; P < 0.001 and r = 0.366; P = 0.007; respectively).

† Didier Borderie and Charles Chapron contributed equally to the direction of this work.

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LIMITATIONS, REASONS FOR CAUTION: Inclusion of only surgical patients may constitute a possible selection bias. In fact, our control group involved women who underwent surgery for benign gynaecological conditions. This specificity of our control group may lead to biases stemming from the fact that some of these conditions, such as fibroids, ovarian cysts or tubal infertility, might be associated with altered peritoneal proteins oxidative stress markers.

WIDER IMPLICATIONS OF THE FINDINGS: We demonstrate the existence of a significantly increased protein oxidative stress status in peritoneal fluid from women with endometriosis especially in cases of DIE with intestinal involvement. This study opens the way to future more mechanistic studies to determine the exact role of protein oxidative stress in the pathogenesis of endometriosis. Even if an association does not establish proof of cause and effect, these intrinsic biochemical characteristics of endometriosis may lead to the evaluation of therapeutic approaches targeting oxidative imbalance.

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Key words: thiols / AOPP / nitrates/nitrites / oxidative stress / endometriosis

Introduction

Endometriosis is a chronic gynaecologic disorder defined by the presence of endometrial tissue, glands and stroma, outside of the uterine cavity (Giudice and Kao, 2004). This unknown origin disease represents a public health issue affecting 10–15% of women in their reproductive age. The two primary consequences of endometriosis are pain and infertility (de Ziegler et al., 2010). The implantation and proliferation of endometrial tissue can affect all organs, including the primarily peritoneum, ovary or pelvic organs and occasionally bowel, ureter, bladder or lungs (Sampson, 1927). It is now accepted that there are three different phenotypes of endometriosis, graded from the least to the most severe: (i) superficial peritoneal endometriosis (SUP), (ii) ovarian endometrioma (OMA) and (iii) deeply infiltrating endometriosis (DIE). DIE is the most aggressive form of the disease defined by the involvement of the muscularis propria regardless of the anatomical location (Chapron et al., 2010a,b). Histologically, endometriosis is a heterogeneous lesion with growth of endometrial gland and stroma, surrounded by an important inflammatory and fibrotic process induced by the ectopic location of endometrium (Anaf et al., 2000; Itoya et al., 2003; Yuge et al., 2007). Subsequent chronic inflammation results in an amplification loop, and in a self-supporting survival of endometriotic lesions (Yuge et al., 2007). The aetiology of endometriosis is complex and actually still poorly understood; however, there is growing evidence that inflammation plays central roles in the development and progression of endometriosis (Santulli et al., 2012a,b). In fact, endometriosis is marked by an inflammatory process associated with overproduction of a wide range of inflammatory mediators such as prostaglandins, metalloproteinases, cytokines and chemokines (Bulun, 2009). In addition, reactive oxygen species (ROS) and free radicals promote the growth and adhesion of endometrial cells in the peritoneal cavity, leading to disease establishment and its symptoms, pain and infertility (Jackson et al., 2005; Carvalho et al., 2012).

Oxidative stress occurs when there is a disrupted balance between ROS production and the antioxidant defense (Agarwal et al., 2005). Inadequate antioxidant protection or excess production of ROS may alter the cellular oxidative balance creating a condition known as oxidative stress (Lemarechal et al., 2007). After its constitution, ROS may interact with other molecules to disrupt many cellular components and processes. Cellular targets of ROS are lipids, proteins, nucleotides and carbohydrates (Slater, 1984; Lemarechal et al., 2007; Tsukahara, 2007). Cell damage repair involves both enzymatic and non-enzymatic antioxidants. Excess production or impaired elimination of ROS leads to increased oxidative stress which has been associated with several diseases. Chronic inflammatory diseases, including atherosclerosis (Schisterman et al., 2001), as well as pre-eclampsia (Brewer et al., 2013; Huang et al., 2013) and male and female infertility (Agarwal et al., 2005), have been associated with increased oxidative stress (Lemarechal et al., 2007; Kavian et al., 2012). In neoplastic conditions, the balance of ROS production controls tumour cell proliferation (Laurent et al., 2005; Alexandre et al., 2006; Trachootham et al., 2009; Gorini et al., 2013), as well as the metastatic potential of tumour cells (Ishikawa et al., 2008). Previous studies report that, oestrogen as well as oestrogen metabolites act as pro-oxidants to generate ROS (Bolton, 2002). Oestrogen-induced ROS play important roles in cell proliferation, migration, invasion and cell transformation, by increasing genomic instability and by transducing signal through redox sensitive transcription factors (Okoh et al., 2011).

Various sources of evidence support the role of oxidants in the development of endometriosis, a metastatic benign pathology (Borghese et al., 2010), as endometriotic cells show higher endogenous oxidative stress with increased ROS production and alterations in ROS detoxification pathways (Ngo et al., 2009; Leconte et al., 2011).

However, despite evidence that oxidative stress may play a role in endometriosis (Agarwal et al., 2005), to date no study has explored the peritoneal fluid protein oxidative status. Investigating the mechanisms that underlie oxidative stress associated with endometriosis may be useful for determining specific pathways of oxidative stress in vivo and for establishing the consequences of oxidant stress exposure, facilitating the comprehension of this enigmatic disease.

