High serum follistatin levels in women with ovarian endometriosis

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BACKGROUND: Follistatin is an activin-binding protein produced by several tissues, including endometrium and endometriotic implants. We aimed to quantify follistatin in patients with ovarian endometriosis and investigate its value as a diagnostic marker.

METHODS: Women undergoing laparoscopic excision of ovarian endometrioma (n = 52) or other benign ovarian cysts (n = 52) were studied, plus women with non-ovarian endometriosis (n = 11) and healthy controls (n = 27). Serum was collected from all subjects, and peritoneal and cystic fluid from a subset with endometrioma. Follistatin was measured by enzyme-linked immunosorbent assay. The diagnostic accuracy of follistatin to detect endometrioma was evaluated by receiver operating characteristic (ROC) curve and compared with cancer antigen (CA)-125.

RESULTS: Serum follistatin was increased in women with ovarian endometrioma (2080 ± 94 pg/ml) compared with controls (545 ± 49 pg/ml, P < 0.001), other benign ovarian cysts (795 ± 60 pg/ml, P < 0.001) or non-ovarian endometriosis (1271 ± 115 pg/ml, P < 0.001). Cystic fluid showed a higher concentration of follistatin (9850 ± 4461 pg/ml) than peritoneal fluid (1885 ± 261 pg/ml, P < 0.001) and serum (P < 0.001). Follistatin levels detected 48/52 cases of endometrioma (92% sensitivity) at 1433 pg/ml cut-off, corresponding to 92% specificity. CA-125 detected only 44% of endometriomas with 90% specificity. ROC curve comparison showed follistatin was more accurate than CA-125 to discriminate women with endometrioma either from controls or women with other benign ovarian cysts (P < 0.0001).

CONCLUSIONS: Serum follistatin is increased in women with endometriosis and allows clear distinction between endometrioma and other benign ovarian cysts. Follistatin has the sensitivity and specificity to become a useful clinical marker of ovarian endometrioma.

Key words: follistatin / endometriosis / CA-125 / ovarian cyst / diagnostic accuracy

Introduction

Follistatin is a monomeric glycoprotein that binds to activin with high affinity and exerts most of its physiological effects via the neutralization of activin (de Winter et al., 1996; Kumar, 2005). Follistatin circulates in two major isofoms: a full-length molecule composed of 315 amino acids (FS315), and a short isoform of 288 amino acids (FS288) generated by alternative splicing of the Fst gene. Although first isolated from the follicular fluid, follistatin is produced by a number of non-ovarian sources, such as the pituitary gland, placenta and endometrium (Florio et al., 2003; Muttukrishna et al., 2004).

The activin/follistatin system is thought to act primarily as a local growth regulator system controlling proliferation, differentiation and apoptosis of many cell types in an autocrine and paracrine manner (Krnet et al., 2006). Of particular interest is the full expression of the activin/follistatin system in human endometrium (Jones et al., 2002b), a tissue with fast renewal and complex differentiation. Activin A stimulates the decidualization of endometrial stromal cells (Jones et al., 2002a) and aberrant expression of the activin/follistatin axis has been observed in the endometria of women with recurrent miscarriage (Prakash et al., 2006), anovulatory bleeding (Reis et al., 2007) and endometriosis (Rombauts et al., 2006).

Endometriosis is a gynecological condition in women of reproductive age, which primarily produces infertility and pain. It is defined as the presence of viable endometrial glands and/or stroma outside the uterine cavity—mainly on the pelvic peritoneum, the ovaries, the rectovaginal septum and more rarely at other sites. Endometriomas are invaginations of ovarian surface epithelium containing endometrial tissue, commonly referred to as ovarian cysts lined by endometrial tissue (Giudice and Kao, 2004).
This ectopic tissue produces both activin A and follistatin (Florio et al., 1998; Reis et al., 2001; Torres et al., 2007). Activin A does not appear to be overexpressed in endometriotic cells and remains unchanged in the peritoneal fluid (PF) and peripheral circulation of women with endometriosis (Florio et al., 1998; Reis et al., 2001). Follistatin, by contrast, is up-regulated in endometriotic implants compared with normal endometrium (Torres et al., 2007). This fact led us to hypothesize that follistatin might reach the circulation and reflect the presence of endometriosis, thus providing a new marker for endometriosis. Due to the limited diagnostic performance of cancer antigen (CA)-125, the carbohydrate antigen currently used to detect endometriosis (Mol et al., 1998; Somigliana et al., 2002), alternative serum markers with higher sensitivity, specificity and predictive value could help in the early detection and monitoring of disease progression as well as response to treatment (Gupta et al., 2006).

