Peritoneal endometriotic lesions differentially express a haptoglobin-like gene*

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A unique glycoprotein, called endometriosis protein-I (ENDO-I), has previously been shown to be synthesized and secreted by rat and human endometriotic explants. Furthermore, the N-terminal amino acid sequence analysis showed that ENDO-I shares homology with haptoglobin. The present study was designed to determine this sequence of ENDO-I cDNA from human peritoneal endometriotic lesions and to determine the relative expression of the ENDO-I gene in several human tissues. Poly A-enriched RNA was isolated and reverse-transcribed. To determine the sequence of ENDO-I cDNA, a polymerase chain reaction was performed on cDNA from human endometriotic lesions and a gene-specific primer based on the human haptoglobin sequence. A similar procedure was followed to assess the relative expression of the ENDO-I gene in various tissues. Glyceraldehyde-3-phosphate-dehydrogenase was used as an internal control. ENDO-I gene expression was quantified by densitometry. Sequence analysis of ENDO-I cDNA identified 873 nucleotides that displayed 99.4% homology with the human haptoglobin β-chain. Relative expression of ENDO-I mRNA by peritoneal endometriotic lesions was 19-fold greater than peritoneum, 28-fold greater than endometrioma and 37-fold greater than eutopic endometrium (P < 0.01). Haptoglobin-like ENDO-I may be associated with localized angiogenesis and altered immune response involved with the aetiology/pathophysiology of endometriosis.

Key words: endometriosis/endometrium/gene expression/haptoglobin-like protein/peritoneum

Introduction

Endometriosis is a common gynaecological disease characterized by the presence of both endometrial glands and stroma outside the uterine cavity. Active endometriotic lesions exhibit an invasive growth pattern (Nakamura et al., 1993) and intense vascularization (Nisolle et al., 1993). Yet, despite these obvious similarities to tumours, the disease is not neoplastic. Women affected with this disorder suffer a variety of symptoms such as dyspareunia, dysmenorrhoea, abdominal pain and infertility. Several theories, all involving interaction between endometrial and peritoneal cells, have been proposed to explain the aetiopathology of pelvic endometriosis (Bontis and Vavilis, 1997). A growing body of evidence shows that qualitative and quantitative differences in secretory products occur between endometriotic tissue and its uterine counterpart (Sharpe et al., 1991; 1993; Sharpe and Vernon, 1993; Noble et al., 1996; Tseng et al., 1996). Using a rat model for endometriosis, we have reported that ectopic uterine tissues uniquely synthesize and secrete two proteins, endometriosis protein-I (ENDO-I) and endometriosis protein-II (ENDO-II), in comparison with eutopic uterine tissues (Sharpe and Vernon, 1993). These two proteins have been subsequently detected in human endometriotic explant and cell culture media (Sharpe et al., 1993). ENDO-II has been recently identified as tissue inhibitor of metalloproteinases-1 (Sharpe-Timms et al., 1995).

ENDO-I is a complex acidic glycoprotein (M, 40 000–55 000; pl 4.0–5.2), as visualized by two-dimensional sodium dodecyl sulphate–polyacrylamide electrophoresis (SDS–PAGE) (Sharpe and Vernon, 1993; Sharpe et al., 1993). The synthesis of ENDO-I by rat endometriotic implants does not depend upon the stage of the reproductive cycle or the steroid treatment. We have recently demonstrated that rat ENDO-I is a haptoglobin (Hp)-like protein (Sharpe-Timms et al., 1998). Amino acid sequence analysis of three of the multiple ENDO-I isoforms showed 100% homology with the first 16 amino acids of the N-terminal of the rat Hp β-chain. Furthermore, the sequence of a partial cDNA cloned from rat endometriotic tissue was 99.9% identical to the β-subunit of rat Hp over a 859 nucleotide overlap.

This study reports for the first time the molecular characterization of an Hp-like gene, ENDO-I, in human pelvic endometriosis. For this purpose, we determined the sequence of a partial cDNA from a partial ENDO-I transcript from pelvic endometriotic lesions and studied the differential expression of the ENDO-I gene in several human tissues, including eutopic endometrium from controls with no pelvic disease, eutopic endometrium from women with endometriosis, eutopic endometrium from women with adenomyosis, peritoneum from controls with no pelvic disease, and endometrioma.

