Diagnostic value of serum activin A and follistatin levels in women with peritoneal, ovarian and deep infiltrating endometriosis

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BACKGROUND: Activin A is a growth factor, produced by the endometrium, whose actions are modulated by the binding protein follistatin. Both proteins are detectable in the peripheral serum and their concentrations may be increased in women with endometriosis. The present study was designed to evaluate whether serum levels of activin A and follistatin are altered, and therefore have a potential diagnostic value, in women with peritoneal, ovarian and deep infiltrating endometriosis.

METHODS: We performed a multicenter controlled study evaluating simultaneously serum activin A and follistatin concentrations in women with and without endometriosis. Women with endometriosis (n = 139) were subdivided into three groups: peritoneal endometriosis (n = 28); ovarian endometrioma (n = 61) and deep infiltrating endometriosis (n = 50). The control group (n = 75) consisted of healthy women with regular menstrual cycles. Blood samples were collected from a peripheral vein and assayed for activin A and follistatin using commercially available enzyme immunoassay kits.

RESULTS: The ovarian endometrioma group had serum activin A levels significantly higher than healthy controls (0.22 ± 0.01 ng/ml versus 0.17 ± 0.01 ng/ml, P < 0.01). None of the endometriosis groups had serum follistatin levels which were significantly altered compared with healthy controls; however, levels found in the endometrioma group (2.34 ± 0.32 ng/ml) were higher than that in the deep endometriosis group (1.50 ± 0.17 ng/ml, P < 0.05). The area under the receiver operating characteristic curve of activin A was 0.700 (95% confidence interval: 0.605–0.794), while that of follistatin was 0.620 (95% confidence interval: 0.510–0.730) for the diagnosis of ovarian endometrioma. The combination of both markers into a duo marker index did not improve significantly their diagnostic accuracy.

CONCLUSIONS: The present study demonstrated that serum activin A and follistatin are not significantly altered in peritoneal or deep infiltrating endometriosis and have limited diagnostic accuracy in the diagnosis of ovarian endometrioma.

Key words: endometriosis / endometrium / infertility / inhibins and activins

Introduction

Endometriosis is a relatively common, distressing, and some times disabling, disease defined by the presence of ectopic implants resembling the uterine mucosa both histologically and functionally. Because the endometriotic lesions host chronic inflammatory processes and may induce peritoneal adhesions and pelvic anatomy distortion (Bulun, 2009; Giudice, 2010), endometriosis is a major cause of pelvic pain and infertility. Nevertheless, its diagnosis is not easy, for the symptoms are inconclusive, and the standard diagnostic method is the histological examination of the lesions, which requires invasive access to the peritoneal cavity through laparoscopy or laparotomy (Bulun, 2009; Giudice, 2010; de Ziegler et al., 2010).

A valuable diagnostic approach to endometriosis is the use of biochemical serum markers, such as cytokines, hormones and growth factors. The rationale behind this is that increased circulating...
levels of such substances could result from the direct release by the endometriotic tissue into bloodstream and/or the inflammatory response associated with the disease leaving some biochemical fingerprint. Such diagnostic markers are needed especially for minimal and mild forms of endometriosis, which are not assessable to physical examination or to image techniques, and may not justify the surgical approach (Vercellini, et al., 2009a,b; Somigliana et al., 2010). Current biochemical methods, however, have suboptimal diagnostic accuracy for all forms of endometriosis.

Activin A is a member of the transforming growth factor (TGF) β superfamily and is produced by the healthy endometrium (Leung et al., 1998; Jones et al., 2000) and also by endometriosis (Reis et al., 2001). Activin A promotes the complex process of decidualization whereby the endometrium undergoes a process of transformation during each menstrual cycle in preparation for embryo implantation, a complex process named decidualization (Jones et al., 2002). Thus, activin A potentially allows uterine receptivity for the implanting embryo (Florio et al., 2003), and aberrant expression of activin A 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).