In the present study, for the first time, we assayed protein oxidative stress markers in peritoneal fluid samples from a large series of women with endometriosis. The concentrations of these markers in the samples were compared with those from endometriosis-free women according to the histological phenotype of endometriosis lesions (SUP, OMA and DIE) and in surgically investigated controls without endometriosis. Our secondary objective was to evaluate the concentrations of peritoneal fluid protein oxidative stress markers with respect to type of endometriosis and the anatomical distribution of DIE lesions.

Materials and Methods

Patients

The local ethics committee (CCPPRB: Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale) of Paris Cochin approved the
study protocol. From January 2011 to December 2012, a series of 373 patients were recruited after providing informed written consent. A thorough surgical examination of the abdomino-pelvic cavity was performed in each study participant. Ovarian malignancy and borderline tumours were not included in this study. No women in this study had a previous history of myomectomy, autoimmune or inflammatory diseases.

Women were allocated to two groups according to the surgical findings (Chapron et al., 2011): the endometriosis group (n = 150) consisted of subjects with histologically proven endometriosis, and the control group (n = 85) of women without any macroscopic endometriotic lesion, as checked during a thorough examination of the abdomino-pelvic cavity.

During surgery, endometriosis was staged and scored (total, implants and adhesions scores) according to the revised American Fertility Society (rAFS) Classification (AFS, 1985). In addition, because different types of endometriosis (SUP, OMA and DIE) are frequently associated with infertility (Somigliana et al., 2007), according to previously described classification, patients were assigned to the group corresponding to the most severe (worst) lesion, in the following order from the least to the most severe: SUP, OMA and DIE (Chapron et al., 2010a, b). By definition, DIE patients were graded from the least to the most severe DIE lesion as follows: uterosacral ligament(s), vagina, bladder, intestine and ureter (Chapron et al., 2006). The patient’s most severe localization was considered for grading.

A previous study revealed a significant increase in the protein oxidative stress status and a reduced antioxidant capacity in sera from women with uterine leiomyoma (Santulli et al., 2013). Overall, several reports have suggested the role of oxidative stress in the development of uterine leiomyomas (Chioj and Hu, 1999; Fokonski et al., 2000; Pejcic et al., 2006; Vural et al., 2012). According to these previous studies, because uterine leiomyomas display higher endogenous oxidative stress with a significant increase in protein oxidative stress status and reduced antioxidant capacity, women with macroscopic uterine leiomyoma during abdomino-pelvic surgical exploration were excluded from the study.

Women without any evidence of a uterine leiomyoma lesion were allocated to the control group (n = 85). Among the control group women, the indications for surgery were non-endometriotic benign ovarian cysts, pelvic pain and tubal infertility.

The study analysis used a prospectively managed database (Chapron et al., 2011). For each patient, personal history data were obtained during face-to-face interviews conducted by the surgeon during the month preceding surgery. We used a highly structured previously published questionnaire (Chapron et al., 2010a, b). The following data were recorded: age, parity, gravidity, height, weight, body mass index (BMI), existence of gynaecologic pain symptoms [dysmenorrhoea, deep dyspareunia, non-cyclic chronic pelvic pain (NCCPP)] (Fauconnier et al., 2002) and gastrointestinal (GI) (Dousset et al., 2010) and lower urinary (LU) tract symptoms (Chapron et al., 2010a, b). Pain intensity was evaluated preoperatively using a 10-cm visual analogue scale (VAS) (Huskisson, 1974). Women without hormonal treatment were cycling women (in proliferative or secretory phase of the menstrual cycle) without any hormonal treatment (including progestinic, estro-progestinic or GnRH analogues) in the last 6 months before the surgery (Santulli et al., 2014). Oral contraceptive (OC) use was defined as the use of OC for at least 6 months before surgery (Chapron et al., 2011; Santulli et al., 2014). In both groups, women under hormonal treatment used identical therapies: antigonadotropic oral contraceptive.

Collection of peritoneal fluid samples

Peritoneal fluids were collected by aspiration from the cul-de-sac during laparoscopy in 235 women. Only peritoneal fluids without dilution were retrieved (Santulli et al., 2012a, b). In case of haemorrhage after the insertion of trocars, samples were not collected to avoid blood contamination. The peritoneal fluids were centrifuged at 800 g for 12 min at 4 °C, and peritoneal fluid supernatants were collected. Aliquots of those samples were stored at −70 °C until needed for analysis.

Measurement of stress oxidative parameters

Thiols

Thiols were determined with Ellman’s reagent (Hu et al., 1993). We mixed 50 µl of peritoneal fluid with 1.0 ml of 0.1 M Tris, 10 mM EDTA, pH 8.2. We determined the absorbance at 412 nm, and then added 40 µl of 10 mM Ellman’s reagent (Sigma) in methanol to the sample. The absorbance obtained before the addition of Ellman’s reagent was subtracted from that obtained after incubation with Ellman’s reagent. A control containing Ellman’s reagent only was included, and the concentration of thiols was calculated using a molar extinction coefficient of 13 600 M⁻¹ cm⁻¹ at 412 nm.