Thus, the aims of the present study were: (1) to quantify the concentration of follistatin and CA-125 in the serum of women with ovarian endometrioma and other benign cysts; (2) to evaluate the follistatin levels in the cystic content and PF of a subset of patients with ovarian endometriotic cyst; (3) to investigate the use of follistatin as a marker in the differential diagnosis of benign ovarian cysts.

**Subjects and Methods**

**Subjects**

The study evaluated four groups of subjects (Table I). Groups A and B comprised reproductive age women who underwent laparoscopic excision of benign ovarian cysts detected by ultrasound. Indications for laparoscopy followed our routine protocol, which includes: a persistent, large (≥5 cm) or complex pelvic mass without evidence of malignancy and/or pelvic pain not responding to medication. The subjects were enrolled prospectively between September 2004 and August 2006 at the University of Siena academic hospital. Group A included 52 consecutive patients diagnosed as having ovarian endometrioma, of whom 47 had only endometrioma and five had also small foci of peritoneal endometriosis. All patients were classified as stage III or IV according to the American Society for Reproductive Medicine revised classification of endometriosis (1997). Group B included consecutive patients with other benign ovarian cysts (n = 52), including serous (n = 17) and mucinous (n = 18) cystadenomas, dermoid cysts (n = 7) and hemorrhagic corpora lutea cysts (n = 10).

All patients were eumenorrheic and normoestrogenic and 90% of them reported regular menstrual cycles. A positive ultrasound test for endometrioma was computed when the examiner described a cyst or mass with thick walls, regular margins, and homogeneous low echogenicity of fluid (Eskenazi et al., 2001). Diagnosis was confirmed by histological examination of all cysts. All surgical interventions and sample collections were performed during the follicular phase of menstrual cycle.

In addition, we included a group of patients with stage I or II pelvic endometriosis without evidence of ovarian localization (non-ovarian endometriosis, n = 11, Group C). Finally, a normal control group (n = 27) comprised healthy women with regular menstrual cycles who requested intrauterine or surgical contraception (Group D). Pelvic examination and transvaginal ultrasound were performed prior to inclusion in the study and were all normal.

Informed consent was obtained from all subjects prior to inclusion in the study, which was approved by the local Human Investigation Committee. All subjects who had used steroid hormones during the past 3 months and those known to have pituitary, thyroid, renal, liver or adrenal disorders were not included in the study.

**Fluid sampling**

Blood samples were drawn from a peripheral vein immediately before anesthesia was administered, and were allowed to clot at room temperature. In a representative subset of patients with endometriosis (n = 15) with the same clinical and demographic characteristics as the whole endometriosis group, PF was obtained by aspiration of cul de sac fluid at the time of laparoscopy, although cystic fluid was collected by needle aspiration. None of the PF samples was contaminated with blood. All fluid samples were centrifuged at 400 × g for 10 min, and aliquots of the supernatants were stored at −20°C until follistatin assay.

**Follistatin assay**

Follistatin concentrations were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA). All samples were run in duplicate. Briefly, the assay diluent (100 μl), samples and standards (100 μl) were added to a 96-well antibody-coated plate, which was sealed and incubated for 3 h at 4°C. The plate was then washed with wash buffer, banged dry on paper towel, and incubated with horseradish peroxidase conjugated second antibody for a further 2 h at 4°C. After further washing, substrate solution (tetramethylbenzidine) was added for

| Table I Clinical and demographic data for women in the study |
|-------------------|-------------------|-------------------|-------------------|-------------------|
| Group | A—Endometrioma | B—Other cysts | C—Non-ovarian endometriosis | D—Control |
| N | 52 | 52 | 11 | 27 |
| Age (year) | 34 ± 6 | 32 ± 4 | 33 ± 5 | 33 ± 7 |
| Parity | 0 (0–2) | 0 (0–2) | 0 (0–2) | 0 (0–3) |
| BMI (kg/m²) | 24 ± 2 | 24 ± 3 | 24 ± 3 | 25 ± 4 |
| ASRM stage | III–IV | – | I–II | – |

ASRM: American Society for Reproductive Medicine.
Age and BMI are given as mean ± SE; parity is given as median and range.
30 min at room temperature and the reaction was stopped by adding 2 N sulfuric acid, then absorbance was read at 450 nm.

