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# Evaluation of a panel of 28 biomarkers for the non-invasive diagnosis of endometriosis

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**BACKGROUND:** At present, the only way to conclusively diagnose endometriosis is laparoscopic inspection, preferably with histological confirmation. This contributes to the delay in the diagnosis of endometriosis which is 6–11 years. So far non-invasive diagnostic approaches such as ultrasound (US), MRI or blood tests do not have sufficient diagnostic power. Our aim was to develop and validate a non-invasive diagnostic test with a high sensitivity (80% or more) for symptomatic endometriosis patients, without US evidence of endometriosis, since this is the group most in need of a non-invasive test.

**METHODS:** A total of 28 inflammatory and non-inflammatory plasma biomarkers were measured in 353 EDTA plasma samples collected at surgery from 121 controls without endometriosis at laparoscopy and from 232 women with endometriosis (minimal–mild $n = 148$; moderate–severe $n = 84$), including 175 women without preoperative US evidence of endometriosis. Surgery was done during menstrual ($n = 83$), follicular ($n = 135$) and luteal ($n = 135$) phases of the menstrual cycle. For analysis, the data were randomly divided into an independent training ($n = 235$) and a test ($n = 118$) data set. Statistical analysis was done using univariate and multivariate (logistic regression and least squares support vector machines (LS-SVM) approaches in training- and test data set separately to validate our findings.

**RESULTS:** In the training set, two models of four biomarkers (Model 1: annexin V, VEGF, CA-125 and glycodelin; Model 2: annexin V, VEGF, CA-125 and sICAM-1) analysed in plasma, obtained during the menstrual phase, could predict US-negative endometriosis with a high sensitivity (81–90%) and an acceptable specificity (68–81%). The same two models predicted US-negative endometriosis in the independent validation test set with a high sensitivity (82%) and an acceptable specificity (63–75%).

**CONCLUSIONS:** In plasma samples obtained during menstruation, multivariate analysis of four biomarkers (annexin V, VEGF, CA-125 and sICAM-1/or glycodelin) enabled the diagnosis of endometriosis undetectable by US with a sensitivity of 81–90% and a specificity of 63–81% in independent training- and test data set. The next step is to apply these models for preoperative prediction of endometriosis in an independent set of patients with infertility and/or pain without US evidence of endometriosis, scheduled for laparoscopy.

**Key words:** endometriosis / non-invasive diagnosis / plasma biomarkers / multiplex immunoassay

# Introduction

Endometriosis is defined as the presence of endometrial-like tissue outside the uterine cavity and associated with a constellation of symptoms, including chronic pelvic pain, dysmenorrhoea, dyspareunia, dyschezia and subfertility (Sinaii et al., 2008; Falcone and Lebovic, 2011). Endometriosis affects 6–10% of women of reproductive age in the general population; however, its prevalence is 35–50% in women with pain, infertility or both (Snesky and Liu, 1980; Houston, 1984; Cramer, 1987; Giudice and Kao, 2004). Endometriosis can appear as peritoneal lesions, ovarian endometriotic cysts and deeply infiltrative endometriosis (DIE; Nisolle and Donnez, 1997) and can be classified into four stages: minimal, mild, moderate and severe (ASRM, 1997).

Endometriosis does not always provide a visible handicap, despite its often crippling effects, and thus is not widely and sufficiently recognized by the general public, many general practitioners and some gynaecologists. As reported by a survey completed by 7025 women with endometriosis, 65% of the women with endometriosis were misdiagnosed with another condition, and 46% had to see five doctors or more before they were correctly diagnosed (European Endometriosis Alliance, 2006; Mihalyi et al., 2010).

At present, the only way to conclusively diagnose endometriosis is through laparoscopic inspection, preferably with histological confirmation (Kennedy et al., 2005), which explains the diagnostic delay of endometriosis (between the onset of symptoms and a diagnosis) of 6–11 years (Hadfield et al., 1996; Husby et al., 2003; Nnoaham et al., 2011).

So far, it has not been possible to predict the presence of endometriosis based on symptoms, clinical examination, imaging techniques or blood tests, as outlined below.

Firstly, the diagnosis of endometriosis based on symptoms is unreliable.

Although the association between endometriosis and symptoms such as dysmenorrhoea, non-menstrual pelvic pain, dyspareunia and infertility is widely accepted (Fauconnier and Chapron, 2005; Ballard et al., 2008; Sinaii et al., 2008; Falcone and Lebovic, 2011), the predictive value of these symptoms for the diagnosis of endometriosis is limited (Eskenazi et al., 2001; Chapron et al., 2005; Meuleman et al., 2009). Moreover, there is no correlation between severity of endometriosis (rAFS; American Fertility Society Classification) and the type or severity of pain symptoms (Kennedy et al., 2005).

Secondly, routine vaginal examination alone may be insufficient to detect endometriosis prior to laparoscopy (Hudelist et al., 2011), as in many women with endometriosis no abnormality is detected during clinical examination (D'Hooghe and Hill, 2006).

Thirdly, transvaginal ultrasound (TVU) is an adequate diagnostic method to detect ovarian endometriotic cysts, but does not rule out peritoneal endometriosis, endometriosis-associated adhesions (Moore et al., 2002; Kennedy et al., 2005) and some locations of DIE (Dessole et al., 2003; Bazot et al., 2004; Bazot et al., 2009).

Fourthly, no blood tests exist for the diagnosis of endometriosis (Kennedy et al., 2005). Although CA-125, cytokines, angiogenic and growth factors are differentially expressed in the peripheral blood of women with endometriosis when compared with controls (reviewed by Othman et al., 2008; May et al., 2010), so far neither a single biomarker nor a panel of biomarkers has been validated as a non-invasive test for endometriosis (May et al., 2010), possibly because most studies included limited numbers of patients and limited assessment of different cycle phases and endometriosis stages (May et al., 2010). Studies evaluating a panel of biomarkers (Gagne et al., 2003; Somigliana et al., 2004; Agic et al., 2008; Seeber et al., 2008; Mihalyi et al., 2010) are also limited with respect to the number of biomarkers analysed, the statistics used (univariate statistical analysis) and the lack of validation in an independent test set of patients.

In a clinical practice dealing with women with subfertility with or without pain, a non-invasive test of endometriosis with a high sensitivity would allow the identification of those women with endometriosis who could benefit from laparoscopic surgery reported to improve these symptoms, i.e. increase fertility and decrease pain (Kennedy et al., 2005; D'Hooghe et al., 2006). As endometriosis can be progressive in up to 50% of women (D'Hooghe and Debrock, 2002), early non-invasive diagnosis has the potential to offer early treatment and prevent progression. Ideally, decreased levels of such a test during/after treatment would also correlate with decreased pelvic pain and increased fertility. Such a test would be useful to women especially with endometriosis which is not diagnosed by TVU. The current study was done according to the QUADAS (Quality Assessment of Diagnostic Accuracy Studies) criteria (Whiting et al., 2003; May et al., 2010) with multivariate analysis of 28 different biomarkers in biobanked plasma samples from a large cohort ($n = 353$) of well phenotyped women with subfertility and/or pain to develop (based on the training data set) and validate (based on the independent test set) a non-invasive diagnostic test with a high sensitivity (80% or more).