Advanced oxidation protein products

Advanced oxidation protein products (AOPP) were quantified as described previously (Witko-Sarsat et al., 1998). We placed 200 µl of peritoneal fluid diluted 1:5 in phosphate-buffered saline into each well of a 96-well microtitre plate and added 20 µl of acetic acid to each well. For the standards, we added 10 µl of 1.16 M potassium iodide (Sigma, St Louis, MO, USA) to 200 µl of chloramine-T solution (0–100 µmol/l) (Sigma) in a well and then added 20 µl of acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm against a blank consisting of 200 µl of phosphate-buffered saline, 10 µl of 1.16 M potassium iodide and 20 µl of acetic acid. AOPP concentrations are expressed as micromoles/litre of chloramine-T equivalents.

Carbonyl groups

Protein carbonyl groups were detected and quantified using 2,4-dinitrophenylhydrazine (DNPH) (Reznick and Packer, 1994). Briefly, 0.5 ml of peritoneal fluid (1 mg protein/ml) were treated with 0.5 ml 10 mM DNPH in 2 M HCl, or with 0.5 ml 2 M hydrochloric acid (HCl) alone for the blank. Samples were incubated for 1 h at room temperature in the dark, and then treated with 10% trichloroacetic acid and centrifuged. The pellet was washed three times in ethanol/ethyelacetate and solubilized in 1 ml of 6 M guanidine in 20 mM potassium phosphate, adjusted to pH 2.3 with trifluoroacetic acid; the resulting solution was incubated at 37 °C for 15 min. The carbonyl concentration was determined from the difference in absorbance at 370 nm between DNPH-treated and HCl-treated samples, with ε₃₇₀ = 22 000 M⁻¹ cm⁻¹. The carbonyl content is expressed as nanomoles of carbonyl per milligram of protein.

Nitrates plus nitrates

The nitrate concentration was determined with a spectrophotometric assay using oxidation catalysed by cadmium metal which converts nitrate to nitrite as previously described (Sasvay et al., 2002). Briefly, the total nitrite determined therefore corresponds to nitric oxide (NO) production. Protein was first removed from the medium by incubation with zinc sulphate. Cadmium metal was then added and the medium was incubated overnight with shaking in the dark. The next day, the solution was mixed and sulphanilamide and N-1 naphthylethylendiamine was added. These compounds form a coloured complex with nitrite, which can be determined by spectrophotometry (Dyndatech Instruments, Guernsey). The detection limit was 0.04 µmol/l.

Statistical analysis

All data were collected in a computerized database and analysed by SPSS software (SPSS, Inc., Chicago, IL, USA). When women with endometriosis and control women were analysed, we used Student’s t-test for quantitative
variables and Pearson’s χ² or Fisher’s exact test for qualitative variables as appropriate.

Considering the non-Gaussian distribution of stress oxidative parameters, statistical analysis between two groups was performed with the Mann–Whitney U-Test. When more than two groups were compared, we used Kruskal–Wallis test. When group medians were significantly different by Kruskal–Wallis test (P < 0.05), post hoc pairwise comparisons were performed using the Dunn’s Multiple Comparison Test.

Correlations between peritoneal fluid stress oxidative parameters levels and anatomical characteristics of disease severity, measured with semiquantitative variables, were examined using the non-parametric Spearman’s rank correlation test. P < 0.05 was considered statistically significant.

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**Results**

**Patients and controls**

There were 150 endometriosis-affected women and 85 disease-free women recruited for this study (Fig. 1).

According to the endometriosis surgical classification, based on the location of the worst lesion, the 150 histologically proven endometriotic patients were classified as follows: 55 (36.7%) SUP, 31 (20.7%) OMA (right: 8; left: 16; bilateral: 7) and 64 (42.7%) DIE. The patients’ distribution according to the worst lesion of DIE was: 22 (34.4%) uterosacral ligament(s), 6 (9.4%) vagina, 8 (12.5%) bladder, 26 (40.6%) intestine and 2...
(3.1%) ureter. DIE patients (n = 64) presented a total of 142 histologically proved DIE lesions distributed as follows: 65 uterine serosal lesions (45.8%), 19 vaginal lesions (13.4%), 11 bladder lesions (7.7%), 45 intestine lesions (31.7%) (1 intestinal lesion in 19 patients and more than 1 intestinal lesion in 9 patients) and 2 ureteral lesions (1.4%) (unilateral lesions in 2 patients). Among DIE patients, the mean (± SD) number of nodules per patient was 2.2 ± 1.3 (range 1–6); 21 women (32.8%) presented a unifocal DIE lesion and 43 women (67.2%) presented multifocal lesions. The patient’s distribution according to rAFS stage was as follows: 50 (33.3%) Stage I; 27 Stage II (18.0%); 36 (24.0%) Stage III and 37 (24.7%) Stage IV. The mean total, implants and adhesions rAFS scores were, respectively, 22.5 ± 25.1, 11.9 ± 12.1 and 10.5 ± 17.3.

Among the 85 endometriosis-free women, the indications for surgery are resumed as follows: non-endometriotic benign ovarian cysts (30–35.3%), pelvic pain (10–11.8%), tubal infertility (37–43.5%) and others (8–9.4%). There were no differences in age, infertility and hormonal status between the study and control groups (Table I).