This assay uses a pair of monoclonal antibodies raised against recombinant human FS300 and detects both FS288 and FS315 with 95% recovery. However, when applied to serum samples it reflects essentially FS315 because this isoform accounts for >90% of the follistatin in circulation (Schneyer et al., 2004). The assay measures total (free + conjugated) serum follistatin and has a detection limit of 29 pg/ml, with a linear detection range from 250 to 16 000 pg/ml. There is no significant cross reactivity with α2-macroglobulin, inhibins, activins, or other members of the transforming growth factor beta superfamily. The intra- and inter-assay coefficients of variation are <3.0 and <9.0%, respectively.

CA-125 assay
CA-125 analysis was performed using the Cobas Core CA-125 enzyme-immunoassay analysis kit (Roche, Basel, Switzerland). The sensitivity of the assay was <1 U/l, and the intra- and inter-assay variations were <5.6 and 7.8%, respectively.

Statistical analysis
Data were tested for normality using the Kolmogorov–Smirnov test. Unless otherwise stated, data were normally distributed and therefore expressed as mean ± SD for reference values or mean ± SE for group comparisons. Differences between groups were tested by one-way analysis of variance (ANOVA). If a significant overall difference was found, the post hoc Newman–Keuls test was computed for multiple comparisons. Because CA-125 had a skewed distribution in the endometriosis group, the median is presented and ANOVA was carried out on log-transformed data, which had normal distribution. Statistical significance was set at \( P < 0.05 \).

Receiver operating characteristic (ROC) curves were obtained with their respective areas under the curves and 95% confidence intervals and compared by the method of Hanley and McNeil (1983), using the ‘Analyze-it’ software package for Microsoft Excel®. Sensitivity, specificity and likelihood ratios (LR) were calculated with 95% confidence intervals, using the best cut-off points indicated by the ROC curve analyses. In order to simulate typical clinical uses of the diagnostic tests, we evaluated the accuracy of the markers to distinguish cases of ovarian endometrioma from (i) other benign ovarian cysts and (ii) absence of ovarian disease. The first scenario applies to patients with a benign ovarian cyst detected by ultrasound, whereas the second one refers to the screening of asymptomatic women.

Results
As shown in Fig. 1, serum follistatin concentrations were increased in women with ovarian endometrioma (2080 ± 94 pg/ml) compared with controls (545 ± 49 pg/ml, \( P < 0.001 \)) and to those with other benign ovarian cysts (795 ± 60 pg/ml, \( P < 0.001 \)). The group with non-ovarian endometriosis had intermediate serum follistatin concentrations (1271 ± 115 pg/ml) which were lower than those found in women with endometrioma (\( P < 0.001 \)) and above the other groups (\( P < 0.01 \)). CA-125 levels were increased in the women with endometrioma (50 ± 6 U/l, median 36 U/l) compared with controls (23 ± 2 U/l, \( P < 0.01 \)) and other cysts (25 ± 3 U/l, \( P < 0.001 \)), but not in women with non-ovarian endometriosis (44 ± 15 U/l, median 28 U/l).

The cystic fluid of endometrioma showed a significantly higher concentration of follistatin \((9850 ± 4461 \text{ pg/ml})\) than the PF \((1885 ± 261 \text{ pg/ml}, P < 0.001)\) and serum \((P < 0.001, \text{Fig. 2})\).

As shown by the ROC curves in Fig. 3, follistatin was more accurate than CA-125 to discriminate women with endometrioma either from normal controls or from women with other benign ovarian cysts \((P < 0.01, \text{ANNOVA and Newman–Keuls test})\). CA, cancer antigen.


\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{Serum levels of follistatin (A) and CA-125 (B) in patients with endometrioma \((n = 52)\), non-ovarian endometriosis \((n = 11)\) and other benign ovarian cysts \((n = 52)\). The control group \((n = 27)\) is represented by a bar indicating mean ± SD. The dotted line indicates the 95th percentile of the control group. Significant differences were observed for both markers between women with endometrioma and the remaining groups \((P < 0.01, \text{ANNOVA and Newman–Keuls test})\).}
\end{figure}
52 and 44% of the cases of endometrioma with the cut-offs corresponding to <10% false positives in the normal controls and women with other ovarian cysts, respectively. Accordingly, the LR for negative test results was significantly lower with follistatin than with CA-125 (Table II). The ultrasound detection rate for endometriomas was 73% (95% confidence interval: 63–80%).