Materials and methods

Human tissues

Human tissues were obtained from informed volunteer patients routinely presenting to the physicians in the Department of Obstetrics...
The use of rats in this project was approved by the Institutional Review Board, Health Sciences Section. Specimens were acquired as previously described (Sharpe-Timms et al., 1996). Endometrial tissues were obtained at the time of hysterectomy or laparotomy, endometrial biopsy samples (P) were obtained from women without pelvic disease. Peritoneal tissue specimens (P) were obtained from patients with endometriosis. Peritoneal tissue specimens (P) were obtained from patients with pelvic disease.

Endometrial tissues were obtained from women without pelvic or endometrial disease (UE-C), from patients with adenomyosis (UE-A) with no pelvic disease, and patients with endometriosis (UE-E). Pelvic endometriotic lesions (PE) and endometrioma samples (OE) were taken from patients with endometriosis. Peritoneal tissue specimens (P) were obtained from women without pelvic disease.

and Gynecology at the University of Missouri School of Medicine, as approved by the Institutional Review Board, Health Sciences Section.

Specimens were acquired as previously described (Sharpe et al., 1993). Endometrial tissues were obtained at the time of hysterectomy or using a endometrial suction curette (Pipelle; Unimar, Wilton, CT, USA). Pelvic endometriotic lesions, endometriomata, and peritoneum were obtained at the time of laparotomy or laparoscopy. The presence of endometriosis was confirmed histologically. Most of the patients, including women who contributed peritoneal endometriotic lesions, were not taking any sex steroid hormones or steroid modulating medication at the time of the surgery. Eutopic endometrium specimens number 6 and 7 were obtained from patients receiving progestational medication. Normal peritoneal sample number 2 was contributed by a patient undergoing oestrogenic therapy.

### Rat tissues

The use of rats (n = 4) in this project was approved by the University of Missouri Animal Care and Use Committee. Rats were maintained in accordance with the National Institutes of Health guidelines. Healthy rats without endometriosis were killed and the liver was rapidly excised and frozen in liquid nitrogen. The frozen specimens were stored at –80°C until analysis. Rat liver was used as a source of Hp mRNA. The relative amount of ENDO-I transcript in pelvic endometriotic lesions was compared with the relative level of rat hepatic Hp mRNA (see below).

### RNA isolation

Tissues were homogenized in total RNA lysis buffer (Qiagen, Valencia, CA, USA) containing 1% of 2-mercaptoethanol (Sigma, St Louis, MO, USA) and stored at –80°C until analysis; 20–50 mg of tissue (wet weight) was used as the starting material for RNA isolation. From the lysates, total nucleic acids were extracted by adding 1/10 of the lysate volume of 5% w/v sodium N-laurylsarcosinate (Sigma), followed by sequential phenol:chloroform:isoamylalcohol (25:24:1 v/v) and chloroform:isoamylalcohol (24:1 v/v) extractions. Total nucleic acids were precipitated with 0.15 volume of 2 M sodium acetate (Sigma) and 2.5 volumes of ethanol. PolyA+ RNA selection from the total nucleic acids was carried out with the Micro-FastTrack kit (Invitrogen, San Diego, CA, USA) following the manufacturer’s instructions. The yield was ~20 ng of polyA+ selected RNA per mg of tissue.

### Determination of ENDO-I transcript levels in polyA+ selected RNA by a semi-quantitative reverse transcriptase–polymerase chain reaction (RT–PCR) procedure

All mRNA in a given sample was reverse-transcribed with an adaptor primer (Gibco BRL, Gaithersburg, MD, USA) as described by Sharpe-Timms et al. (1998). For reverse transcription, 250 ng of polyA+ selected RNA was used. ENDO-I gene expression in the different tissues was analyzed by semi-quantitative RT–PCR using glycerol-dodehyde-3-phosphate dehydrogenase (GAPDH) as internal standard. The composition of the PCR reactions for GAPDH, ENDO-I and rat Hp were as described by Sharpe-Timms et al. (1998), except that the sequence of the gene-specific primer for human ENDO-I was 5’GATTCCAAGGCCAGCTTTCCTGCGAGGCT3’ corresponding to amino acids 7–16 of the β-chain of human Hp (Maeda, 1985). The GAPDH primers annealed to two different exons of the GAPDH gene, thus providing a control for genomic DNA contamination (Ercolani et al., 1988).

