Clinical management of endometriosis
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Chapter 8

Performance of peripheral (serum and molecular) blood markers for diagnosis of endometriosis

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ABSTRACT

Purpose: To quantify the mRNA levels of MMP-3, MMP-9, VEGF and Survivin in peripheral blood and the serum levels of CA-125 and Ca19-9 in women with and without endometriosis and to investigate the performance of these markers to differentiate between deep and ovarian endometriosis.

Methods: A case control study enrolled a series of 60 patients. Twenty controls have been matched with 20 cases of ovarian and 20 cases of deep endometriosis. Univariable and multivariable performance of serum CA125 and CA19-9, mRNA for Survivin, MMP9, MMP3 and VEGF genes have been evaluated by means of ROC curves and logistic regression, respectively.

Results: No difference in markers’ concentration was detected between ovarian and deep endometriosis. In comparison with controls, serum CA125 and CA19 yielded the better sensitivity followed by mRNA for Survivin gene (81.5, 51.9 and 7.5% at 10% false positive rate, respectively). Multivariable estimated odds of endometriosis yielded a sensitivity of 87% at the same false positive rate.

Conclusions: A combination of serum and molecular markers could allow a better diagnosis of endometriosis.
INTRODUCTION

Endometriosis is one of the most frequent benign gynaecological diseases that cause pelvic pain, dysmenorrhea, dyspareunia and infertility in about 10–20% of the female population of reproductive age [1]. The disease severity is assessed by simply describing the findings at surgery or quantitatively, using a classification system such as the one developed by the American Society for Reproductive Medicine [2]. However, there is an agreement that such classification systems for endometriosis are subjective and correlate poorly with intensity of symptoms. Anatomic classification, based on the depth of infiltration of endometriotic nodules is now largely accepted by many authors [3–5]. Deeply infiltrating endometriosis, implants of endometriosis that penetrates 5 mm under the peritoneal surface [6], constitutes a major concern for the gynaecologist in view of the greater severity of the symptoms and its therapeutic complexity [7, 8].

Currently, for a definitive diagnosis of endometriosis, visual inspection of the pelvis at laparoscopy is the gold standard investigation [9]. However, this diagnostic procedure depends on the expertise of the surgeon and therefore is intrinsically inaccurate. The diagnostic laparoscopy, as well, is associated with an approximately 3% risk of the minor complications, such as nausea or shoulder tip pain, and a risk of major complications, such as bowel perforation and vascular damage [10, 11].

Several previous studies have suggested methods of non-invasive diagnosis of endometriosis. Most of these methods involved searching for a marker (or a combination of markers) in the peripheral blood of patients with endometriosis that was believed to be involved in the pathophysiology of the disease.

From the pathophysiological point of view, it has been suggested that the ectopic implants of endometrial cells are rich in angiogenic growth factors that, together with metalloproteinases (MMPs), have an important role in the survival and invasion of these cells [12]. Survivin, as well, was demonstrated to play a role in the development of endometriosis [13].

Matrix metalloproteinases (MMPs) are a family of enzymes involved in extracellular matrix remodelling [12, 14–17]. Several previous studies have demonstrated that ectopic and eutopic endometrium show altered expression of mRNA of MMPs, indicating that these enzymes play an important role in the pathogenesis of endometriosis [14, 18, 19].

Vascular endothelial growth factor (VEGF) is a heparin-binding glycoprotein with potent angiogenic and vascular permeability activities. Growing evidence supports its role in the development of the disease [20]. In previous studies, it has been observed a significant increase in VEGF and its mRNA expression in endometriotic tissues and eutopic endometrium of patients with endometriosis compared to the controls [18, 12–24]. CA 125 and CA 19-9 are glycoproteins whose concentrations are elevated in patients with ovarian tumours [25]. CA 125 is of limited diagnostic value in the diagnosis of endometriosis [26, 27]. Serum levels of the antigen CA 19-9 have been
shown to increase in accordance with the advancement of the clinical stage of endometriosis either alone or when used with CA 125 [28].