### Protein oxidative stress markers

AOPP, nitrates/nitrites, thiols and protein carbonyls were measured in peritoneal fluid of all the 235 women studied. Peritoneal fluids protein concentrations were not different between women with and without endometriosis. Concentrations of peritoneal AOPP were significantly higher in endometriosis patients than in the control group (median, 128.9 μmol/l; range, 0.3–1180.1 versus median, 77.8 μmol/l; range, 0.8–616.1; P < 0.001). In a similar manner, concentrations of peritoneal nitrates/nitrites were higher in endometriosis patients than in the control group (median, 24.8 μmol/l; range, 1.6–681.6 versus median, 18.5 μmol/l; range, 1.6–184.5; P < 0.05). In contrast, concentrations of peritoneal fluids thiols and carbonyls were not different between the endometriosis and control groups (thiols: median, 118.2 μmol/l; range, 8.4–461.0 versus median, 102.3 μmol/l; range, 1.6–222.7; P = 0.055; and carbonyls: median, 1.5 nmol/mg; range, 0.0–24.6 versus median, 1.2 nmol/mg; range, 0.0–27.3; P = 0.327).

The percentages of women without and with oral contraceptive use, and for whom knowledge of hormonal treatment use was unknown, were similar in the endometriosis and controls groups (P = 0.456) (Table I). We studied the effect of hormonal treatment on peritoneal oxidative stress markers. Subgroup analysis failed to show any difference between endometriotic women with and without hormonal treatment in peritoneal fluids AOPP, thiols, nitrates/nitrites or protein carbonyls (P = 0.494; P = 0.054; P = 0.918; P = 0.538; respectively) (Supplementary data, Table SII). In the control group after subgroup analysis, there was also no effect of hormonal treatment on peritoneal fluids Aopp, thiols, nitrates/nitrites or protein carbonyls (P = 0.436; P = 0.054; P = 0.477; P = 0.328; respectively) (Supplementary data, Table SII).

Among cycling women (n = 74), no differences were found in the percentages of women in the proliferative and the secretory phases in the endometriosis group when compared with controls [endometriosis: 23/43 (53.5%) in the proliferative phase and 20/43 (46.5%) in the secretory phase versus controls: 17/31 (54.8%) in the proliferative phase and 14/31 (45.2%) in the secretory phase, respectively; P = 0.920] (Supplementary data, Table SII). We studied the effects of menstrual cycle phases on peritoneal oxidative stress marker levels in both study groups. Among cycling endometriotic women, we failed to show any effect of menstrual cycle phases on peritoneal fluids AOPP, thiols, nitrates/nitrites and protein carbonyls (P = 0.074; P = 0.148; P = 0.199; P = 0.190; respectively) (Supplementary data, Table SII). Subgroup analysis in control women also showed no difference between the proliferative and the secretory phases on peritoneal fluids AOPP, thiols and protein carbonyls (P = 0.606; P = 0.153; P = 0.055; respectively) (Supplementary data, Table SII). In control women, peritoneal fluid nitrates/nitrites levels were significantly higher during the proliferative phase of the menstrual cycle when compared with the secretory phase (P = 0.023) (Supplementary data, Table SII).

According to the surgical classifications, Fig. 2 depicts the mean concentrations of AOPP, nitrates/nitrites, thiols and protein carbonyls in the peritoneal fluid of SUP, OMA, DIE and controls patients. Peritoneal fluids protein AOPP and nitrates/nitrites were significantly different among the subgroups (P < 0.001 and P < 0.001; respectively) whereas peritoneal fluids thiols and protein carbonyls levels were not significantly different among subgroups (P = 0.051 and P < 0.511; respectively) (Table II). A post hoc test showed a significant increase in peritoneal AOPP concentrations in DIE versus controls (P < 0.001) and in DIE versus SUP (P < 0.05) (Table II and Fig. 2). In addition, a post hoc test showed a significant increase in peritoneal nitrates/nitrites concentrations in DIE versus controls (P < 0.05) (Table II and Fig. 2).

We further studied peritoneal fluids concentrations in endometriotic women according to rAFS Stages I–IV classification (Supplementary...
We failed to show any difference in peritoneal fluids thiols and protein carbonyls levels according to rAFS stages (P = 0.056 and P = 0.215; respectively) (Table III). Peritoneal fluids AOPP were significantly different among groups (P < 0.001) (Table III). In addition, we found that nitrates/nitrites were, in a similar manner, significantly different among groups (P = 0.023) (Table III). A post hoc test showed a significant increase in peritoneal AOPP concentrations in endometriosis Stage II–IV versus controls (P < 0.01) or Stage II versus Stage I (P < 0.01) (Table III and Supplementary data, Fig. S1). In addition, a post hoc test showed a significant increase in peritoneal nitrates/nitrites concentrations in endometriosis Stage IV versus controls (P < 0.05) (Table III and Supplementary data, Fig. S1).