### PCR parameters

The amount of template cDNA and number of cycles needed to perform the PCR reaction in linear phase for both target and reference amplicon were determined (El-Husseini et al., 1994; Nicoletti and Sassy-Pringent, 1996). Cycling conditions were as follows: (i) ENDO-I: 1 min at 94°C followed by 5 cycles of 94°C for 30 s and 72°C for 5 min; 5 cycles of 94°C for 30 s and 70°C for 5 min; then 22, 25 or 28 cycles of 94°C for 30 s and 68°C for 5 min; followed by a 10-min final extension at 68°C. (ii) GAPDH: 96°C for 30 s, 54°C to 49°C for 30 s and 72°C for 90 s plus 5 s autoextension for 22, 25 or 28 cycles.

The same type of experiments were used to assess the linear range for the amount of template cDNA, therefore 0.5, 1 or 2 µl of original cDNA solution were amplified for 25 cycles. The PCR products from these control experiments were fractionated in the same ethidium bromide-stained agarose gel electrophoresis to avoid inter-experimental differences between gel staining and photographic exposure, and photographed under a UV light source (Image Analysis Software, Alpha Innotech Corporation, San Leandro, CA, USA). The 1 kb DNA ladder was obtained from Gibco BRL. Once the linear range was established, 1 µl of each original cDNA solution was amplified for 25 cycles under the PCR conditions described above. Parallel experiments under identical experimental conditions were carried out for all the samples included in this study. PCR products were resolved by ethidium bromide-stained agarose gel electrophoresis, photographed under a UV light source, and transferred to nylon membranes (Micro Separations Inc, Westborough, MA, USA) by
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Figure 1. Comparison of ENDO-I and human haptoglobin (Hp) cDNA sequences. ENDO-I nucleotide sequence demonstrated 99.4% identity with nucleotides 368–1222 of the β-subunit of human Hp, which corresponds to the coding sequence and 3' untranslated region. Vertical lines = identical match. Bold = polyadenylation signal.

capillary elution (Sambrook et al., 1989). Electronic pictures of ethidium bromide-stained gels were digitized into colour-scale images using the ImageQuant program (Molecular Dynamics, Sunnyvale, CA, USA). The ethidium bromide-stained DNA products were quantified by densitometry. Results were expressed as the ratio between the amount of the ENDO-I amplification product over the GAPDH amplification product for each sample. These normalized values were called ENDO-I relative transcript levels.

Southern blot analysis of the ENDO-I PCR products

The specificity of ENDO-I amplification was tested by Southern blot analysis. The nylon membranes were hybridized with a 250 bp-cDNA fragment that spanned nucleotides 39–288 of the human ENDO-I sequence (shown below). The probe was labelled by PCR in a final volume of 20 µl containing 0.1 ng of electro-eluted ENDO-I PCR product as template; 20 mM Tris–HCl (pH 8.4 at 22°C); 1.5 mM MgCl2; 82.5 nM of dATP; 82.5 nM of dGTP; 82.5 nM of dTTP; 25 µCi of [α-32P]dCTP (3000 Ci/mmol; NEN, Boston, MA, USA) and 0.125 IU of Taq polymerase (Sigma). Forward primer sequence was 5’CATCACTCTCTCATAAAGAC3’ and reverse primer sequence was 5’TCCCTCCTTTAGCTTTTCCC3’. Touchdown cycling conditions were as follows: 25 cycles of 96°C for 30 s, 44–38°C for 2 min and 72°C for 90 s plus 5 s autoextension; followed by a 5 min final extension at 72°C. Blots were blocked for 1 h at 65°C in blocking solution (Promega, Madison, WI, USA), hybridized at 65°C for 16 h in high stringency solution (Promega) containing the radio-labelled probe, and washed twice at room temperature in 5× sodium chloride/sodium citrate (SSC), 0.5% SDS, twice at 37°C in 1× SSC, 0.5% SDS and three times at 65°C in 0.1× SSC, 0.1% SDS, for 15 min each time. The radioactive filters were exposed to X-ray film (Kodak, Rochester, NY, USA) for 2 h.