# Materials and Methods

## Selection of plasma samples from the LUFC endometriosis research biobank

Since 1999, a biobank has been developed based on the collection and storage of plasma samples after signed informed consent from women undergoing laparoscopic surgery at the Leuven University Fertility Center (LUFC). For each patient, detailed clinical information is available in the electronic file of the patient, including age, cycle phase at surgery, detailed surgery report with scoring and staging according to the classification of the ASRM (1997), medication use, data of preoperative ultrasound (US). All patients had signed a written informed consent and the study protocol was approved by the Commission for Medical Ethics of the Leuven University Hospital Belgium.

The electronic biobank database of the LUFC was searched for all plasma samples that had both the necessary minimal volume (2.5 ml) combined with the following essential clinical information of the patient at the time of sample collection (age, indication for surgery (infertility and/or pain), stage and score of endometriosis (ASRM, 1997) and menstrual cycle phase. Plasma samples from patients using hormonal medication (combined oral contraceptive pill or progestins or GnRH analogues) and from patients operated within 6 months prior to the time of sample collection were excluded.

The first comparison was of controls (endometriosis was excluded laparoscopically by an experienced endometriosis surgeon), versus all stages of endometriosis. Endometriosis patients were then divided into three groups, minimal–mild endometriosis, moderate–severe endometriosis and US-negative endometriosis. Histological confirmation of endometriosis was available for the majority (202/232, 87.1%) of the endometriosis

patients included in our study. A total of 353 plasma samples met our inclusion criteria and were randomly divided into an independent training- and test set, with equal distribution of controls (34%) and endometriosis (66%) patients in both data sets using a stratified random sample selection step. The training set population included samples from 235 patients (80 controls and 155 endometriosis (102 *minimal–mild endometriosis*; 53 *moderate–severe endometriosis*). The independent test set population included samples from 118 patients (41 controls and 77 endometriosis (46 *minimal–mild endometriosis*; 31 *moderate–severe endometriosis*). Training- and test set plasma samples were collected during the menstrual ($n = 57$; $n = 26$), luteal ($n = 92$; $n = 43$) and follicular ($n = 86$; $n = 49$) phases of the cycle, respectively.

As a non-invasive diagnostic test would be especially useful in women with endometriosis which is not diagnosed by TVU, as mentioned in the section Introduction, a subset analysis was done on samples collected from the 175 women with laparoscopically confirmed endometriosis without evidence of endometriosis on a preoperative gynaecological US. For this subset, the training set population included 117 US-negative endometriosis patients (99 *minimal–mild endometriosis*, 18 *moderate–severe endometriosis*) and 81 controls. The independent test set included 58 US-negative endometriosis patients (47 *minimal–mild endometriosis*, 11 *moderate–severe endometriosis*) and 40 controls. For this subset analysis, both training- and test set plasma samples were collected during the menstrual ($n = 40$; $n = 27$), luteal ($n = 78$; $n = 33$) and follicular ($n = 80$; $n = 38$) phases of the cycle, respectively.

Plasma samples had been collected at the time of surgery (according to the standard operation procedure) in EDTA tubes, centrifuged at 3000 rpm for 10 min at 4°C, aliquoted, labelled and stored at −80°C till analysis. The time interval between sample collection and storage in the −80°C freezer was maximum 1 h.

## Selection and measurement of target biomarkers

After an extensive literature search, 28 plasma biomarkers were selected based on their potential role in the pathogenesis of endometriosis, differential expression in endometriosis patients compared with controls (reviewed by May *et al.*, 2010) and commercial availability of the assays. Table I shows the complete list of biomarkers analysed in this study according to their biological function (glycoproteins, inflammatory and non-inflammatory markers, adhesion molecules, angiogenic and growth factors).

The following multiplex and single immunoassay technologies were used: Bio-Plex Protein Array System (Bio-Rad Laboratories, Hercules, CA, USA) was used for the measurement of IL-1beta, IL-4; IL-6; IL-8; IL-10, IL-17, TNF-alpha, RANTES, NGF, b-FGF, IFN-gamma, MIF, MCP-1, VCAM, VEGF, M-CSF, HGF. Multiplexing sandwich-ELISA system of Aushon Biosystems Search Light Assay Services (Woburn, USA) was used for the measurement of osteopontin, IGFBP-3 and leptin. Single ELISAs were used for the measurement of sICAM-1 and follistatin (R&D Systems, Minneapolis, USA), annexin V (American Diagnostica, Inc., Stamford, USA), IL-21 (Bender Med Systems, Vienna, Austria) and glycodelin (Bioserv Diagnostics, Rostock, Germany). Plasma concentrations of CA-125, CA-19-9 and hsCRP were measured by automated immunoassays (Roche, Vilvoorde, Belgium).

Since the commercially available glycodelin ELISA kit (Bioserv Diagnostics, Rostock, Germany) has been validated only in serum samples, an additional analytical validation step on plasma was performed to validate the use of the glycodelin ELISA kit in plasma. An intra-assay variation was between 12.6 and 15.3%. The inter-assay coefficient of variation was between 6.8 and 18.8%. The recovery range of 10 samples in the spike-recovery experiment was between 82 and 120%. The glycodelin ELISA (Bioserv Diagnostics, Rostock, Germany) showed a good linearity [a slope of 0.96 and a Spearman correlation coefficient of 0.92 ($P = 0.0013$)] between the observed and expected levels of glycodelin in plasma. The data of analytical validation of glycodelin ELISA kit (Bioserv Diagnostics, Rostock, Germany) on EDTA plasma showed that the assay is accurate for EDTA plasma.

In an additional methodology study (Vodolazkaia *et al.*, 2011) we confirmed that the hsCRP assay was superior to the classical CRP assay for the detection of low CRP levels (indicating subclinical inflammation in the plasma of endometriosis patients) and for the diagnosis of moderate–severe endometriosis. The hsCRP assay was used for the measurement of CRP in the entire study population.

**Table I Complete list of analysed biomarkers.**

| Biological groups | Biomarkers | Study |
|---|---|---|
| Glycoprotein markers | CA-125, CA 19–9; follistatin | Mol *et al.* (1998); Matalliotakis *et al.* (1998); Agic *et al.* (2008); Kurdoglu *et al.* (2009); Florio *et al.*, 2009) |
| Inflammatory markers | IL-1beta, IL-6, IL-8, IL-17, IL-21, RANTES, TNF-alpha, IFN-gamma, MCP-1, MIF, CRP, OPN | Pizzo *et al.* (2002); Mihalyi *et al.* (2010); Zhang *et al.* (2005); Ouyang *et al.* (2008); Khorram *et al.* (1993); Abrao *et al.* (1997); Morin *et al.* (2005); Cho *et al.* (2009) |
| Non-inflammatory markers | IL-4, IL-10, annexin V | Antsiferova *et al.* (2005), Kyama *et al.* (2011) |
| Adhesion molecules | sICAM-1, VCAM-1 | Barrier and Sharpe-Timms (2002) |
| Angiogenic and growth factors | VEGF, NGF, FGF-2, Leptin, IGFBP-3, glycodelin (PP-14), M-CSF, HGF | Telimaa *et al.* (1989); Matalliotakis *et al.* (2003); Tokushige *et al.* (2006a,b); Bourlev *et al.* (2006); Kim *et al.* (2000); Zong *et al.* (2003) |

## Statistical analysis

As mentioned above, all samples were randomly divided into a training set (70%) and in a test set (30%), and data were analysed separately for each set using univariate and multivariate statistical analyses. Undetectable amounts of a target molecule measured were considered to be one-half the limit of quantification for statistical analysis. IL-4, NGF-beta and M-CSF were not detectable in >90% of the samples and have been excluded from the statistical analysis.