Subgroup analysis in endometriotic women (Fig. 3), according to the surgical classification of endometriosis, clearly displayed a large increase in peritoneal AOPP and nitrates/nitrites, in DIE women with intestinal involvement (n = 28) when compared with DIE women without intestinal involvement (n = 36) (AOPP: median, 308.7 μmol/l; range, 0.3–1180.0 versus median, 145.4 μmol/l; range, 8.6–831.1; P = 0.001; and nitrates/nitrites: median, 55.9 μmol/l; range, 10.7–681.6 versus median, 22.7 μmol/l; range, 1.9–199.8; P < 0.001). No statistical
Increased oxidative stress markers in deep endometriosis

Table II  Peritoneal fluid oxidative stress parameters in women with endometriosis and controls.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 85)</th>
<th>SUP (n = 55)</th>
<th>OMA (n = 31)</th>
<th>DIE (n = 64)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOPP (µmol/l)</td>
<td>77.8 (0.8–616.1)</td>
<td>95.6 (18.0–839.6)</td>
<td>119.7 (34.5–771.3)</td>
<td>174.0 (0.3–1180.0)(^{a,b})</td>
<td>&lt;0.001(^c)</td>
</tr>
<tr>
<td>Thiols (µmol/l)</td>
<td>102.3 (1.6–222.7)</td>
<td>132.2 (25.7–379.2)</td>
<td>118.9 (8.4–225.7)</td>
<td>108.5 (14.8–461.0)</td>
<td>0.05(^c)</td>
</tr>
<tr>
<td>Nitrates/nitrates (µmol/l)</td>
<td>18.5 (1.6–184.5)</td>
<td>24.1 (1.6–73.2)</td>
<td>22.3 (1.6–64.2)</td>
<td>26.8 (1.9–681.6)(^d)</td>
<td>0.020(^c)</td>
</tr>
<tr>
<td>Carboxyls (nmol/mg)</td>
<td>1.2 (0.1–27.3)</td>
<td>1.6 (0.1–7.6)</td>
<td>1.2 (0.1–19.2)</td>
<td>2.1 (0.1–24.6)</td>
<td>0.510(^c)</td>
</tr>
</tbody>
</table>

Data, median (range); AOPP, advanced oxidation protein products; SUP, superficial peritoneal endometriosis; OMA, endometrioma; DIE, deeply infiltrating endometriosis.

\(^a\)Statistically different from control women (P < 0.001).
\(^b\)Statistically different from SUP (P = 0.05).
\(^c\)Statistical analysis was performed using Kruskal–Wallis test. Post hoc test were performed using the Dunn’s Multiple Comparison Test.
\(^d\)Statistically different from control women (P < 0.05).

Table III  Peritoneal fluid oxidative stress parameters in women with endometriosis and controls according to rAFS stage.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 85)</th>
<th>Stage I (n = 50)</th>
<th>Stage II (n = 27)</th>
<th>Stage III (n = 36)</th>
<th>Stage IV (n = 37)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOPP (µmol/l)</td>
<td>77.8 (0.8–616.1)</td>
<td>92.3 (17.9–458.8)</td>
<td>139.9 (43.6–839.6)(^{a,b})</td>
<td>138.6 (34.5–1180.0)(^a)</td>
<td>147.0 (8.6–876.9)(^a)</td>
<td>&lt;0.001(^c)</td>
</tr>
<tr>
<td>Thiols (µmol/l)</td>
<td>102.3 (1.6–222.7)</td>
<td>123.4 (25.7–259.4)</td>
<td>126.9 (60.3–216.5)</td>
<td>118.2 (8.4–461.0)</td>
<td>99.8 (21.5–245.4)</td>
<td>0.056(^c)</td>
</tr>
<tr>
<td>Nitrates/nitrates (µmol/l)</td>
<td>18.5 (1.6–184.5)</td>
<td>24.7 (1.6–73.2)</td>
<td>26.8 (1.9–140.3)</td>
<td>19.5 (1.6–101.4)</td>
<td>43.7 (1.9–681.6)(^d)</td>
<td>0.023(^c)</td>
</tr>
<tr>
<td>Carboxyls (nmol/mg)</td>
<td>1.2 (0.1–27.3)</td>
<td>1.7 (0.1–24.6)</td>
<td>1.3 (0.1–5.9)</td>
<td>1.2 (0.1–19.2)</td>
<td>2.3 (0.6–7.2)</td>
<td>0.215(^c)</td>
</tr>
</tbody>
</table>

Data, median (range); AOPP, advanced oxidation protein products; SUP, superficial peritoneal endometriosis; OMA, endometrioma; DIE, deeply infiltrating endometriosis.

\(^a\)Statistically different from control women (P < 0.01).
\(^b\)Statistically different from Stage I (P < 0.01).
\(^c\)Statistical analysis was performed using Kruskal–Wallis test. Post hoc test were performed using the Dunn’s Multiple Comparison Test.
\(^d\)Statistically different from control women (P < 0.05).

Figure 3  Peritoneal fluids oxidative stress parameters [A. AOPP (µmol/l); B, nitrates/nitrates (µmol/l)] among endometriosis women according to the existence of DIE with intestinal involvement. AOPP and nitrates/nitrates in DIE women with intestinal involvement (n = 28) are compared with that in DIE women without intestinal involvement (n = 36). Statistical analysis was performed using the Mann–Whitney U. Peritoneal fluids oxidative stress parameters are represented as median with interquartile range. P < 0.05 was considered statistically significant.