Relying on previous studies that demonstrated increased expression of mRNA of MMPs, VEGF and Survivin in the ectopic and eutopic endometrium in women with endometriosis [14, 18, 29–33], we were prompted to quantify the mRNA levels of MMP-3, MMP-9, VEGF and Survivin in peripheral blood and the serum levels of CA-125, CA19-9 in women with and without endometriosis. The specific aim of this study was to investigate the univariable and multivariable performances of these markers to diagnose or exclude the endometriosis and to differentiate between deep and ovarian endometriosis.

PATIENTS AND METHODS

Patient recruitment

The subjects enrolled in this study were women of reproductive age (between 26 and 40 years) undergoing laparoscopy for suspected endometriosis or non-malignant conditions (myoma, tubal ligation, and ovarian biopsy) in the Minimally Invasive Gynecological Surgery Unit, S. Orsola-Malpighi Hospital, University of Bologna between February 2007 and May 2008. At the time of surgery, pelvic organs were examined carefully for the presence and extent of endometriosis. Exclusion criteria were: (i) a suspected or ascertained diagnosis of systemic pathologies (malignancies, autoimmune diseases, and liver diseases), (ii) pregnancy. Patients routinely gave an informed consent for the use of their data for research purposes. The study was approved by the Institutional Review Board of the University of Bologna.

Overall, 60 consecutive patients satisfied both our inclusion and exclusion criteria. Forty of them were found to have endometriosis. They were divided, according to surgical and histological diagnosis, in three groups: patients with DIE (Group A), with ovarian endometriosis (Group B). Patients without endometriosis were used as controls (Group C). We considered as DIE all the implants of endometriosis that penetrates[5 mm under the peritoneal surface [6].

Blood samples

Peripheral venous blood samples were obtained from the patients in the follicular phase of the menstrual cycle immediately before laparoscopy. The quantitative detection of CA 125 and CA 19-9 was performed using a commercially available chemiluminescent immunometric assay provided by Roche Diagnostics GmbH (Germany) to be analysed with the Elecsys Analyzer. The concentrations of CA 125 and CA 19-9 were expressed as IU/ml. Sensitivity for both CA 125 and CA 19-9 was 0.6 IU/ml.

Another blood sample was collected from each woman and treated for RNA extraction within 1 hour of being drawn. They were lysed by diluting 2 mL of whole blood with 2 ml of phosphate
buffered saline (PBS) and adding 4 mL of lysis solution (Applied Biosystems, CA, USA). The lysates were stored at -80°C until automated RNA extraction.

**Automated RNA extraction**

Total RNA was extracted from peripheral blood samples using the ABI Prism 6100 nucleic acid Prep Station (Applied Biosystems, CA, USA). This instrument is engineered to yield a high-quality RNA free of cross-contamination. Total RNA extracted from 2 mL of peripheral blood was subjected to standard ethanol precipitation, and the pellet was resuspended in 20 μL of RNase-free water. The quality of RNA samples was determined by electrophoresis through agarose gels. The extracted RNA samples, then, were stored at -80°C.

**Real-time reverse transcription polymerase chain reaction**

The concentration of each total RNA sample was determined by NanoDrop TM 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). All RNA samples were then diluted at 20 ng/μL. 25 μL of total RNA was reversetranscribed using high-capacity cDNA archive kits (Applied Biosystems, Foster City, CA), according to the manufacturer’s protocol. Gene-specific primers for qRT-PCR analysis were designed using Primer Express software (Applied Biosystems).

An SYBR Green PCR Master Mix (Applied Biosystems) was used with 5 ng of cDNA and with 100–400 nM of each primer. A negative control without any cDNA template was run with every assay. All PCR reactions were performed using the ABI PRISM 7900 Sequence Detection System (PE Applied Biosystems). Each cDNA sample was tested in duplicate. The target mRNA gene was quantified by measuring the Ct (threshold cycle) to determine the relative expression while glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to normalize the data. All samples were resolved in a 2% agarose gel to confirm the PCR specificity.

