

# Non-invasive diagnosis of endometriosis based on a combined analysis of six plasma biomarkers

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**BACKGROUND:** Lack of a non-invasive diagnostic test contributes to the long delay between onset of symptoms and diagnosis of endometriosis. The aim of this study was to evaluate the combined performance of six potential plasma biomarkers in the diagnosis of endometriosis.

**METHODS:** This case–control study was conducted in 294 infertile women, consisting of 93 women with a normal pelvis and 201 women with endometriosis. We measured plasma concentrations of interleukin (IL)-6, IL-8, tumour necrosis factor-alpha, high-sensitivity C-reactive protein (hsCRP), and cancer antigens CA-125 and CA-19-9. Analyses were done using the Kruskal–Wallis test, Mann–Whitney test, receiver operator characteristic, stepwise logistic regression and least squares support vector machines (LSSVM).

**RESULTS:** Plasma levels of IL-6, IL-8 and CA-125 were increased in all women with endometriosis and in those with minimal–mild endometriosis, compared with controls. In women with moderate–severe endometriosis, plasma levels of IL-6, IL-8 and CA-125, but also of hsCRP, were significantly higher than in controls. Using stepwise logistic regression, moderate–severe endometriosis was diagnosed with a sensitivity of 100% (specificity 84%) and minimal–mild endometriosis was detected with a sensitivity of 87% (specificity 71%) during the secretory phase. Using LSSVM analysis, minimal–mild endometriosis was diagnosed with a sensitivity of 94% (specificity 61%) during the secretory phase and with a sensitivity of 92% (specificity 63%) during the menstrual phase.

**CONCLUSIONS:** Advanced statistical analysis of a panel of six selected plasma biomarkers on samples obtained during the secretory phase or during menstruation allows the diagnosis of both minimal–mild and moderate–severe endometriosis with high sensitivity and clinically acceptable specificity.

**Key words:** plasma biomarkers / endometriosis / secretory / menstrual phase / non-invasive diagnosis

## Introduction

Endometriosis is defined as the presence of endometrial-like tissue outside the uterus. It results often in subfertility and pain, occurs mainly in women of reproductive age (16–50 years) and has a progressive character in at least 50%, but the rate and risk factors for progression are unknown (D'Hooghe *et al.*, 2006). Endometriosis can be

classified into four stages: minimal, mild, moderate and severe (ASRM, 1997). More advanced endometriosis can be deeply invasive behind the cervix and invade into the rectovaginal septum, obliterating the pouch of Douglas partially or completely, or can present as ovarian endometriotic cysts (endometrioma). The stage of endometriosis is positively correlated with the degree of subfertility, but not as clearly as with the degree of pelvic pain (D'Hooghe

and Debrock, 2002; Fauconnier and Chapron, 2005; Kennedy *et al.*, 2005).

The diagnosis of endometriosis can be suspected in women with pelvic pain and/or subfertility, although endometriosis may be completely asymptomatic (Kennedy *et al.*, 2005). Clinical detection of abdominal or pelvic pain can be suggestive of endometriosis. Vaginal ultrasound is an adequate diagnostic method to detect ovarian endometriotic cysts and deeply infiltrative endometriotic noduli, but does not rule out peritoneal endometriosis or endometriosis-associated adhesions. The gold standard for the diagnosis of endometriosis is laparoscopic inspection, ideally with histological confirmation (Kennedy *et al.*, 2005).

Development of a non-invasive diagnostic test for endometriosis would have a groundbreaking impact on the patients' quality of life, on the efficacy of available treatment as well as on the cost of endometriosis. However, a recent survey completed in 7025 women with endometriosis (European Endometriosis Alliance, 2006) demonstrated that 65% of the women with endometriosis were first misdiagnosed with another condition, and 46% had to see five doctors or more before they were correctly diagnosed, resulting in an average delay of 8 years between the onset of symptoms and the diagnosis of endometriosis (Zondervan *et al.*, 1999; Ballard *et al.*, 2006).