### *Univariate statistical analysis*

Data are presented as median and interquartile range. A $P$-value of $<0.05$ was considered statistically significant. Differences in biomarkers levels were evaluated using the Mann–Whitney test and the Kruskal–Wallis test with *post hoc* Dunn analysis in the training- and the test data set separately.

A receiver operating characteristic (ROC) curve analysis was performed to determine the diagnostic performance of each biomarker separately. The optimal cut-offs levels resulting in the highest sensitivity at the acceptable specificity (>50%) in the training set were validated on the independent test set.

In our study, the area under the ROC curve (AUC) was calculated and evaluated based on previously published guidelines (Akobeng, 2007; Bossuyt, 2009). The clinical value of a laboratory test with AUC values between 0 and 0.5, 0.5–0.7, 0.7–0.9 or >0.9 can be defined as zero, limited, moderate and high, respectively (Bossuyt, 2009). Taking into account our clinical perspective on the requirements for a diagnostic test for endometriosis, as explained in the section Introduction and published before (D'Hooghe *et al.*, 2006), our data analysis focused on the need for a diagnostic test with a high sensitivity (>80%) and an acceptable specificity (>50%).

*Multivariate statistical analysis*

Multivariate analysis was carried out to identify whether a panel of biomarkers could increase the sensitivity and specificity of the non-invasive test for endometriosis when compared with univariate analysis. We implemented and applied univariate and multivariate biomarker selection methods and used the selected biomarkers in the multivariate classification to assess their performances. Two fundamentally different classifiers—multivariate logistic regression and the least squares support vector machines (LS-SVM)—were used, as published before (Mihalyi *et al.*, 2010). When compared with multivariate logistic regression, LS-SVM is less sensitive to the influence of irrelevant features as it has an internal mechanism to minimize their effect. An agreement between these two classifiers strongly indicates robustness of the selected biomarker panel (Pochet and Suykens, 2006).

## Selection of biomarkers based on the training data set

Three biomarker selection methods were used to obtain the most accurate biomarker panel. For both univariate and multivariate biomarker selection, bootstraps (70% of the training data set, in a stratified manner) were repeatedly thrown out from the training data set within the loop for 500 times, randomizing the whole training set before every iteration (François *et al.*, 2007). In each run, the biomarkers selection method has been applied on bootstrap sample to collect corresponding statistics, with only the biomarkers significant across repetitions being kept.

When the univariate biomarker selection scheme was applied, only biomarkers that were significant according to the Mann–Whitney test in 70% and more randomizations were selected (univariate approach; Supplementary data, Tables SI and SII).

When the multivariate biomarker selection scheme was applied, two approaches based on multivariate stepwise logistic regression with Akaike information criteria were used to account for possible correlation between biomarkers. The Akaike information criteria were chosen due to the robustness for the prediction (Agresti, 2002). In the first approach, only the biomarkers with high frequency of appearance in regression models in all runs (70% and more randomizsations) were considered for feeding the classification step (Multivariate approach 1; Supplementary data, Tables SI and SII).

In the second approach, all of multivariate logistic regression models containing the most frequent biomarkers as determined in the first approach have been selected [Multivariate approach 2; Table VI, Supplementary data, Tables SI and SII)]. After this, all biomarkers figuring in the best among these models were considered informative.

## Classification and validation

Using the biomarkers selected in the previous step, we applied two classification algorithms (multivariate logistic regression and LS-SVM) on the independent training- and test set separately to estimate several measures of performance—accuracy, area under the ROC curve, sensitivity, specificity, positive (PPV) and negative predictive values (NPV), positive and negative likelihood ratio (LR) and diagnostic odds ratio (DOR).

# Results

## Clinical characteristics of study population

The characteristics of the controls and the endometriosis patients are shown in Table II.

## Univariate analysis

The training and test data set were analysed separately, firstly regardless of the cycle phase then secondly according to menstrual cycle phase (menstrual, follicular or luteal). Thirdly, we analysed the performance of single biomarkers for the diagnosis of US-negative endometriosis.

*All endometriosis versus controls*

*Analysed with all menstrual cycle phases combined*. In the training data set, the plasma levels of IGFBP-3, CA-125, CA 19-9 and glycodelin were significantly higher, whereas the plasma levels of IL-1beta, IFN-γ, TNF-alpha, Leptin and sICAM-1 were decreased in women with endometriosis compared with controls. Significantly elevated levels of CA-125, glycodelin, and significantly decreased levels of Leptin in women with endometriosis compared with controls were also observed in the independent test set (Table III).

*Analysed according to menstrual cycle phase*. During the menstrual phase, increased plasma levels of CA-125 and glycodelin were detected in the training data set in women with endometriosis compared with controls. CA-125 was also significantly increased in the independent test set (Table III). During the follicular phase, plasma levels of IGFBP-3, IL-21, CA-125 and glycodelin were significantly higher in women with endometriosis compared with controls in the training data set. Significantly elevated levels of CA-125 (during follicular and luteal phases) and glycodelin (during follicular phase) in women with endometriosis compared with controls were confirmed in the independent test set (Table III).

*US-negative endometriosis versus controls*

*Analysed with all menstrual cycle phases combined*. In the training data set, the plasma levels of VEGF, IGFBP-3, CA-125, CA 19-9 and glycodelin were significantly higher, whereas the plasma levels of sICAM-1 were decreased in women with US-negative endometriosis compared with controls. In the test data set, CA-125 and glycodelin levels were also significantly higher in women with US-negative endometriosis than in controls (Table IV).

*Analysed according to menstrual cycle phase*. In the training data set, increased plasma levels of IGFBP-3, CA-125, glycodelin (during the follicular cycle phase), CA-125 and CA 19-9 (during the luteal cycle phase) were detected in women with US-negative endometriosis