**Statistical analysis**

The distributions of the demographic characteristics and circulating biochemical and mRNAs marker concentrations were analyzed. Median values were stratified retrospectively according to the severity of endometriosis (ovarian and deep infiltrating endometriosis). The non-parametric analysis of variance (Kruskal–Wallis test) and Dunn post hoc test were used for comparisons among and between groups. The sensitivity and false positive rate (FPR) were calculated for each available marker using a univariate receiver operating characteristic (ROC) curve. A multivariable analysis was performed using logistic regression to calculate the odds for each woman for classification as a case. Finally, a ROC curve for the calculation of multivariable sensitivity was built using, as the test variable, the estimated odds for endometriosis for each woman in the series by means of logistic regression. A comparison of the multivariable ROC curves was performed according to the method
of DeLong. Statistical analysis was carried out by means of the Statistical Package for the Social Sciences (SPSS) software version 15.0 (SPSS Inc., Chicago, USA).

RESULTS
The three study groups were homogeneous with regard to median age and median BMI (Table 1). Significant differences were noted in CA125, CA19-9, MMP3 and Survivin among the three groups of patients (Table 2). Since no differences have been noted for the two subtypes of endometriosis, the two groups were therefore unified for further analysis. Receiver operating characteristic (ROC) curves analysis identified CA 125 and CA19-9 as markers with the highest sensitivity followed by Survivin (Table 3). Finally by logistic regression output, the combination of the three variables (CA 125, Ca19-9 and Survivin) yielded a higher sensitivity than that obtained by single marker analysis (Table 3; Fig. 1). The comparison of the DR obtained by CA125 alone and by means of the combined model yielded a p-value of 0.442.

<table>
<thead>
<tr>
<th>Marker</th>
<th>DIE (Group A)</th>
<th>Ovarian endometriosis (Group B)</th>
<th>Endometriosis-Free (Group C)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA 125 (IU/ml)</td>
<td>43 (9-1098)</td>
<td>37 (14-773)</td>
<td>8.4 (0-98)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CA 19-9 (IU/ml)</td>
<td>14 (2-622)</td>
<td>36 (1-160)</td>
<td>6 (1-31)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Survivin**</td>
<td>0.36 (0.2-1.0)</td>
<td>0.36 (0.1-2.9)</td>
<td>1 (0.1-1.9)</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>MMP3**</td>
<td>1.56 (0.2-5.5)</td>
<td>1.42 (0.3-6.5)</td>
<td>1 (0.1-1.9)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>VEGF-A**</td>
<td>1.04 (0.6-1.9)</td>
<td>1.12 (0.5-1.9)</td>
<td>1 (0.1-1.9)</td>
<td>0.581</td>
</tr>
<tr>
<td>MMP9**</td>
<td>0.76 (0.1-5.6)</td>
<td>1.14 (0.1-5.6)</td>
<td>1 (0.1-1.9)</td>
<td>0.676</td>
</tr>
</tbody>
</table>

*Mann-Whitney U Test
Data are expressed as median (Min-Max)
DIE: Deep Infiltrating Endometriosis, BMI: Body Mass Index

Table 1: Baseline clinical characteristics of study groups

*Kruskal-Wallis test, **expressed as relative quantification. DIE: Deep infiltrating endometriosis, MMP3: matrix metalloproteinase3, VEGF: vascular endothelial growth factor, MMP9: matrix metalloproteinase 9
Table 2: Markers distribution in the three groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>AUC</th>
<th>SE</th>
<th>p-value</th>
<th>95% CI</th>
<th>Sensitivity at 10% False positive rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA 125</td>
<td>0.914</td>
<td>0.051</td>
<td>&lt;0.001</td>
<td>0.813</td>
<td>1.015</td>
</tr>
<tr>
<td>CA 19-9</td>
<td>0.819</td>
<td>0.066</td>
<td>0.001</td>
<td>0.691</td>
<td>0.948</td>
</tr>
<tr>
<td>Survivin</td>
<td>0.697</td>
<td>0.076</td>
<td>0.015</td>
<td>0.549</td>
<td>0.846</td>
</tr>
<tr>
<td>All</td>
<td>0.968</td>
<td>0.023</td>
<td>&lt;0.001</td>
<td>0.922</td>
<td>1.012</td>
</tr>
</tbody>
</table>

Table 3: Output of ROC curve for each of the variable measure

![ROC curve](image)

**AUC**: Area under the curve, **SE**: Standard error, **CI**: confidence interval

**Figure1**: ROC curve output for calculation of multivariable sensitivity obtained using the estimated odds for endometriosis for each woman in the series by means of logistic regression. X axis demonstrates the false positive rate and Y axis demonstrates the sensitivity.