Sequence analysis of ENDO-I cDNA

Amplified PCR product of ENDO-I from one of the peritoneal endometriosis samples was eluted from a polyacrylamide gel (Sambrook et al., 1989) and analysed by automated sequence analysis, using the ENDO-I gene-specific primer as a sequencing primer. The reactions were run by the University of Missouri DNA Core Facility using Applied Biosystems Prism Dye-Deoxy terminator FS chemistry and analysed in the Applied Biosystems 377 automated DNA sequencer (PE Applied Biosystems, Foster City, CA, USA). The result was compared with known sequences in computerized data banks (Genetics Computer Group, Madison, WI, USA).
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Figure 2. Representative amplification of target cDNA as a function of the number of polymerase chain reaction (PCR) cycles for (A) ENDO-I and (B) GAPDH. Poly A± selected mRNA from pelvic endometriotic lesion number 3 was reverse transcribed. Of the reverse transcription reaction, 1 µl was used to: (A) amplify ENDO-I cDNA for 22, 25 or 28 cycles; (B) amplify GAPDH cDNA for 22, 25 or 28 cycles. Inserts: ethidium bromide-stained agarose gel electrophoresis of the actual PCR products.

Figure 3. Representative amplification of the target cDNA as a function of the amount of template cDNA. All the mRNA were reverse-transcribed simultaneously in the same reaction using a universal poly (dT) primer. 0.5, 1 and 2 µl of the same cDNA solution was used to amplify (A) ENDO-I and (B) GAPDH for 25 cycles in two separate reactions. Inserts: ethidium bromide-stained agarose gel electrophoresis of the actual polymerase chain reaction (PCR) products.

Statistical analysis
A logarithmic transformation of the ratio ENDO-I/GAPDH was performed to homogenize variances and normalize the distribution (Sokal and Rohlf, 1981). One-way analysis of variance was performed to detect statistical differences in ENDO-I expression among the various tissue types, followed by a Tukey’s post-hoc test for all pairwise multiple comparisons.

Results
Specimen characteristics
Table I summarizes the procedure followed to obtain the specimens, the histological date of the endometrium, and age of the patients. Eutopic endometrium from controls, eutopic endometrium from women with adenomyosis and peritoneum were taken from patients without pelvic disease. Eutopic endometrium from women with endometriosis and peritoneal endometriotic lesions were taken from patients with moderate to severe endometriosis. Endometriomatas were chocolate cysts in three of the four cases studied.

Human ENDO-I cDNA sequence analysis
A partial cDNA from one of the samples of human peritoneal endometriotic lesions was obtained. The PCR yielded a band of the expected size (950 bp). The sequence of the ENDO-I cDNA had 99.4% homology to the β-chain of human Hp cDNA over 873 nucleotides (Figure 1). Beyond the last nucleotide of the 3’ untranslated region, several adenylate residues corresponding to the poly A tail were detected. There were only five mismatches with human Hp cDNA sequence at nucleotides 98, 157, 172, 190 and 797 of the ENDO-I cDNA sequence. The four glycosylation sites at Asn 23, 46, 50, and 80 of the Hp β-chain were conserved in human ENDO-I.

Semi-quantitative RT–PCR validation
PCR amplification of GAPDH yielded a 320 bp fragment. This corresponded with a product originating in the mRNA. No contaminating genomic DNA, which would have been visualized as a 633 bp band, was detected in any of the samples tested. In addition, no amplification of GAPDH or ENDO-I was obtained when reverse transcriptase was omitted from the samples (data not shown). A series of PCR reactions was carried out to determine the linear amplification range as a function of the number of cycles and the amount of template. The amount of template was expressed as µl of cDNA solution because the same solution was used to amplify the target gene and the internal standard for a given sample. Amplification
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Figure 4. Representative differential expression of ENDO-I mRNA by eutopic endometrium from controls (UE-C), eutopic endometrium from women with endometriosis (UE-E) and pelvic endometriotic lesions (PE). (A) Ethidium bromide-stained agarose gel electrophoresis showing the reverse transcription–polymerase chain reaction (RT–PCR) analysis of ENDO-I and GAPDH for UE-C (lanes 1–2), UE-E (lanes 3–6), and PE (lanes 7–11). Lanes 3 and 7 and 4 and 8 are matching samples. M indicates size markers. (B) Southern blot analysis of ENDO-I PCR products shows specific hybridization between the radio-labelled ENDO-I 250 bp-probe and the amplified 950 bp-bands.