**Table II Clinical characteristics of study population.**

| | Controls | Endometriosis patients | |
|---|---|---|---|
| | | All | US negative |
| Numbers | 121 | 232 | 175 |
| Age (years) | | | |
| Mean (SD) | 31.7 (5.28) | 31.2 (4.02) | 31.2 (4.11) |
| Median (range) | 32 (19–46) | 31 (24–44) | 31 (24–44) |
| Symptoms | | | |
| Subfertility (*n*) | 117 (76/41) | 213 (142/71) | 159 (108/51) |
| Dysmenorrhoea (*n*) | 61 (39/22) | 148 (98/50) | 103 (73/30) |
| Dyspareunia (*n*) | 17 (8/9) | 66 (42/24) | 46 (34/12) |
| Chronic pelvic pain (*n*) | 10 (7/3) | 20 (12/8) | 14 (7/7) |
| Dyschezia (*n*) | 5 (3/2) | 20 (14/6) | 6 (4/2) |
| Minimal–mild endometriosis (*n*) | | 148 (102/46) | 146 (99/47) |
| Moderate–severe endometriosis (*n*) | | 84 (53/31) | 29 (18/11) |
| Cycle phase | | | |
| Menstrual (*n*) | 27 (19/8) | 56 (38/18) | 40 (29/11) |
| Follicular (*n*) | 46 (29/17) | 89 (57/32) | 72 (44/28) |
| Luteal (*n*) | 48 (32/16) | 87 (60/27) | 63 (44/19) |
| Cycle information | | | |
| Regular cycle (*n*) | 89 (57/32) | 173 (117/56) | 126 (90/36) |
| Irregular cycle (*n*) | 24 (16/8) | 28 (17/11) | 24 (12/12) |
| Missing data (*n*) | 8 (5/3) | 31 (21/10) | 25 (15/10) |
| Other pelvic pathology | | | |
| Adhesions without endometriosis (*n*) | 37(28/9) | | |
| Post-operative (*n*) | 15 (12/3) | | |
| Post PID (*n*) | 9 (6/3) | | |
| Unknown aetiology (*n*) | 13 (10/3) | | |
| Myoma (*n*) | 12 (8/4) | 7 (6/1) | 6 (3/3) |
| Parasalpingeal cyst (*n*) | 19 (13/6) | 21 (14/7) | 21 (10/11) |
| Hydrosalpinx (*n*) | 9 (6/3) | 4 (3/1) | |

Data are total (training/test).

compared with controls. In the test data set, higher plasma CA-125 levels (during the follicular phase) were observed in women with US-negative endometriosis than in controls. In the training set, plasma levels of IL-1beta, IL-6, IFN-γ, TNF-alpha (during the follicular cycle phase) and sICAM-1 (during the menstrual cycle phase) were lower in women with US-negative endometriosis than in controls, but these data were not confirmed in the test set (Table IV).

*Diagnostic performance of single biomarkers for the diagnosis of US-negative endometriosis (Table V)*. Univariate ROC curve analysis was performed for the significantly different biomarkers in the training set to identify the discriminative power of each biomarker separately. Optimal cut-off levels for each of these biomarkers, resulting in the highest sensitivity at acceptable specificity (>50%; D'Hooghe *et al.*, 2006) in the training set, were subsequently validated on the independent test set (Table V).

In the training set, the best discriminative ability for the diagnosis of US-negative endometriosis, based on the highest AUC (area under the ROC curve) and highest sensitivity at a specificity of at least >50%, was obtained using sICAM-1 (during the menstrual phase), glycodelin (during the follicular phase) and CA-125 (during the follicular phase and independently of the cycle phase; Table V). In the independent test set, these results were only validated for CA-125 (during the follicular phase; Table V). At a cut-off plasma level of CA-125 >11.5 U/ml (during the follicular phase), US-negative endometriosis was diagnosed with a sensitivity of 76% and a specificity of 60% in the training set, and with a sensitivity of 86% and a specificity of 63% in the test set (Table V).

## Multivariate analysis

### *Multivariate analysis: all data*

Supplementary data, Tables SI and SII show selected models based on multivariate logistic regression and LS-SVM analysis (respectively) of all data, presented separately for training- and test set. Overall, the best results were obtained in the menstrual phase (for *minimal–severe*

**Table III** Levels of plasma biomarkers for endometriosis (all stages) versus controls.

| Biomarker | Phase of cycle | Training set | | | Test set | | |
|---|---|---|---|---|---|---|---|
| | | Controls | Endometriosis | *P** value | Controls | Endometriosis | *P** value |
| IL-1beta (pg/ml) | All | 0.78 (0.53–1.03) | 0.66 (0.4–0.9) | 0.045 | 0.43 (0.31–0.93) | 0.65 (0.41–0.94) | NS |
| IL-8 (pg/ml) | All | 3.0 (2.13–3.7) | 3.0 (2.0–4.2) | NS | 2.3 (0.96–3.03) | 2.9 (1.9–3.9) | 0.05 |
| IFN-γ (pg/ml) | All | 99 (48.4–134.4) | 67 (46.4–114.1) | 0.024 | 72 (38–120) | 72 (38–114.5) | NS |
| RANTES (pg/ml) | All | 2530 (2096–2798) | 2459 (2013–2986) | NS | 2196 (1704–2497) | 2487 (1887.5–2922.6) | 0.01 |
| TNF-α (pg/ml) | All | 51 (37.1–66.1) | 43 (32.2–56.0) | 0.02 | 40 (31.4–69.4) | 42 (34.5–58.5) | NS |
| VEGF (pg/ml) | All | 0.47 (0.18–10.5) | 4.94 (0.18–13.75) | 0.02 | 0.71 (0.18–4.16) | 2.84 (0.18–9.84) | NS |
| Leptin (pg/ml) | All | 8070 (4523–13 062) | 5575 (2971–12 524) | 0.04 | 9320 (6120–14090) | 6274 (2470–12 385) | 0.02 |
| IGFBP-3 (pg/ml) | All | 197 300 (78 240–274 800) | 230 950 (155 890–282 670) | 0.008 | 210 600 (99 410–271 670) | 229 300 (135 925–286 520) | NS |
| sICAM-1 (ng/ml) | All | 240 (212–270) | 228 (200–258) | 0.04 | 246 (217–289) | 227 (209–256) | NS |
| CA-125 (U/ml) | All | 12 (9.8–18.0) | 20 (16–34) | <0.0001 | 14 (10–19) | 21 (14–32) | <0.0001 |
| CA 19–9 (IU/ml) | All | 9 (6.0–13.3) | 11 (7–18) | 0.03 | 9 (5–14) | 10 (7–20) | NS |
| Follistatin (pg/ml) | All | 1750 (1441–2241) | 1710 (1282–2499) | NS | 1670 (1263–1843) | 1827 (1504–2898) | 0.006 |
| Glycodelin (ng/ml) | All | 16 (7.6–30.9) | 34 (14.2–60.0) | <0.0001 | 16 (7.7–31.2) | 29.2 (10.6–58.7) | 0.02 |
| IL-8 (pg/ml) | Menstrual | 2.8 (1.7–3.0) | 3.1 (2.0–4.5) | NS | 1.1 (0.8–2.2) | 3.6 (2.4–3.9) | 0.01 |
| CA-125 (U/ml) | Menstrual | 13 (11–24) | 25 (19.3–37) | 0.0006 | 16.5 (15–17) | 44 (18.3–61) | 0.02 |
| Follistatin (pg/ml) | Menstrual | 1814 (1467–2555) | 1710 (1308–2569) | NS | 1279 (1168–1453) | 1722 (1559–2086) | 0.02 |
| Glycodelin (ng/ml) | Menstrual | 26 (14.2–32.1) | 68 (45.3–118.9) | 0.009 | 46.7 (31.6–84.5) | 59.4 (25.3–137.7) | NS |
| TNF-α (pg/ml) | Follicular | 50 (37.3–67.6) | 39 (31.4–47.5) | 0.03 | 41 (32.5–48.7) | 40.8 (33.5–50.5) | NS |
| Leptin (pg/ml) | Follicular | 4933 (3677–9305) | 5518 (3010–9965) | NS | 12 545 (9257–14 770) | 6146 (2986–9899) | 0.002 |
| IGFBP-3 (pg/ml) | Follicular | 196 940 (71 875–249 140) | 239 940 (189 589–315 690) | 0.005 | 218 440 (119 663–248 071) | 251 970 (143 093–306 700) | NS |
| IL-21 (pg/ml) | Follicular | 39 (39–85.2) | 81 (39–149) | 0.03 | 103 (39–193) | 85 (39–140) | NS |
| CA-125 (U/ml) | Follicular | 11 (9–14) | 18 (13–35) | <0.0001 | 12 (10–19) | 20.5 (13–26.3) | 0.003 |
| Glycodelin (ng/ml) | Follicular | 9.4 (6.0–15.7) | 20.2 (8.8–43.6) | 0.007 | 8.9 (3.7–14.8) | 16.24 (9.16–45.40) | 0.01 |
| CA-125 (U/ml) | Luteal | 14.5 (10.5–19.5) | 20 (16–30) | 0.002 | 13.5 (10.75–20.25) | 19 (14–28) | 0.03 |
| Follistatin (pg/ml) | Luteal | 1777 (1653–2357) | 1845 (1538–2511) | NS | 1819 (1496–2125) | 2437 (1700–3996) | 0.02 |