Difference was highlighted in thiols and carboxyls peritoneal concentrations between DIE women with and without intestinal involvement (thiols: median, 106.4 µmol/l; range, 14.8–461.0 versus median, 115.3 µmol/l; range, 21.5–344.2; P = 0.439; and carboxyls: median, 1.36 nmol/mg; range, 0.3–9.9 versus median, 2.13 nmol/mg; range, 0.1–24.6; P = 0.853).
Clinical and surgical correlations with peritoneal fluids AOPP, thiols, nitrates/nitrites and protein carbonyls are reported in Table IV. We found a significant correlation between pelvic pain symptoms scores and peritoneal protein oxidative stress markers in women with endometriosis. Table IV show significant correlations between AOPP and dysmenorrhoea ($r = 0.245; P = 0.005$), deep dyspareunia and GI symptoms ($r = 0.305; P = 0.001$). In addition, we found a significant correlation between AOPP peritoneal levels and surgical characteristics of disease severity according to rAFS total score ($r = 0.238; P = 0.005$), rAFS adhesion score ($r = 0.228; P = 0.007$) and rAFS implant score ($r = 0.189; P = 0.027$) (Table IV). Nitrates/nitrites peritoneal levels significantly correlated only with GI symptoms ($r = 0.267; P = 0.005$) (Table IV). No significant correlations were shown in control women. Correlations between anatomical characteristics of DIE women with peritoneal fluids protein oxidative parameters are reported in Fig. 4. Peritoneal fluid AOPP and nitrites/nitrites levels correlated with the anatomical features corresponding to the extent of the DIE with intestinal involvement. Figure 4 depicts positive peritoneal fluids AOPP correlations with intestinal DIE features against the total number of intestinal DIE lesions ($r = 0.464; P < 0.001$). In addition, we found a positive significant correlation between peritoneal fluids nitrites/nitrites levels and the total number of intestinal DIE lesions ($r = 0.366; P = 0.007$) (Fig. 4).

**Discussion**

We believe that this is the first report which has analysed protein peritoneal fluids oxidative status in women with endometriosis according to the surgical classification of endometriosis in SUP, OMA and DIE. We found significantly increased concentrations of peritoneal protein AOPP and nitrites/nitrites in patients with endometriosis compared with control women without endometriosis. In contrast, peritoneal thiols and carbonyls levels were not significantly different in cases of endometriosis. According to the surgical classification, peritoneal fluids protein AOPP and nitrites/nitrites were significantly increased in DIE when compared with controls ($P < 0.001$ and $P < 0.05$; respectively). In addition, we

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**Table IV** Correlation analysis of peritoneal protein oxidative stress markers levels with clinical and surgical data in women with endometriosis ($n = 150$).

<table>
<thead>
<tr>
<th>Measurements</th>
<th>AOPP</th>
<th>Thiols</th>
<th>Nitrates/nitrites</th>
<th>Carbonyls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Correlation</td>
<td>P-value</td>
<td>Correlation</td>
<td>P-value</td>
</tr>
<tr>
<td>Dysemorrhoea</td>
<td>0.245</td>
<td>0.005</td>
<td>0.190</td>
<td>0.053</td>
</tr>
<tr>
<td>Deep dyspareunia</td>
<td>0.150</td>
<td>0.099</td>
<td>0.016</td>
<td>0.862</td>
</tr>
<tr>
<td>NCCP</td>
<td>0.157</td>
<td>0.073</td>
<td>-0.028</td>
<td>0.753</td>
</tr>
<tr>
<td>GI symptoms</td>
<td>0.305</td>
<td>0.001</td>
<td>0.052</td>
<td>0.571</td>
</tr>
<tr>
<td>LU tract symptoms</td>
<td>-0.034</td>
<td>0.710</td>
<td>0.058</td>
<td>0.526</td>
</tr>
<tr>
<td>Total score rAFS</td>
<td>0.238</td>
<td>0.005</td>
<td>-0.096</td>
<td>0.264</td>
</tr>
<tr>
<td>Implants score rAFS</td>
<td>0.189</td>
<td>0.027</td>
<td>-0.104</td>
<td>0.226</td>
</tr>
<tr>
<td>Adhesions score rAFS</td>
<td>0.228</td>
<td>0.007</td>
<td>-0.056</td>
<td>0.519</td>
</tr>
</tbody>
</table>

All the analysis were performed using the Spearman Rank correlation test. Pain intensity was evaluated preoperatively using a 10-cm VAS. rAFS: according to The Revised American Fertility Society Classification of Endometriosis.
Increased oxidative stress markers in deep endometriosis

Clearly show among endometriotic women with DIE that the existence of a more severe disease with intestinal involvement was associated with significantly higher peritoneal AOPP and nitrates/nitrites levels. Finally, our study indicates a significant correlation between protein peritoneal oxidative markers and intrinsic anatomical characteristics of the severity of deep infiltrating endometriosis. Elevated peritoneal concentrations of AOPP and nitrates/nitrites were associated with the existence of more extended intestinal DIE that was more often multifocal.