So far, non-invasive approaches such as ultrasound, magnetic resonance imaging or blood tests have not yielded sufficient power for the diagnosis of endometriosis (Chen *et al.*, 1998; Mol *et al.*, 1998; Zondervan *et al.*, 1999; Harada *et al.*, 2002; Somigliana *et al.*, 2004; Kennedy *et al.*, 2005; Ballard *et al.*, 2006). However, most studies evaluating biomarkers for the diagnosis of endometriosis have shown various limitations: low patient number, mostly assessment of only one biomarker, univariate analysis only if multiple biomarkers were tested, or lack of consideration for biomarker variability according to menstrual cycle phase (O'Shaughnessy *et al.*, 1993; Tabibzadeh *et al.*, 1995a, b; Abrao *et al.*, 1997; Bon *et al.*, 1999; Harada *et al.*, 2002; Somigliana *et al.*, 2004; Xavier *et al.*, 2005, 2006).

The objective of the current study was to evaluate whether the combined analysis of various potential biomarkers in a large, well-defined patient population can be accurate for the diagnosis of endometriosis, using stepwise logistic regression analysis and least squares support vector machines (LSSVMs).

## Materials and Methods

### Patients and plasma samples

Plasma samples were collected after obtaining written informed consent from women undergoing laparoscopic surgery for subfertility with or without pain at the Leuven University Fertility Center (LUFC) since 1999. Our study had received approval from the Commission for Medical Ethics (Leuven University Hospital) before its initiation. Prior to anaesthesia induction, 4 × 4 ml blood was collected, 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 at maximum 1 h. For each patient, relevant information (e.g. date of collection, identification code, clinicopathological data) was entered in the electronic biobank database of the LUFC.

In 2005, the electronic biobank database of the LUFC was searched for all plasma samples that had both the necessary minimal volume (2.5 ml) and the required clinical information of the patient at the time of sample

**Table I** Distribution of study samples according to stage of endometriosis and menstrual cycle phase

Cycle phase	Stage of endometriosis		
	Controls (Stage 0)	Stage I–II	Stage III–IV
Menstrual	19	25	15
Proliferative	36	60	23
Secretory	38	47	31
Total per stage	93	132	69
Total in study	294		

collection [age, stage and score of endometriosis (ASRM, 1997), menstrual cycle phase determined according to Noyes *et al.* (1950) criteria, current medication and number and type of previous operations]. Patients were divided into three groups according to the presence and degree of endometriosis: controls (normal pelvis), minimal–mild endometriosis and moderate–severe endometriosis. A total of 320 plasma samples were identified as meeting our inclusion criteria. Subsequently, the following samples were excluded for analysis: samples collected from women who were on hormonal medication at the time of collection, who had been operated within 6 months prior to the time of collection or who had other pelvic inflammatory disease or general diseases at the time of collection. After this exclusion, a total of 294 plasma samples were included in our study (Table I).

### Selection and measurement of target biomarkers

After an extensive literature search, six plasma biomarkers were selected based on earlier reports that their plasma concentration showed significant differences between women with and without endometriosis. These molecules, interleukin (IL)-6, IL-8, tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), CA-125, CA-19-9 and high-sensitivity C-reactive protein (hsCRP), are suggested to be involved in the development and/or progression of endometriosis as autocrine/paracrine factors or as products of immunocompetent cells promoting vascularization and/or supporting survival and proliferation of ectopic endometrial cells through various mechanisms (Mihalyi *et al.*, 2005; Kyama *et al.*, 2006; Debrock *et al.*, 2006; Kyama *et al.*, 2008).

Plasma concentrations of IL-6, IL-8 and TNF- $\alpha$  were determined by using commercially available ELISA kits (BD Biosciences, Erembodegem, Belgium) according to the manufacturer's instructions. Plasma concentrations of CA-125, CA-19-9 and hsCRP levels were measured by automated assays on a Roche Modular P or Modular E170 instruments (Roche, Vilvoorde, Belgium) at the central laboratories of the University Hospitals Leuven (Gasthuisberg, Leuven).