Data are presented for significantly different biomarkers in training and/or test sets as the median and interquartile range (interquartile range indicates a range from 25th to 75th percentile).
*Mann–Whitney test.

**Table IV Levels of significantly different plasma biomarker for US-negative endometriosis versus controls.**

| Biomarker | Phase of cycle | Training set | | | Test set | | |
|---|---|---|---|---|---|---|---|
| | | Controls | US-negative endometriosis | *P** value | Controls | US-negative endometriosis | *P** value |
| MCP-1 (pg/ml) | All | 39 (26.2–51.4) | 38 (26–54.1) | NS | 42 (34–50) | 33 (23–42) | 0.004 |
| VEGF (pg/ml) | All | 0.32 (0.18–8.14) | 5.7 (0.18–12.84) | 0.003 | 0.71 (0.18–11.22) | 1.7 (0.2–7.0) | NS |
| Leptin (pg/ml) | All | 7615 (4605–12 630) | 6230 (3010–11 803) | NS | 9890 (4559–15 246) | 5440 (2890–12 385) | 0.03 |
| IGFBP-3 (pg/ml) | All | 177 790 (78 535–257 826) | 245 640 (158 665–287 158) | 0.001 | 214 520 (102 979–293 235) | 223 300 (140 858–288 076) | NS |
| sICAM-1 (ng/ml) | All | 244 (217–285) | 227 (207–261) | 0.045 | 244 (213–267) | 234.5 (207.9–260.8) | NS |
| CA-125 (U/ml) | All | 12 (10–20) | 18 (13–30) | <0.0001 | 13.5 (9.8–15) | 19 (15–25) | <0.0001 |
| CA 19–9 (IU/ml) | All | 9 (5–14) | 10 (7–17) | 0.04 | 9 (6–13.3) | 8 (6–14) | NS |
| Glycodelin (ng/ml) | All | 14 (8–31) | 31 (13–52) | 0.0002 | 22.4 (6.8–32.4) | 37.4 (12.9–79.8) | 0.03 |
| IL-8 (pg/ml) | Menstrual | 2.9 (2.22–3.05) | 3.5 (2.27–4.98) | NS | 1.0 (0.31–1.69) | 2.7 (2.1–3.9) | 0.001 |
| RANTES (pg/ml) | Menstrual | 2332 (1925–2610) | 2632 (2222–2940) | NS | 1997 (1848–2149) | 2340 (2199–2663) | 0.006 |
| VEGF (pg/ml) | Menstrual | 0.82 (0.18–3.5) | 7.91 (0.18–15.26) | NS | 0.175 (0.175–0.175) | 1.92 (0.175–13.22) | 0.044 |
| sICAM-1 (ng/ml) | Menstrual | 260 (232–291.5) | 211 (197–239) | 0.005 | 247 (243–259) | 257 (223.2–271.7) | NS |
| CA-125 (U/ml) | Menstrual | 17 (11–12) | 21 (15–42) | NS | 13.5 (13–15) | 22 (20–31) | 0.002 |
| IL-1beta (pg/ml) | Follicular | 0.89 (0.64–1.23) | 0.63 (0.34–0.88) | 0.01 | 0.66 (0.26–0.96) | 0.65 (0.44–0.82) | NS |
| IL-6 (pg/ml) | Follicular | 10 (5–17.5) | 6 (3.9–9.5) | 0.01 | 6.6 (4.72–9.38) | 4.5 (3.3–10.6) | NS |
| IFN-$\gamma$ (pg/ml) | Follicular | 95 (49–143) | 61 (42.5–88) | 0.02 | 84 (56–109) | 47 (33–94) | NS |
| MCP-1 (pg/ml) | Follicular | 46 (31–62.5) | 32 (24–52.5) | 0.054 | 42 (38–47) | 33 (26–41) | 0.04 |
| TNF-$\alpha$ (pg/ml) | Follicular | 49 (35–72) | 39 (31–44) | 0.03 | 43 (35–49.5) | 36.5 (30.6–46.5) | NS |
| IGFBP-3 (pg/ml) | Follicular | 182 475 (82 236–244 606) | 25 8470 (179 836–312 858) | 0.01 | 225 090 (72 275–259 675) | 251 970 (200 658–324 274) | NS |
| CA-125 (U/ml) | Follicular | 11 (10–15.8) | 18 (12–30.3) | 0.002 | 10.5 (8–14) | 16.5 (13.5–19) | 0.0003 |
| Glycodelin (ng/ml) | Follicular | 8.60 (4.5–12.7) | 24 (8.5–45.4) | 0.0009 | 13.2 (5.7–18.0) | 15.7 (8.9–27.9) | NS |
| IL-8 (pg/ml) | Luteal | 2.83 (1.0–3.6) | 3.3 (2.6–4.2) | NS | 3.5 (2.9–4.1) | 2.2 (1.8–2.5) | 0.01 |
| MCP-1 (pg/ml) | Luteal | 34 (26.7–46.5) | 40 (25.5–52) | NS | 47 (41–67) | 25.5 (18.3–39.7) | 0.002 |
| CA-125 (U/ml) | Luteal | 13.5 (10–21) | 17 (14–23) | 0.04 | 15 (12.5–18.8) | 19 (14–26) | NS |
| CA 19–9 (IU/ml) | Luteal | 7 (4–10) | 10 (7–16.3) | 0.007 | 10.5 (6.3–13.8) | 8 (5.5–17) | NS |

Data are presented for significantly different biomarkers in training and/or test sets as the median and interquartile range (interquartile range indicates a range from 25th to 75th percentile).
*Mann–Whitney test.