The novelty of the topic and the methodological design are both strengths of our study. As far as we know, this is the largest study on peritoneal concentrations of protein oxidative markers ever performed in endometriotic patients. The sample size of this study may have resulted in reducing selection bias and minimizing errors in statistical values. Even though previous studies are present in the literature studying the relationship between oxidative stress and endometriosis (Carvalho et al., 2012), none of the studies have focused on protein oxidative status (AOPP, nitrates/nitrites, carbonyls and thiols) in peritoneal fluids of women with endometriosis. We included in this study only patients with complete surgical exploration of the abdomino-pelvic cavity. Considering the heterogeneous character of endometriosis, we focused on patients with well-defined phenotypes. According to the surgical classification, patients were classified as SUP, OME or DIE. This classification describes the disease phenotype more accurately than the rAFS classification which can encompass in the same stage different types of endometriosis lesions including SUP, OMA and DIE. For homogeneity of the study, after thorough examination of the abdomino-pelvic cavity during surgery, only women with benign gynaecological conditions, without any evidence of macroscopic visual endometriosis, were allocated to the control group. According to a previous study, uterine leiomyoma display a significantly increased protein oxidative stress status and reduced antioxidant capacity (Santulli et al., 2013), therefore women with uterine leiomyoma were excluded of the study.

In spite of all precautions, our study may be still subject to certain shortcomings and/or biases. The study was conducted in a referral centre specialized in endometriosis surgery. Women with OMA or DIE operated in our centre might have particularly severe forms of endometriosis as reflected by the high proportions of women with DIE (42.7%), the high number of DIE patients presenting intestinal lesions (43.7%) and the high percentage of women with a previous history of surgery for endometriosis (21.3%). This referral bias for women with severe lesions might have amplified the difference in protein oxidative stress status levels between endometriosis patients and controls. Additionally, our study included only women with histologically proven endometriosis; women with asymptomatic forms of endometriosis and women undergoing ART without surgical intervention were therefore not analysed in our study. We also recognize that there is no ideal control group for studying peritoneal stress oxidative parameters in women with endometriosis. Our control group involved women operated for benign gynaecological conditions and some of these conditions (tubal infertility or ovarian cysts), might also be associated with altered peritoneal protein oxidative stress markers. Speaking against this possibility is the fact that we found clear statistically significant differences between women with and without endometriosis.

Proteins constitute a major component of plasma and peritoneal fluid and both are major targets of oxidation (Jackson et al., 2005; Lemarechal et al., 2006). Oxidation profoundly alters the structure and activity of oxidized proteins compared with their native forms. Therefore, the evaluation of oxidative proteins in these biological fluids is related to the intensity of free radical reactivity. Previous studies have shown increased levels of protein oxidation markers in a wide range of pathologic conditions in humans, including diabetes mellitus, atherosclerosis, rheumatoid arthritis, systemic sclerosis and neurodegenerative syndromes (Stadtman, 2001; Allanore et al., 2004; Lemarechal et al., 2006).

In peritoneal fluid, oxidative stress takes place in inflammatory cells with cellular debris serving as a substrate, and derived products of this process move to the systemic circulation in serum/plasma. Accordingly, peritoneal fluid might be more vulnerable than serum to the effects of oxidative stress (Jackson et al., 2005). Furthermore, serum oxidative stress levels may reflect oxidative status due to other causes in addition to endometriosis, while measures in peritoneal fluid provide a more localized measure of oxidative stress related to endometriosis.

Thus, the examination of individual peritoneal fluids proteins for oxidative markers may be helpful in the establishment of specific oxidative stress pathways in vivo and in determining the potential functional effects of oxidant stress exposure. In this study, we assessed the protein redox balance in peritoneal fluids by evaluating: (i) indirect oxidative markers as total protein carbonyl groups and AOPP; (ii) thiols concentrations that provide an indirect reflection of the anti-oxidative defenses (Lemarechal et al., 2006) and (iii) total nitrates/nitrites levels which reflect activity of NO synthase and the creation of NO radicals (Moshage et al., 1995). The lifespan of NO is relatively short. It is converted within seconds to nitrate and nitrite in an oxygenated environment such as peritoneal fluid. Nitrate and nitrite, distributed into the peritoneal fluid, reflect the NO action in the peritoneal fluids. We show a significant increase in total nitrates/nitrites in peritoneal fluids of women with, when compared with women without, endometriosis. In addition, the most elevated total nitrates/nitrites levels were observed in cases of intestinal involvement and correlate with the multifocality of intestinal DIE lesions. Even if an association does not constitute a proof of cause and effect, this first result suggests that NO plays a role in the pathogenesis of endometriosis, especially in the most aggressive form with intestinal involvement. Our findings of increased NO activity are consistent with prior evidence of increased peritoneal macrophage activation in women with endometriosis (Osborn et al., 2002). We can speculate that peritoneal fluid NOware derived from peritoneal macrophages as previously stated (Osborn et al., 2002). Several studies have documented that women with endometriosis display increased peritoneal fluid volumes and peritoneal macrophage numbers (Bacci et al., 2009; Berbic et al., 2009; Capobianco and Rovere-Querini, 2013; Itoh et al., 2013). In cases of endometriosis, peritoneal macrophages display several features of activation with an increased ability to phagocytize (Lebovic et al., 2004). Furthermore, Wu et al. verified that peritoneal macrophages are activated in endometriosis. These authors observed that peritoneal macrophages from endometriosis patients produce more NO in vitro after lipopolysaccharide treatment (Wu et al., 1999).

Additionally, Osborn et al. (2002) performed in vitro stimulations of macrophages from women with endometriosis and found a strong correlation between the number of peritoneal macrophages and the total amount of peritoneal fluid NO.