Follistatin and inhibins are naturally occurring activin antagonists. Follistatin is a single chain glycoprotein (Jeno et al., 1987) found in multiple tissues. Its production occurs in a coordinated way with activin A, and it is the major regulator of activin bioactivity. Follistatin binds to activin A with high affinity and blocks its interaction with the activin receptor ActRlI (De Winter et al., 1996). Follistatin also binds and modulates the actions of several other members of the TGF-β family, such as other activin isoforms, myostatin and certain bone morphogenetic proteins. Follistatin is abundantly expressed in human endometrium and endometriotic tissue at all phases of menstrual cycle (Florio et al., 2003, 2004; Torres et al., 2007).

Considering that activin A and follistatin genes are transcribed and their products are fully expressed by human endometrium and endometriosis, we have previously investigated whether their concentrations in biological fluids are altered in women with endometriosis. Activin A was measured in the peritoneal fluid and was not significantly altered (Florio et al., 1998), whereas no controlled study has assessed activin A levels in the peripheral serum of women with endometriosis. Follistatin was found to be consistently increased in the peritoneal fluid of women with ovarian endometriotic cysts, but only mildly increased in non-ovarian disease (Florio et al., 2009).

The present multicenter controlled study was designed to simultaneously measure serum activin A and follistatin concentrations in women with the three major forms of endometriosis: peritoneal, ovarian and deep infiltrating disease (Revised American Society for Reproductive Medicine, 1997; Chapron et al., 2003; Bulun, 2009). The study aims were (i) to evaluate whether average serum levels of activin A and follistatin are altered in women with each specific form of endometriosis and (ii) to investigate the diagnostic value of serum activin A and follistatin levels in women with peritoneal, ovarian and deep infiltrating endometriosis.

**Methods**

Subjects were enrolled prospectively for this study between March 2008 and May 2010 at the academic hospitals of Siena, Italy and Belo Horizonte, Brazil. In addition, retrospective cases were retrieved from the biological sample banks of the endometriosis units from academic medical centers in São Paulo, Brazil and Milan, Italy. Informed consent was obtained from all subjects prior to inclusion in the study, which was approved by the local Human Investigation Committees.

The study evaluated four groups of subjects (Table I). The control group (n = 75) consisted of healthy, asymptomatic women, with regular menstrual cycles and documented ovulation, who requested intrauterine or surgical contraception or underwent ICSI due to an isolated male factor. None of the subjects reported current or recent use of steroid hormones. Pelvic examination and transvaginal ultrasound were performed prior to inclusion in the study and all results were normal. Laparoscopy was not performed systematically, but when available, it showed normal results.

Women with endometriosis (n = 139) were subdivided into three groups according to laparoscopy results (Table I): peritoneal endometriosis (n = 28), defined by the presence of only superficial peritoneal foci of endometriosis; ovarian endometrioma (n = 61), defined by the presence of at least one ovarian cyst lined by endometriotic tissue (Giudice and Kao, 2004) regardless of the presence of superﬁcial peritoneal foci of endometriosis and deep infiltrating endometriosis (n = 50), defined by the presence of lesions in at least one of the following locations: (i) bladder, when lesions involved the bladder muscularis propria; (ii) uterosacral ligament; (iii) vagina, when lesions involved the anterior rectovaginal pouch, posterior vaginal fornix or retroperitoneal area between the anterior rectovaginal pouch and posterior vaginal fornix and (iv) intestine, when lesions involved the muscularis propria of the bowel (Chapron et al., 2003). Laparoscopies were performed by experienced surgical teams and diagnoses were confirmed by histological examination of biopsies from representative endometriotic lesions. All patients were eumenorrheic and normoestrogenic and 92% of them reported regular menstrual cycles. Those reporting current or recent (up to 6 months) use of steroid hormones or GnRH analogs by the time of blood sampling were not included in the present study.

**Blood sampling**

All blood samples, either prospective or from biological sample banks, were collected from a peripheral vein immediately before administering anesthesia for laparoscopy, and were allowed to clot at room temperature. Blood was centrifuged at 400g for 10 min at room temperature and the serum was separated with a disposable pipette, transferred to a cryoresistant tube and stored at −80°C for 3–84 months (average 26 months). The aliquots used in this study had never been thawed before.