Figure 5. Representative differential expression of ENDO-I mRNA by peritoneum (P), endometrioma (OE), eutopic endometrium from women with adenomyosis (UE-A), and a pelvic endometriotic lesion (PE). (A) Ethidium bromide-stained agarose gel electrophoresis showing the reverse transcription–polymerase chain reaction (RT–PCR) analysis of ENDO-I and GAPDH for P (lane 1), OE (lanes 2–3), UE-A (lanes 4–5), and PE as positive control (lane 7). M indicates size markers. (B) Southern blot analysis of ENDO-I PCR products shows specific hybridization between the radio-labelled ENDO-I 250 bp-probe and the amplified 950 bp bands.

Differential expression of ENDO-I gene by human tissues

Figure 4 shows differential expression of ENDO-I gene in representative samples of several human tissues. ENDO-I transcripts levels in polyA+ selected RNA were significantly greater ($P < 0.01$) in pelvic endometriotic lesions than eutopic endometrium from controls and eutopic endometrium from women with endometriosis, belonging to proliferative and secretory stages of the menstrual cycle. Lanes 3 and 7 and 4 and 8 were matching samples of eutopic endometrium and pelvic endometriotic lesions from two different patients.

Since only one gene-specific primer was used to amplify ENDO-I cDNA, the specificity of the PCR reaction was tested by Southern blot analysis. A 250 bp fragment that spanned nucleotides 39–288 of the human ENDO-I partial cDNA was used as probe. This probe started nine nucleotides downstream from the end of ENDO-I forward primer. The analysis showed.
that the 950 bp bands were specific and genuine amplification products from ENDO-I cDNA (Figures 4B and 5B).

Figure 6 shows relative transcript levels of ENDO-I in the various tissues. The highest ENDO-I relative transcript level was present in pelvic ectopic endometrium and was significantly higher than the average level of ENDO-I transcript in eutopic endometrium, peritoneum and endometrioma ($P < 0.01$). No statistical difference was detected between eutopic endometrium, peritoneum and endometrioma. Average relative ENDO-I mRNA levels in pelvic endometriotic lesions were 19-fold greater than ENDO-I transcript levels in peritoneum, 28-fold times greater than ENDO-I transcript levels in endometrioma and 37-fold greater than ENDO-I transcript levels in eutopic endometrium. All the ENDO-I/GAPDH ratios for each particular experimental group were within 2 SD of the mean. Therefore, sample type (i.e. hysterectomy, laparoscopy or endometrial biopsy samples) and hormonal therapy in three specimens did not influence the results.

**Relative expression of ENDO-I in human pelvic endometriotic lesions and Hp in rat liver**

The expression level of ENDO-I mRNA in human pelvic endometriotic lesions was compared with the expression of $Hp$ mRNA in rat liver. This experiment allowed us to determine the relative amount of ENDO-I in human peritoneal endometriotic lesions and assess whether the transcript levels were of physiological significance (Figure 7). The relative expression of ENDO-I mRNA in human pelvic endometriosis was very similar to $Hp$ levels in non-stimulated rat liver.

**Discussion**

This study confirms and expands what we have previously reported for rat endometriosis regarding the expression of $ENDO-I$ (Sharpe and Vernon, 1993; Sharpe-Timms et al., 1998) and demonstrates for the first time the expression of a $Hp$-like gene by human peritoneal endometriotic lesions. In addition, it helps validate the rat endometriosis model, as the surgically-induced disorder shares biochemical markers with the naturally occurring disease.

Human $ENDO-I$ cDNA coding and 3’ untranslated regions are almost identical to the $β$-chain of human $Hp$. More studies are needed, however, to completely define the identity of $ENDO-I$. The five mismatches found in $ENDO-I$ cDNA sequence, compared with $Hp$ cDNA sequence, may be due to $Taq$ DNA polymerase errors or to genuine differences between the two cDNA. We have used an authentic internal standard ($GAPDH$) to make corrections because of the differences in the quality of the RNA samples or variations in the PCR efficiency. The 320 bp DNA fragment obtained by amplification of the $GAPDH$ template, the adenylate residues from the polyA tail detected beyond the last nucleotide of the 3’ untranslated region of $ENDO-I$ cDNA, and the lack of amplification of both genes when the reverse transcriptase was omitted ruled out genomic contamination.