**Table V Univariate ROC analysis for US-negative endometriosis.**

| Biomarker | Cycle phase | Cut-off | Training set | | | Test set | | |
|---|---|---|---|---|---|---|---|---|
| | | | AUC | Sensitivity% | Specificity% | AUC | Sensitivity% | Specificity% |
| CA-125 | All | >12.5 U/ml | 0.67 | 78 | 51 | 0.77 | 83 | 42.5 |
| Glycodelin | All | >18 ng/ml | 0.66 | 66 | 61 | 0.64 | 62 | 43 |
| VEGF | All | >1.5 pg/ml | 0.62 | 66 | 56 | 0.51 | 50 | 60 |
| IGFBP-3 | All | >210 ng/ml | 0.64 | 66 | 59 | 0.54 | 56 | 45 |
| sICAM-1 | All | <243 ng/ml | 0.58 | 63 | 51 | 0.53 | 56 | 50 |
| CA 19–9 | All | >9.5 IU/ml | 0.59 | 55 | 58 | 0.53 | N/A | N/A |
| sICAM-1 | Menstrual | <254.6 ng/ml | 0.76 | 83 | 59 | 0.60 | 71 | 30 |
| IL-1beta | Follicular | <0.9 pg/ml | 0.67 | 78 | 50 | 0.50 | 82 | 38 |
| IL-6 | Follicular | <10 pg/ml | 0.67 | 76 | 50 | 0.59 | 73 | 19 |
| IFN-γ | Follicular | <76 pg/ml | 0.66 | 74 | 60 | 0.61 | 68 | 63 |
| TNF-α | Follicular | <45.6 pg/ml | 0.65 | 78 | 57 | 0.60 | 68 | 38 |
| IGFBP-3 | Follicular | >200 ng/ml | 0.67 | 69 | 60 | 0.64 | 73 | 31 |
| Glycodelin | Follicular | >9.0 ng/ml | 0.73 | 74 | 57 | 0.61 | 70 | 36 |
| CA-125 | Follicular | >11.5 U/ml | 0.71 | 76 | 60 | 0.85 | 86 | 63 |
| CA-125 | Luteal | >13.5 U/ml | 0.64 | 80 | 50 | 0.62 | 79 | 29 |
| CA 19–9 | Luteal | >7.5 IU/ml | 0.68 | 73 | 56 | 0.55 | N/A | N/A |

N/A, not applicable; AUC, area under the ROC curve; ROC, receiver operating characteristic.

endometriosis and *minimal–mild* endometriosis) and in the follicular phase (for *moderate–severe* endometriosis).

Using multivariate logistic regression analysis, *endometriosis (all stages)* was diagnosed with a model combining VEGF, annexin V and CA-125 levels of plasma obtained during the menstrual phase with a 71% sensitivity/67% specificity (LR+ 2.1; LR− 0.43; AUC of 0.69) in the training set and with a 85% sensitivity/75% specificity (LR+ 3.4; LR− 0.2; AUC of 0.80) in the test set (Supplementary data, Table SI). Using LS-SVM analysis of the same samples and biomarkers, endometriosis (all stages) was diagnosed with a 94% sensitivity/55% specificity (LR+ 2.1; LR− 0.11; AUC of 0.83) in the training set and with a 89% sensitivity/62.5% specificity (LR+ 2.4; LR− 0.18; AUC of 0.84) in the test set (Supplementary data, Table SII).

Using multivariate logistic regression analysis, *minimal–mild* endometriosis was diagnosed with a model combining IL-6, IL-10, VEGF, HGF and sICAM-1 levels of plasma obtained during the menstrual phase with a 84% sensitivity/75% specificity (LR+3.4; LR− 0.21; AUC of 0.80) in the training set and with a 88% sensitivity/63% specificity (LR+ 2.3; LR− 0.2; AUC of 0.75) in the test set (Supplementary data, Table SI). However, using LS-SVM analysis this model performed poor in the diagnosis of *minimal–mild* endometriosis with a 77% sensitivity/51% specificity (LR+ 1.6; LR− 0.46; AUC of 0.67) in the training set and with a 50% sensitivity/89% specificity (LR+ 4.0; LR− 0.57; AUC of 0.69) in the test set (Supplementary data, Table SII).

Using multivariate logistic regression analysis, *moderate–severe* endometriosis was diagnosed with a model combining CA-125 and glycodelin levels of plasma obtained during the follicular phase with a 90% sensitivity/75% specificity (LR+ 3.5; LR− 0.13; AUC of 0.82) in the training set and with a 83% sensitivity/78% specificity (LR+ 3.8; LR− 0.2; AUC of 0.81) in the test set (Supplementary data, Table SI). Using LS-SVM analysis of the same samples and biomarkers, *moderate–severe* endometriosis was diagnosed with a 95% sensitivity/92% specificity (LR+ 12.9; LR− 0.05; AUC of 0.97) in the training set and with a 93% sensitivity/82% specificity (LR+ 5.3; LR− 0.08; AUC of 0.93) in the test set (Supplementary data, Table SII). When the selection of biomarkers was performed by univariate analysis, the model combining CA-125, glycodelin, hsCRP and follistatin (during the follicular phase) only slightly improved the diagnostic performance of the test for *moderate–severe* endometriosis (compared with the diagnostic performance of the two biomarker model (CA-125 and glycodelin; Supplementary data, Tables SI and SII).

### *Multivariate analysis: US-negative endometriosis*

The subanalysis of samples collected from women with laparoscopically confirmed endometriosis without evidence of endometriosis on preoperative gynaecological US is presented in Table VI.

Using multivariate logistic regression analysis, US-negative endometriosis was diagnosed with a model combining menstrual phase plasma levels of four biomarkers (VEGF, annexin V, CA-125 and sICAM-1) with a 81% sensitivity/77% specificity (LR+ 3.4; LR− 0.25; AUC of 0.79) in the training set and with a 82% sensitivity/75% specificity (LR+ 3.3; LR− 0.24; AUC of 0.78) in the test set (Table VI). Using LS-SVM analysis of the same samples and biomarkers, US-negative endometriosis was diagnosed with a 86% sensitivity/68% specificity (LR+ 2.7; LR− 0.2; AUC of 0.86) in the training set and with a 82% sensitivity/75% specificity (LR+ 3.3; LR− 0.24; AUC of 0.81) in the test set (Table VI).

Substituting sICAM-1 by glycodelin in the model with four biomarkers produced similar results. Indeed, using multivariate logistic regression analysis, US-negative endometriosis was diagnosed with a model combining menstrual phase plasma levels of four biomarkers (VEGF,

**Table VI** **Selected best predictive models for the diagnosis of US negative endometriosis based on the multivariate logistic regression and LS-SVM models.**

| Model | Method biomarkers selection | Method classification | Cycle phase | AUC training/ test | Sensitivity% training/test | Specificity% training/test | Accuracy% training/ test | PPV% training/ test | NPV% training/ test | LR+ training/ test | LR− training /test | DOR training/ test |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| VEGF, annexin V, CA-125, glycodelin | Multivariate approach 2 | Multivariate logistic regression | Menstrual | 81/78 | 81/82 | 81/75 | 81/79 | 90/82 | 68/75 | 4.3/3.3 | 0.23/0.24 | 18.7/13.8 |
| VEGF, annexin V, CA-125, glycodelin | Multivariate approach 2 | LS-SVM | Menstrual | 85/84 | 90/82 | 68/63 | 81/74 | 81/75 | 81/71 | 2.8/2.2 | 0.15/0.29 | 18.7/7.6 |
| VEGF, annexin V, CA-125, sICAM-1 | Multivariate approach 2 | Multivariate logistic regression | Menstrual | 79/78 | 81/82 | 77/75 | 79/79 | 86/82 | 68/75 | 3.4/3.3 | 0.25/0.24 | 13.6/13.8 |
| VEGF, annexin v, CA-125, sICAM-1 | Multivariate approach 2 | LS-SVM | Menstrual | 86/81 | 86/82 | 68/75 | 79/79 | 81/82 | 77/75 | 2.7/3.3 | 0.20/0.24 | 13.5/13.8 |

Multivariate approach 2: multivariate selection of models that contain the most frequent biomarkers (threshold of 70%).

annexin V, CA-125 and glycodelin) with a 81% sensitivity/81% specificity (LR+ 4.3; LR− 0.23; AUC of 0.81) in the training set and with a 82% sensitivity/75% specificity (LR+ 3.3; LR− 0.24; AUC of 0.78) in the test set (Table VI).