**Activin A and follistatin assays**

Activin A and follistatin concentrations were measured using commercial quantitative sandwich enzyme immunoassay kits (R&D Systems, Minneapolis, MN, USA). All samples were handled blindly by one author (SL) who holds expertise in laboratory testing, and assays were run in duplicate. Briefly, the assay diluent (100μl), samples and standards (100μl) were added to a 96-well antibody-coated plate, which was sealed and incubated for 3 h at room temperature (activin A) or at 4°C (follistatin). The plate was then washed with wash buffer, blotted dry on paper toweling and incubated with horseradish peroxidase conjugated secondary antibody for a further 1 h at room temperature (activin A) or 2 h at 4°C (follistatin). After further washing, substrate solution (tetramethylbenzidine) was added for 30 min at room temperature and the reaction was stopped by adding 2N sulfuric acid, then absorbance was read at 450 nm. The activin A assay uses a pair of monoclonal antibodies raised against recombinant human activin A. The assay has a detection limit of 4 pg/ml with a linear detection range from 15 to 1000 pg/ml. There is no significant cross-reactivity with other members of the TGFβ superfamily. Assay...
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### Statistical analysis

Data distribution was analyzed using histograms, along with symmetry (g1) and kurtosis (g2) measures (Zar, 1996). The null hypothesis of population normality was further tested using the Kolmogorov–Smirnov goodness-of-fit procedure and the Shapiro–Wilk test. All procedures were performed in SPSS 10.1 (SPSS Inc., Chicago, IL, USA) and the results were re-checked in a table of critical values for symmetry and kurtosis measures (Zar, 1996). After confirming normal distribution, data 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. Statistical significance was set at $P < 0.05$.

Receiver operating characteristic (ROC) curves were obtained with 95% confidence intervals and compared by the method of Hanley and McNeil (1983), using the ‘Analyze-it’ software package for Microsoft Excel®. Briefly, the method consists of a non-parametric estimate of the area under the ROC curve for each diagnostic test, with the respective confidence interval, and comparison between paired ROC curves for the same subjects and outcomes, with statistical significance based on the $z$ test (Hanley et al., 1983). In addition, we selected the cut-off levels yielding 90% specificity and calculated their respective sensitivities and positive likelihood ratios, with 95% confidence intervals.

The study sample was sufficient to detect differences in the mean concentrations of serum analytes of at least 0.09 ng/ml (activin A) or 0.7 ng/ml (follistatin) with power (1 − β) = 0.90 and significance level (α) = 0.05. The sample size was also sufficient to estimate the diagnostic accuracy of these markers in the detection of each type of endometriosis, considering expected sensitivity/specificity of 0.80, minimal acceptable lower 95% confidence limit of 0.60 and power of 0.95 (Flahault et al., 2005).

### Results

Activin A was detectable in all samples analyzed. The concentration (mean ± SE) was 0.17 ± 0.01 ng/ml in the control group, 0.19 ± 0.01 ng/ml in the peritoneal endometriosis group, 0.22 ± 0.01 ng/ml in the ovarian endometrioma group and 0.16 ± 0.02 ng/ml in the deep endometriosis. As shown in Fig. 1A, the ovarian

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**Table I Clinical characteristics of the study groups.**