Figure 4 shows the scatter plot of follistatin and CA-125 in women with ovarian endometrioma and women with other benign ovarian cysts. There was no correlation between the two markers (Pearson’s $r^2 = 0.001$ and 0.030 for endometrioma and other cysts, respectively). CA-125 testing produced 76 negative results (left quadrants) of which 29 were false negatives (endometriomas with CA-125 concentrations lower than the cut-off level). Of those 29 patients misdiagnosed by CA-125 testing, 28 had high follistatin levels. In contrast, of 47 patients with non-endometriotic cysts that were correctly identified by low CA-125 levels, only four had false positive follistatin results (Fig. 4, upper left quadrant).

![Figure 2](image-url)  
**Figure 2** Follistatin concentrations in the serum, PF and cystic fluid of women with ovarian endometrioma ($n = 15$). Data are mean ± SE. *P < 0.001 versus serum and PF (ANOVA and Newman–Keuls test). PF, peritoneal fluid.

![Figure 3](image-url)  
**Figure 3** ROC curves of follistatin (black diamonds) and CA-125 (white squares) to the diagnosis of ovarian endometrioma versus no ovarian cyst (A) and versus other benign ovarian cysts (B).

**Table II** Sensitivity, specificity, LR and area under the ROC curve for serum follistatin and CA-125 in the diagnosis of ovarian endometrioma

<table>
<thead>
<tr>
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<th>Endometrioma versus no ovarian cyst</th>
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<th>Endometrioma versus other benign ovarian cysts</th>
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<tbody>
<tr>
<td></td>
<td>Follistatin</td>
<td>CA-125</td>
<td>Follistatin</td>
</tr>
<tr>
<td>Best cut-off point</td>
<td>1025 pg/ml</td>
<td>32 U/l</td>
<td>1433 pg/ml</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>100 (99–100)</td>
<td>52 (38–65)</td>
<td>92 (85–99)</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>96 (89–100)</td>
<td>96 (89–100)</td>
<td>92 (85–99)</td>
</tr>
<tr>
<td>LR of positive test*</td>
<td>25 (4–184)</td>
<td>14 (2–97)</td>
<td>12 (5–31)</td>
</tr>
<tr>
<td>LR of negative test*</td>
<td>0.00 (0.00–0.14)</td>
<td>0.50 (0.37–0.67)</td>
<td>0.08 (0.03–0.21)</td>
</tr>
<tr>
<td>Area under ROC curve</td>
<td>0.993 (0.978–1.000)</td>
<td>0.681 (0.565–0.797)</td>
<td>0.980 (0.960–1.000)</td>
</tr>
</tbody>
</table>

The 95% confidence intervals are given in parenthesis.

* $\text{Sensitivity}/(1-\text{specificity}).$

** $\text{(1-Sensitivity)}/\text{specificity}.$

LR, likelihood ratio; ROC, receiver operating characteristic; CA, cancer antigen.
Discussion

The present study is the first to measure serum follistatin concentration in patients with ovarian endometriosis. We found increased serum follistatin levels in women with endometrioma, but not in women with other types of benign ovarian cysts. Moreover, follistatin was shown to be a potential marker of endometrioma, with much higher sensitivity than the current marker CA-125.

The increased follistatin levels in women with endometrioma suggest that follistatin may be released into circulation by the endometriotic tissue. We have recently described the expression and localization of follistatin in ovarian endometriosis (Torres et al., 2007), and we observed that follistatin was homogenously distributed throughout the cytoplasm of the epithelial cells of endometriotic glands, with a stronger protein and mRNA expression in ovarian endometriosis than in healthy eutopic endometrium (Torres et al., 2007). In the present study, in the subset of patients with endometrioma, the higher concentration of follistatin in the cystic content than in PF serum was consistent with the previous data and suggests a local secretion of follistatin by the endometriotic cells, since transudation of external sources would not explain the much higher concentration of the hormone in the endometriotic cyst.