Using LS-SVM analysis of the same samples and biomarkers, US-negative endometriosis was diagnosed with a 90% sensitivity/68% specificity (LR+ 2.8; LR− 0.15; AUC of 0.85) in the training set and with a 82% sensitivity/63% specificity (LR+ 2.2; LR− 0.29; AUC of 0.84) in the test set (Table VI).

# Discussion

The present study is an important step in the development and validation of a blood test for endometriosis with a high sensitivity and acceptable specificity in a group of patients with subfertility and/or pain with a negative preoperative US. Multivariate analysis based on two models of four biomarkers (annexin V, VEGF, CA-125, glycodelin; annexin V, VEGF, CA-125 and sICAM-1) during the menstrual phase enabled the diagnosis of endometriosis undetectable by US with a high sensitivity (81–90%) at acceptable specificity (63–81%) in an independent training and test data set. This diagnostic performance was better than the diagnostic performance of any single biomarker in our study.

The novelty of our study is based on the design based on QUADAS guidelines (Whiting et al., 2003; May et al., 2010), the large and well-defined patient population ($n = 353$), the large number of evaluated plasma biomarkers ($n = 28$), the advanced multivariate statistical approach to select biomarkers, to classify them by the multivariate logistic regression and LS-SVM, and to validate the models developed in the training set in an independent test set, and the subanalysis of samples from women with negative preoperative US.

The strength of our study is that our design is in accordance with the QUADAS guidelines (Whiting et al., 2003; May et al., 2010) with respect to control group selection and cycle phase correction. In line with these guidelines, sample collection had been performed at a consistent phase of the cycle and results presented were corrected for cycle phases, as recommended (Whiting et al., 2003; May et al., 2010). For instance, three out of nine papers investigating IL-6 failed to adjust for the phase of the menstrual cycle, despite evidence that levels are known to change throughout the cycle (Angstwurm et al., 1997; May et al., 2010).

The choice of an appropriate control group is crucial and depends on the aim of the diagnostic test. In line with the QUADAS guidelines we selected our controls from women with symptoms consistent with endometriosis (such as infertility and/or pelvic pain) but without laparoscopic evidence of endometriosis based on laparoscopic data, obtained by an experienced endometriosis surgeon. Our controls have different pelvic pathology such as non-endometriosis adhesions, myoma, parasalpingeal cyst and hydrosalpinx (Table II).

Interestingly, inflammatory biomarkers such as IL-1beta, IFN-gamma, TNF-alpha and IL-6 were slightly higher in the control group than in the endometriosis group in the training data set. These data were not consistent and not confirmed in the test data set (Tables III and IV), which included a comparable ($P = 0.15$) proportion of patients with non-endometriotic adhesions (9/41, 22%) as the training data set (28/80, 35%; Table II). Nevertheless, this observation could be partially explained by the possibility that controls with non-endometriotic pelvic pathology like adhesions and hydrosalpinx had increased plasma concentrations of inflammatory cytokines. As inflammatory cytokines were not included in the final diagnostic model (Table VI), we speculate that they are not relevant in the discrimination of patients with endometriosis from women with non-endometriotic pelvic pathology due to the similar inflammatory pathways which cause endometriotic and non-endometriotic pelvic pathology and results in pelvic pain and infertility. Moreover, at present, there is no consensus regarding the value of inflammatory factors as biomarkers of endometriosis. Comparable serum IL-6 (Kalu et al., 2007; Socolov et al., 2011), TNF-alpha and IL-1 (Kalu et al., 2007; Othman et al., 2008; Socolov et al., 2011) levels were previously reported in women with and without endometriosis. However, other investigators reported elevated peripheral levels of IL-6 (Bedaiwy et al., 2002; Othman et al., 2008), TNF-alpha (Bedaiwy et al., 2002; Xavier et al., 2006); IFN-gamma (Othman et al., 2008) in endometriosis patients compared with controls. These discrepancies could also be partially explained by the differences in study design (different inclusion criteria and different cycle phases), preanalytical variability in the cytokines levels (due to different types of collected samples (serum versus plasma); different clotting times, different conditions of centrifugation) which could influence the study results. For example, measurable concentrations of inflammatory markers are higher in the serum than in simultaneously collected plasma, due to the release of inflammatory markers during the coagulation process in the serum (Skogstrand et al., 2008). Moreover, biological variability due to functional single nucleotide polymorphisms known to influence protein levels of corresponding circulated proteins can also partially explain the discrepancy in study results, since genetic variants in IL-6 and IFN-gamma genes may influence circulating levels of corresponding proteins (Talar-Wojnarowska et al., 2009; Vallinoto et al., 2010).

The data of our study confirmed the hypothesis (Robin et al., 2009; May et al., 2010) that a panel of biomarkers can improve the sensitivity and specificity of diagnostic test compared with the diagnostic performance of any single biomarker. Our panel of four biomarkers (annexin V, VEGF, CA-125, sICAM-1 or glycodelin) had a better diagnostic performance than any single biomarker in our study. So far, only a limited number of studies have focused on the prediction of endometriosis based on a panel of markers, and these studies were limited by univariate analysis (Somigliana et al., 2004; Agic et al., 2008). Our multivariate statistical approach allowed us to model the relationship between diagnostic categories and all biomarkers simultaneously, taking into account the correlation that may exist between those biomarkers, while univariate analysis only deals with the relationship between one predictor and diagnostic category.

In the present study the two selected panels of four biomarkers (annexin V, VEGF, CA-125, sICAM-1/glycodelin) performed robustly by using two fundamentally different classifiers (multivariate logistic regression and LS-SVM) and could predict US-negative endometriosis with a sensitivity of 81–90% and a specificity of 63–81% (Table VI). Indeed, both methods (multivariate logistic regression and LS-SVM) are widely used in biomarker studies (Robin et al., 2009) and none of these methods is clearly superior when compared with the other (Robin et al., 2009). However, the multivariate logistic regression is more sensitive to feature selection (Pochet and Suykens, 2006; Mihalyi et al., 2010) since it tends to build a classification model that

fits patients from a training set optimally (Pochet and Suykens, 2006), but is not always possible to make good predictions for novel patients from an independent test set, a problem defined as overfitting (Pochet and Suykens, 2006). In contrast, the LS-SVM is less sensitive to feature selection and effect of outliers, preventing the model from overfitting the training data (Pochet and Suykens, 2006; Mihalyi *et al*., 2010). In addition, it has an internal mechanism for modeling non-linearity, giving rise to increased robustness and therefore good performance in an independent test data set (Pochet and Suykens, 2006). Our data show that it is possible to overcome the problem of data overfitting by using the biomarkers selection method, as described in the methodology section. Indeed, the diagnostic performance of the models based on the training set was confirmed on the independent test set by using both classifiers (multivariate logistic regression and LS-SVM).