**DISCUSSION**

The main reason for the emerging interest to find noninvasive diagnostic method is the long interval between first symptoms and the diagnosis of endometriosis. The possible use of a non-invasive diagnostic tool could shorten this time, improve the treatment outcome and reduce recurrence rates.

**Survivin**

Survivin is an inhibitor of apoptosis and is expressed during foetal development and in cancer tissues, but its expression has not been reported in normal adult tissues or benign diseases [34, 35]. Konno et al. [35], demonstrated in their study that Survivin gene and protein expression were detected in normal human endometrium and that Survivin could play an important role in
physiological homeostasis during the normal menstrual cycle. It could be expected that Survivin is also expressed in ectopic endometriotic foci [36].

In our study, we measured the mRNA level of Survivin in the peripheral blood in women with and without endometriosis. The results demonstrated that Survivin in patients with endometriosis is lower when compared with women without endometriosis.

To the best of our knowledge, this is the first report to demonstrate Survivin mRNA expression in the peripheral blood of patients with endometriosis.

**CA 125, CA 19 and their combination with Survivin**

CA 125 levels are commonly applied in diagnostic and monitoring ovarian cancer; however, as a single marker, it is of limited diagnostic value in the diagnosis of endometriosis [26, 27]. Somigliana et al., found significantly higher level of CA 125 in the serum of patients with endometriosis than in controls and reported sensitivity and specificity of 27 and 97%, respectively. Serum levels of the antigen CA 19-9 have been shown to increase in accordance with the advancement of the clinical stage of endometriosis [27]. Kurdoglu et al. [28], demonstrated in their prospective cohort study that serum CA 19-9 is a valuable marker in the diagnosis of endometriosis, and it may be used to predict the patients with severe endometriosis when used with CA 125. In our study, we found significantly higher levels of both CA125 and CA19-9 and lower levels of mRNA for Survivin gene in the serum of women with endometriosis. The combination of Survivin, CA19-9 and CA 125 showed a sensitivity of 87% at a 10% false positive rate and therefore with a reasonable discrimination to identify cases.

**Matrix metalloproteinases**

It is known that the MMP family plays a role in the pathogenesis of endometriosis. The establishment of ectopic sites of endometrial growth is an invasive event linked among other mechanisms to altered expression of MMPs. Gilabert-Estelle’s et al. [18] showed that the mRNA expression of MMP3 was significantly higher in the endometrium from women with endometriosis than in controls, confirming previous reports to the same effect [29–31]. Our data indicate that there was a significant difference in the level of MMP-3 mRNA in peripheral blood from women with endometriosis than from controls.

Regarding the mRNA expression of MMP9, it has been observed that both uterine endometrium and ectopic endometriotic tissue from women with endometriosis express significantly higher levels of MMP9 mRNA than endometrium from normal women [32, 33]. Our data demonstrate that there is no significant difference in mRNA expression levels of MMP9 in peripheral blood from women with endometriosis when compared to controls.
**Vascular endothelial growth factor**

Some studies have actually reported no significant difference in VEGF serum levels in patients with endometriosis compared with controls [19, 37]. In contrast, other authors have shown a significant increase in circulating levels of VEGF among women with endometriosis [38–40]. In our study, we found no significant modulation of VEGFA mRNA levels in the peripheral blood of patients with endometriosis compared to controls. Although VEGF seems to play a pivotal role in the local implantation and development of endometriotic lesions, its peripheral blood mRNA level seems not to be a suitable blood marker of the disease.

In our opinion, the results of this study should be validated by wider data collection. It is possible that peculiar mRNA markers could discriminate properly between ovarian and deep infiltrating endometriosis.

In conclusion, we quantified the sensitivity of known markers for endometriosis like CA125 and CA19-9. We further introduced and compared the performance of a molecular marker named Survivin. The integration of mRNA dosage could improve the diagnosis and the evaluation of endometriosis.
REFERENCES