The possible role of follistatin in the pathogenesis of endometriosis is still uncertain, but some key events implicated in the development of the disease, such as endometrial differentiation, mesothelial invasion, immune system modulation and angiogenesis, are modulated by the activin/follistatin system. By antagonizing activin A, follistatin has been shown to inhibit stromal cell decidualization in vitro (Jones et al., 2002a). Elevated follistatin levels could therefore antagonize activin actions and putatively impair endometrial receptivity. However, women with endometriosis show a paradoxical increase of endometrial activin A production (Rombauts et al., 2006), which might compensate for any increased levels of follistatin in systemic circulation. Follistatin is thought to participate in many inflammatory disorders as part of a short-loop feedback system modulating and blocking the effects of activin A (Jones et al., 2004), and also to stimulate angiogenic responses both in vivo (McCarthy and Bicknell, 1993; Kozian et al., 1997) and in vitro (Kozian et al., 1997; Panopoulou et al., 2005). Although it is still premature to propose any therapeutic approach for endometriosis based on follistatin and related molecules, the existence of all types of activin receptor (Florio et al., 1998) makes it possible that endometriotic cells are actually modulated by this hormone system and that the local production and secretion of follistatin may influence the disease progression (Ferreira et al., 2008).

Compared with CA-125 levels in the same group of patients, follistatin demonstrated a much higher sensitivity in the detection of endometrioma, at cut-off points specific enough not only to avoid false positive results among healthy women, but also to distinguish individuals with endometrioma from those with other benign ovarian cysts. CA-125 has been the standard serum marker for endometriosis over the past two decades, despite its limited diagnostic performance. A meta-analysis of 23 studies involving 2866 patients has come to the conclusion that CA-125 detects only half of the cases of endometriosis stage III/IV with appropriate specificity (Mol et al., 1998). Other non-invasive methods, such as transvaginal ultrasound and pelvic examination, usually achieve high sensitivity for ovarian endometriosis (Eskenazi et al., 2001), although their false positive rate is still of concern. In our study, the detection rate of endometrioma with ultrasound was 73%, which is lower than expected, perhaps reflecting the characteristics of the study population.

Nonetheless, future investigation should evaluate the diagnostic accuracy of follistatin for non-ovarian endometriosis and early stage disease: these are conditions for which a serum marker is even more necessary, owing to the poor performance of non-invasive methods (Mol et al., 1998; Eskenazi et al., 2001). The preliminary data of the present study suggest that serum follistatin levels are modestly but significantly increased in stage I–II non-ovarian endometriosis, which makes follistatin a promising marker also for these conditions.

There are many practical aspects in favor of the use of follistatin to diagnose ovarian endometriosis. The hormone can be measured in serum after blood clotting at room temperature, and does not require sample extraction, radioactive methods, or complicated assay procedures. ELISA follistatin kits are commercially available and the results are reproducible, with acceptable coefficients of variation. In addition, follistatin levels remain stable across the menstrual cycle (Khoury et al., 1995), do not show significant diurnal variation (Foster et al., 2005), and are not affected by menopause (Khoury et al., 1995; Kettel et al., 1996). However, this apparent stability in physiological conditions does not exclude the possibility that some women with endometriosis have a specific rise of serum follistatin levels during the menstrual period, as observed for CA-125 (Abrão et al., 1997), and this remains to be investigated.

The test specificity might be affected by pathological conditions that elevate serum follistatin levels, such as septicemia (Michel et al., 2003), acute severe hepatitis and acute liver failure (Lin et al., 2006). A modest serum follistatin increase, within the normal range, has been observed in some post-menopausal women with epithelial ovarian cancer (Menon et al., 2000), and a small increase that does
not cross the cut-offs proposed in the present study for the detection of endometrioma has been found in women with polycystic ovary syndrome (Eldar-Geva et al., 2001).

In conclusion, serum follistatin levels are increased in women with ovarian endometriosis. The endometriotic tissue itself is a plausible source of the extra follistatin circulating in women with endometrioma, and follistatin seems to fulfill the requirements of sensitivity, specificity and reproducibility in order to become a useful clinical marker of late stage ovarian endometriosis. However, further studies, including a blind validation in a cohort series, will be required to establish the predictive value and to support the clinical use of follistatin in the diagnosis and/or screening of endometriosis.

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