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 75)</th>
<th>Endometriosis: peritoneal (n = 28)</th>
<th>Endometriosis: ovarian endometrioma (n = 61)</th>
<th>Endometriosis: deep infiltrating (n = 50)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>33.8 ± 4.4</td>
<td>34.6 ± 5.0</td>
<td>35.0 ± 6.3</td>
<td>34.0 ± 5.6</td>
<td>0.678</td>
</tr>
<tr>
<td>Parity</td>
<td>1.5 ± 1.4</td>
<td>1.0 ± 1.5</td>
<td>0.7 ± 1.2</td>
<td>0.5 ± 1.0</td>
<td>0.704</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.4 ± 3.2</td>
<td>25.3 ± 1.0</td>
<td>22.2 ± 3.1</td>
<td>22.4 ± 3.3</td>
<td>0.396</td>
</tr>
<tr>
<td>ASRM score</td>
<td>3.7 ± 2.5</td>
<td>3.2 ± 2.5</td>
<td>75.4 ± 52.0</td>
<td>23.1 ± 25.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Stages I–II (%)</td>
<td>100</td>
<td>20</td>
<td>45</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Stages III–IV (%)</td>
<td>0</td>
<td>80</td>
<td>55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dysmenorrhea, VAS</td>
<td>6.4 ± 3.1</td>
<td>7.2 ± 2.7</td>
<td>8.9 ± 13.2</td>
<td>0.647</td>
<td></td>
</tr>
<tr>
<td>Acyclic pelvic pain, VAS</td>
<td>3.5 ± 4.1</td>
<td>4.1 ± 3.7</td>
<td>4.7 ± 4.1</td>
<td>0.545</td>
<td></td>
</tr>
<tr>
<td>Deep dyspareunia, VAS</td>
<td>3.3 ± 5.8</td>
<td>3.6 ± 3.6</td>
<td>3.4 ± 3.6</td>
<td>0.987</td>
<td></td>
</tr>
<tr>
<td>Histologic pattern (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glandular</td>
<td>29</td>
<td>50</td>
<td>0.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stromal</td>
<td>86</td>
<td>100</td>
<td>0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed</td>
<td>71</td>
<td>36</td>
<td>0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>57</td>
<td>61</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bowel symptoms (%)</td>
<td>24</td>
<td>38</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary symptoms (%)</td>
<td>0</td>
<td>19</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation.

VAS, visual analog scale; ASRM, American Society for Reproductive Medicine.

*Refers to the proportion of cases showing each histologic pattern. The sum exceeds 100% because the same case may have more than one histologic pattern (Kamergorodsky et al., 2009).
endometrioma group showed serum activin A levels significantly higher than healthy controls. Follistatin was also detectable in all samples analyzed. The concentration was 1.69 ± 0.07 ng/ml in the control group, 2.24 ± 0.42 ng/ml in the peritoneal endometriosis group, 2.34 ± 0.32 ng/ml in the ovarian endometrioma group and 1.50 ± 0.17 ng/ml in the deep endometriosis group. As shown in Fig. 1B, none of the endometriosis groups had serum follistatin levels significantly different from healthy controls, but there were differences between the endometriosis groups, with higher follistatin levels in the peritoneal and ovarian endometriosis groups compared with the deep endometriosis group.

When the two markers were combined to create a duo marker index, it resulted in significantly increased levels in the ovarian endometrioma group compared with the healthy control group and compared with the deep endometriosis group (P < 0.01). In the peritoneal endometriosis group, the duo marker index was similar to the ovarian endometrioma group but did not allow clear distinction from the healthy controls (Fig. 1C).

Figure 1D shows the ROC curves of activin A, follistatin and the activin A × follistatin product in women with peritoneal endometriosis, ovarian endometrioma and deep infiltrating endometriosis, compared with a control group without endometriosis. Different letters indicate statistically significant differences between groups (ANOVA and Newman–Keuls test).

**Table II** Sensitivity and positive likelihood ratios corresponding to 90% specificity in the detection of ovarian endometrioma with serum activin A, follistatin or both.

<table>
<thead>
<tr>
<th>Marker</th>
<th>90% Specificity cut-off</th>
<th>Sensitivity (95% CI)</th>
<th>Positive likelihood ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activin A</td>
<td>&gt;0.25 ng/ml</td>
<td>0.33 (0.21–0.48)</td>
<td>2.78 (1.34–5.74)</td>
</tr>
<tr>
<td>Follistatin</td>
<td>&gt;2.4 ng/ml</td>
<td>0.37 (0.23–0.52)</td>
<td>3.94 (1.78–8.72)</td>
</tr>
<tr>
<td>Duo marker (activin A × follistatin product)</td>
<td>&gt;0.45 (ng/ml)²</td>
<td>0.41 (0.28–0.56)</td>
<td>4.41 (2.03–9.60)</td>
</tr>
</tbody>
</table>

CI, confidence interval.