In a murine model of endometriosis, NO alters the Th1 and Th2 cytokine balance, promoting the growth of endometriotic implants (Mier-Caberra et al., 2013). The strong correlation between oxidative stress and proliferation of cancer cells, along with the increased production of oxidative stress in response to chronic inflammation in endometriosis,
suggests a crucial place for oxidative stress in the regulation of endometriotic cell proliferation (Ngo et al., 2009). In fact, previous studies have shown that in case of deep infiltrating endometriosis, peritoneal fluids display significantly higher levels of the inflammatory and profibrotic interleukin-33 (Santulli et al., 2012a,b). As shown by Choi et al. (2009), the increase in IL-33 levels in DIE stimulates NO production through its cognate receptor ST2.

These speculations should be tempered by the fact that previous studies shows that eutopic endometrium of endometriotic women present increased endothelial nitric oxide synthase (NOS) expression (Ota et al., 1998; Wang et al., 2006) leading to a potential simultaneous activation of eNOS in the ectopic tissues responsible for the increased NO concentrations in peritoneal fluids. However, these studies focused only on eutopic endometria and therefore the effects on peritoneal fluids are actually still unknown. In addition, a more recent study found a significant increase in tissular levels of nitrates/nitrites without an associated elevation of either endothelial or inductible NOS in endometriotic tissues (Wu et al., 2003).

In women afflicted by infertility NO adversely affect sperm, tubal function, embryos and implantation (Osborn et al., 2002) and could participate in molecular mechanisms of endometriosis related pathogenesis of infertile women (de Ziegler et al., 2010). Finally, NO may contribute to painful symptoms. In fact, previous study has clearly identified an important role of oxidative stress in the nociceptive signalling cascade both centrally and peripherally (Wang et al., 2004; Vaculin et al., 2010).

We found significantly increased peritoneal fluids AOPP levels in women with deep infiltrating endometriosis. AOPP mediate inflammatory processes, enhancing the activation of leukocytes and the oxidative stress imbalance (Witko-Sarsatat et al., 1998; Lemarechal et al., 2006). AOPP are a precocious marker of oxidized proteins and circulate for prolonged periods in the blood of patients, as cells only degrade them within hours and days. Hence, the chemical stability of AOPP better reflects the variation of the oxidative imbalance between in women with and without endometriosis. We failed to show any difference in peritoneal protein oxidative stress markers levels according to menstrual cycle phases in the endometriosis group. In contrast, disease-free women display significant changes in nitrates/nitrites peritoneal levels during the menstrual cycle in favour of increased oxidative stress during the proliferative phase (Supplementary data, Table SII). However, only 31 (36.5%) controls and 43 (28.7%) endometriotic women were informative on menstrual cycle phases. This might be a concern, as the oestrogenic balance variation through the menstrual cycle may have an effect on the performance of peritoneal protein oxidative stress marker measurements. Indeed, very few data are available in the literature about the relationship between protein oxidative stress markers and oestrogen. In a previous study, Younis et al. (2012) investigated the activities and relevance of antioxidant enzymes and specific lipid peroxidation according to ovarian controlled hyperstimulation during IVF. The authors found a significant positive correlation between oestrogen levels and the levels of antioxidant enzymes such as superoxide dismutases and glutathione peroxidase after ovarian stimulation in all study participants (Younis et al., 2012). In contrast, a recent study failed to find any differences in AOPP and nitrite levels between pre-and post-menopausal women, pleading for the absence of any positive correlation between serum AOPP, nitrates and oestrogens levels (Victorino et al., 2013). Nevertheless, the role of oestrogen in modulating AOPP, thiols, nitrates/nitrites and protein carbonyls levels is actually still unclear.

We also assessed the value of performing protein oxidative stress markers measurements during hormonal treatment. We found no effect of hormonal treatment on peritoneal fluids AOPP, thiols, nitrates/nitrites and protein carbonyls in either the endometriosis or control group.

These data plead the absence of an impact of hormonal therapy and menstrual cycle phases on peritoneal fluids AOPP, thiols, nitrates/nitrites and protein carbonyls in endometriotic women. In contrast, disease-free women display significant changes in nitrates/nitrites peritoneal levels during the menstrual cycle.

In conclusion, this study for the first time reveals a significant increase in the protein oxidative stress status of peritoneal fluid samples from women with endometriosis especially in cases of DIE with intestinal involvement. These observations confirm the need of more studies as these biochemical characteristics of endometriosis may open the way to the evaluation of therapeutic approaches targeting oxidative imbalance. In view of its very high occurrence and serious impact on women’s lives, endometriosis is responsible for augmented healthcare costs and work loss productivity (Simoens et al., 2012). The oxidative stress status may represent a mechanism which is amenable to treatment or prevention of endometriosis. These novel avenues may help to reduce the health-related morbidity and healthcare costs of endometriosis.

Supplementary data
Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors’ roles
P.S., D.B., F.B. and C.C. conceived and designed the study. All the authors analysed and interpreted the data. S.C., M.F., L.M. and H.L. performed the laboratory tests. F.B. and D.B. supervised and reviewed all the statistical analysis. P.S., C.C. and L.M. contributed to the data collection and performed the surgical procedures. A.-E.M. performed preoperative transvaginal ultrasonography. P.S., D.B. and C.C. contributed to writing the manuscript. All the authors approved the final version of the manuscript.

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Conflict of interest
There are no conflicts of interest.

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