$ENDO-I$ transcript levels are significantly higher in pelvic ectopic endometrium than in several other human tissues tested. Further, this difference was found between ectopic and eutopic endometrium of the same individuals. Augmented expression of $ENDO-I$ mRNA in pelvic endometriotic lesions did not depend on the stage of the reproductive cycle. Although there appeared to be variation between the relative levels of expression of $ENDO-I$ among the peritoneal endometriotic lesions samples, all $ENDO/GAPDH$ ratios for this group were within 2 SD of the mean. Interestingly, the two major components of the pelvic endometriotic lesion, endometrium
and peritoneum have, separately, low levels of ENDO-I transcript. Ovarian endometriomas (i.e. chocolate cysts, in which the endometrial glands and stroma are enclosed in fibrous ovarian tissue) also have low levels of ENDO-I transcript. These findings suggest that some interaction between peritoneum and ectopic endometrium is needed in order to increase the expression of ENDO-I gene. On the other hand, immune cells that infiltrate peritoneal endometriotic lesions (Oosterlynck et al., 1993a) may also play a role in the induction of ENDO-I gene.

As a similar Hp-like protein is also present in non-menstruating species with surgically-induced endometriosis (i.e. rat, Sharpe-Timms et al., 1998), overexpression of ENDO-I mRNA by peritoneal endometriotic lesions may be a consequence rather than the cause of the ectopic development of endometriosis. Therefore, it is possible that ENDO-I does not play a role in the early stages of the disease but later when an association between endometrium and peritoneum is established and a vascular network is developed between the two tissues (Nisolle and Donnez, 1997). Ongoing studies in our laboratory are being conducted to determine a potential role(s) for ENDO-I in the pathogenesis and pathophysiology of endometriosis.

The similarity between ENDO-I and Hp elicits a number of interesting questions about the role of ENDO-I in endometriosis. The role of Hp in haemoglobin metabolism has been known for many years but recently, a number of other biological functions for Hp, such as immunomodulatory properties and angiogenic activity have been reported (for reviews see Langlois and Delanghe, 1996; Dobryszycka, 1997). ENDO-I mRNA levels in pelvic endometriotic lesions are similar to those detected in non-stimulated rat liver. Upon stimulation, Hp mRNA levels rise between four and eight times (Yang et al., 1995). Therefore, the amount of ENDO-I mRNA detected in human endometriotic lesions may be of pathophysiological significance, especially if the protein acts locally.

One current hypothesis is the symptoms associated with endometriosis are the result of local peritoneal inflammation (Bontis and Vavilis, 1997) due to the secretion of certain factors by the endometriotic lesions (Taylor et al., 1997). These factors would locally promote neovascularization and interact with immune cells. Active endometriotic implants have intense vascularization both within and around the tissue (Nisolle et al., 1993). In-vivo (Oosterlynck et al., 1993b) and in-vitro (Taylor et al., 1997) assays have shown that peritoneal fluid from women with endometriosis have more angiogenic activity than peritoneal fluid from normal controls.

Peritoneal natural killer (NK) cell cytotoxicity is significantly decreased in women with endometriosis (Oosterlynck et al., 1992; Ho et al., 1995). Moreover, the NK activity and phytohaemagglutinin-induced lymphocyte proliferation also decreased when normal peripheral blood mononuclear cells were treated with endometriosis patients’ peritoneal fluid (Oosterlynck et al., 1993c). Culture supernatants of rat endometriotic implants had significantly higher inhibitory effects on NK activity than those of uterine endometrium (Mizumoto et al., 1996).

In conclusion, we have demonstrated that ENDO-I, an Hp-like gene, is dramatically up-regulated in human pelvic endometriotic lesions, when compared with eutopic endometrium from women with and without endometriosis, peritoneum and ovarian endometriomas. Local peritoneal production of ENDO-I, a Hp-like protein, may be associated with localized angiogenesis and altered immune phenomena related to endometriosis and thereby contribute to the aetiology and pathophysiology of this most enigmatic disease.

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