Further analysis showed that serum activin A and follistatin concentrations did not correlate with menstrual cycle day, endometriotic cyst diameter, American Society for Reproductive Medicine (ASRM) classification score, age, smoking, symptoms, histological pattern or pain scores in a visual analog scale (data not shown).
Discussion

The discovery of new biomarkers remains a priority in endometriosis research (Rogers et al., 2009). Due to the limited sensitivity and specificity of clinical symptoms (Djalali et al., 2009; Carneiro et al., 2010), the use of non-invasive laboratory methods is paramount to optimize the diagnostic workup while sparing unnecessary laparoscopies. Thus far, cancer antigen (CA)-125 remains the only serum marker of endometriosis widely used in clinical practice, despite its low sensitivity (Duleba, 1997; Mihalyi et al., 2010; Patrelli et al., 2011). Even the combination of CA-125 with multiple circulating markers, such as anticardiolipin antibody, serum amyloid A, interleukins 6 and 8, tumor necrosis factor-alpha, CA-19-9 and high-sensitivity C-reactive protein has not achieved the desired accuracy, because none of these markers are unique to endometriosis (Abrão et al., 1997; Mihalyi et al., 2010).

Activin A and follistatin were investigated in the present study as potential diagnostic markers for peritoneal, ovarian and deep infiltrating endometriosis. These proteins were chosen because they are produced and released by the human endometrium and by endometriotic implants (Florio et al., 1998; Reis et al., 2001; Torres et al., 2007) and can be quantified in the peripheral serum with reliable and affordable methods. In addition, in a preliminary study we had observed increased serum concentrations of follistatin in women with ovarian endometrioma (Florio et al., 2009).

The present study did not reproduce our previous findings of increased serum follistatin levels in women with endometrioma (Florio et al., 2009), and this lack of replication is probably explained by the intrinsic limitations of any statistical inference from population samples. Actually, the present results show only a modest increase in activin A in ovarian endometrioma and a small difference in follistatin concentrations between women with deep infiltrating endometriosis and women with the other forms of endometriosis. Endometriosis is heterogeneous in its presentation and the disease type is a determinant of the production and release of biomarkers (May et al., 2010). A typical example is CA-125, which is much higher in women with endometrioma (Kitawaki et al., 2005) or dense pelvic adhesions (Cheng et al., 2002) compared with other forms of endometriosis. Activin A expression by endometriomas is increased compared with that of healthy proliferative endometrium (Rocha et al., 2011), but there are no comparative studies to clarify whether the local production and release of activin-related proteins changes according to the type of endometriotic lesion. Interestingly, deep infiltrating endometriotic lesions are often undifferentiated (Kamergorodsky et al., 2009), which could affect the local production of activin A and follistatin, resembling which has been observed in the eutopic endometrium of women with dysfunctional bleeding (Reis et al., 2007).

Activin A and follistatin did not achieve an optimal combination of sensitivity and specificity at any cut-off point, and the combination of both markers did not increase the diagnostic performance of either marker alone, as shown by the ROC curve analysis. These findings point to a large variation in biomarker levels among women with apparently the same disease: while a significant number of patients have higher serum levels of activin A and follistatin, which cannot be explained by chance as they differ significantly from controls, another significant number of endometriotic patients do not have any elevation of either marker.

Activin A is highly concentrated in the cystic fluid of ovarian endometriomas (Reis et al., 2001), and is overexpressed in the proliferative phase endometrium of women with endometriosis, but again this difference is not constant (Rocha et al., 2011). Therefore, the low sensitivity of these proteins as diagnostic markers most likely derives from a variable tissue expression which, in turn, reflects the multiple phenotypic presentations of endometriotic lesions. As observed in a systematic review, low sensitivity has been a key obstacle to the accuracy of peripher-}

Authors’ roles

F.M.R. contributed to study design, data analysis and interpretation, and manuscript writing. S.L., M.S.A., P.V. and P.F. contributed to study design, data acquisition and interpretation, and critical revision and approval of the article. A.L.L.R. and C.P.R. contributed to patient selection, laboratory testing, data analysis and final draft approval. F.P. contributed to study conception and design, data analysis and interpretation, and critical revision and approval of the article.

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Conflict of interest

None declared.

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