The relevance of the selected diagnostic panel (annexin V, VEGF, CA-125 and sICAM-1/glycodelin) is confirmed by the fact that the selected biomarkers are involved in apoptosis, angiogenesis, adhesion and tumorogenesis, which are highly related to the pathogenesis of endometriosis.

Annexin V, a marker of apoptosis, has been recently reported by our group to be a promising semi-invasive biomarker for diagnosis of minimal–mild endometriosis (Kyama *et al*., 2011). Indeed, alterations in the regulation of apoptosis in eutopic and ectopic endometrium from women with endometriosis could contribute to the survival of endometrial cells into the peritoneal cavity and development of endometriosis (reviewed by Taniguchi *et al*., 2011).

Glycodelin is an endometrium-derived protein with known angiogenic, immunosuppressive and contraceptive effects, which could contribute to the development of endometriosis and endometriosis-related infertility (reviewed by Seppälä *et al*., 2009). VEGF is one of the main stimuli for angiogenesis and increased vessel permeability, which contributes to the development of endometriotic lesions (Taylor *et al*., 2002, Becker and D'Amato, 2007). sICAM-1 is one of the major adhesion molecules which inhibits natural killer cell-mediated cytotoxicity (Becker *et al*., 1991), resulting in defective immune surveillance and is involved in the implantation and development of endometriotic lesions (Wu and Ho, 2003).

CA-125 is the most extensively investigated and used peripheral biomarker of endometriosis (Gupta *et al*., 2006). CA-125 is produced by endometrial and mesothelial cells and exudes into circulation via the endothelial lining of capillaries in response to inflammation (Bischof, 1993; Zeillemaker *et al*., 1994; reviewed by Gupta *et al*., 2006). However, CA-125 levels in the peripheral blood lack diagnostic power as a single biomarker of endometriosis (Mol *et al*., 1998; Kennedy *et al*., 2005).

We realize that a diagnostic test may do more harm than good, e.g. by subjecting patients to unnecessary or even potentially harmful procedures (Evers and Van Steirteghem, 2009) since the benefits of treating women with asymptomatic endometriosis is unclear (May *et al*., 2010). Therefore, we do not recommend to develop or use a blood test for screening purpose in asymptomatic women. However, up to 45% of subfertile women with a regular cycle whose partner has normal sperm quality, with or without pelvic pain, and with normal clinical examination and a normal pelvic US may have endometriosis (Meuleman *et al*., 2009). A blood test could identify those most likely to have endometriosis or other pelvic conditions and likely to benefit from surgical therapy for both subfertility and pain (Kennedy *et al*., 2005; D'Hooghe *et al*., 2006). In our study, the biomarker panels allowed to rule in these women with a high sensitivity (81–90%) and acceptable specificity (63–81%) and distinguish them from women without endometriosis who had symptoms similar to those with endometriosis (subfertility and/or pain), which is in line with published recommendations (May *et al*., 2010).

In our future work we plan to develop a computer application based on the selected best predictive models (Table VI) that would be freely available for clinical use. The two models, based on the incorporation of measured plasma levels of selected biomarkers (model 1: annexin V, VEGF, CA-125, glycodelin; model 2: annexin V, VEGF, CA-125, sICAM-1) during menstrual cycle phase will be used to identify high-risk groups of patients with a predicted probability of developing endometriosis based on the developed threshold. If the value of predicted probability is greater than the given threshold we conclude that the endometriosis status is positive while in the reverse case we conclude a negative status. Further prospective study is required to validate these models in clinical setting.

Our study is marked by the following limitations.

Firstly, the best diagnostic model was based on the analysis of plasma samples obtained during the menstrual phase. This is not surprising, since it is well known that plasma CA-125 levels in women (O'Shaughnessy *et al*., 1993) and baboons (Falconer *et al*., 2005) with endometriosis are higher during the menstrual phase than during other phases of the cycle. In practice, this could be a limitation since the blood sampling has to be limited to the menstrual cycle phase only.

Secondly, stress factors directly before surgery might have affected plasma biomarker levels, as blood was taken just prior to anaesthesia (as previously described by Mihalyi *et al*., 2010). More research is needed to validate our diagnostic models in plasma samples obtained in an outpatient clinic. However, in our study, the priority was to ensure that the blood sample was taken at the time of surgery in order to have a direct temporal comparison between laparoscopic diagnosis and staging of endometriosis disease and the plasma levels of the biomarkers studied.

Thirdly, we did not evaluate possible diurnal variability in biomarkers levels, as previously observed for serum IL-6 levels (Arvidson *et al*., 1994). From a practical perspective, we were looking for a robust biomarker panel not depending on diurnal variability, as suggested by the validation of our model in an independent test data set.

Fourthly, the selection of control group was based only on the laparoscopically exclusion of endometriosis by an experienced endometriosis surgeon without histological evaluation, which could be a limitation, especially for the patients with non-endometriotic adhesions. Indeed, it is difficult to rule out that women with a normal pelvis or with non-endometriotic adhesions may have microscopic endometriosis, and that laparoscopic absence of endometriosis may be a temporary phenomenon. However, since intraperitoneal adhesions are accepted as aetiologic factors for infertility (Hammoud *et al*., 2004), inclusion of patients with non-endometriosis adhesions based on laparoscopy data in the control group can be justified in a biomarker study for endometriosis.

In conclusion, multivariate analysis of four biomarkers (annexin V, VEGF, CA-125 and sICAM-1/or glycodelin) in plasma samples obtained during menstruation enabled the diagnosis of endometriosis undetectable by US with a sensitivity of 81–90% and a specificity of 63–81% in the independent training- and test data set. The next

research step is to predict the presence of endometriosis with a high sensitivity, using the models presented in this study, in an independent set of patients with infertility and/or pain without US evidence of endometriosis scheduled for surgery, and to compare the predicted with the actual presence of endometriosis. Although our current study is an important step in the development of a blood test with a high sensitivity for the diagnosis of endometriosis in subfertile patients with a normal gynaecological US, new system biology approaches, i.e. proteomics, are needed to identify novel and specific biomarkers of endometriosis to further increase the sensitivity and specificity of a blood test for endometriosis.

## Supplementary data

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

## Authors' roles

A.V., Y.E., D.P., A.M., X.B., C.M.K., A.F., A.B., E.W., O.G., A.K. and T.M.D. were involved in study concept and design. A.V., A.M., X.B., C.M.K., A.F., A.B., D.S., D.H., C.M., K.P. and C.T. were involved in acquisition of data. A.V., Y.E., D.P., X.B., C.M.K., A.F., A.B., O.G., D.S., D.H. and T.M.D. were involved in analysis and interpretation of data. A.V., Y.E., D.P., A.M., X.B., C.M.K., A.F., A.B., D.S., D.H., C.M., K.P., C.T., O.G., E.W. A.K., B.D.M. and T.M.D. were involved in manuscript drafting and critical discussion.

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## Conflict of interest

None declared.

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