### Statistical analysis

Results are expressed as median and range with 95% confidence intervals. Univariate analyses were carried out using the Prizm 4.0 software package (GraphPad, San Diego, CA, USA) using the Mann–Whitney test and Kruskal–Wallis test with Dunn's multiple comparison test. Additionally, receiver operating characteristic (ROC) curves (Hanley and McNeil, 1982) were constructed for each of the individual plasma markers to identify the discriminative power of each marker alone. Undetectable amounts of target molecule measured were considered to be 0 pg/ml for statistical analysis.

Multivariate analysis was done using stepwise logistic regression (SAS 9.1.3 for Windows, Cary, NC, USA) and LSSVM (MATLAB scripts were downloaded from LS-SVMlab version 1.5 <http://www.esat.kuleuven.ac.be/sista/lssvmlab/>), including only variables with significant odds ratios ( $P < 0.05$ ). For LSSVMs, no variable selection was performed. Models were evaluated by their area under the ROC curve (AUC). After having chosen an operating point on the ROC curve corresponding to a specificity of 70% or higher, sensitivity, accuracy, positive predictive value (PPV) and negative predictive value (NPV) were determined. Since the diagnosis of minimal–mild endometriosis with high sensitivity is clinically highly relevant, as argued before (D’Hooghe et al., 2006) and as mentioned in the discussion section, we also calculated the decrease in specificity for a sensitivity of 100% in this subgroup. Model performance was compared using paired (Hanley and McNeil, 1983) and unpaired ROC curve comparisons where appropriate. Unpaired ROC comparisons were done by a permutation test. Briefly, for both groups, the labels of the samples were permuted 1000 times and the distribution of differences in AUC was constructed. Multivariate analysis was also done with LSSVM analysis, based on earlier results from our group that LSSVMs analysis can be used to predict the depth of infiltration in endometrial carcinoma (De Smet et al., 2006). LSSVMs are less sensitive to feature selection since, in contrast to stepwise logistic regression, they have means to prevent the model from overfitting the data. Additionally, they allow the modelling of complex relationships in the data instead of only linear relationships as is the case with multivariate logistic regression.

A  $P$ -value was determined by counting the number of times an AUC difference more extreme than the observed AUC difference is found (North et al., 2002; Good, 2004). All statistical tests were two-sided and differences were considered statistically significant when the  $P$ -value was  $< 0.05$ .

## Results

### Univariate analysis

#### *Controls versus women with endometriosis (including all stages and cycle phases)*

The plasma levels of IL-6, IL-8, CA-125 were significantly higher, whereas the plasma level of free TNF- $\alpha$  was decreased, in women with endometriosis compared with controls regardless of cycle phase [IL-6: 0.71 pg/ml (0–228.8) versus 0.34 pg/ml (0–5.48),  $P < 0.0001$ ; IL-8: 1.77 pg/ml (0–52.12) versus 0.875 pg/ml (0–6.26),  $P < 0.0001$ ; CA-125: 22 U/ml (6–969.0) versus 13.0 U/ml (4.0–47.0),  $P < 0.0001$ ; TNF- $\alpha$ : 0.03 pg/ml (0–24.81) versus 0.44 pg/ml (0–4.88),  $P < 0.0001$ , respectively].

#### *Controls versus women with minimal–mild and moderate–severe endometriosis (including all cycle phases)*

In women with minimal–mild endometriosis, plasma levels of IL-6, IL-8, CA-125 were increased, and those of TNF- $\alpha$  were decreased, compared with controls [IL-6: 0.70 pg/ml (0–24.43) versus 0.34 pg/ml (0–5.48),  $P < 0.0001$ ; IL-8: 1.6 pg/ml (0–52.12) versus 0.875 pg/ml (0–6.26),  $P = 0.0003$ ; CA-125: 17.0 U/ml (6–969) versus 13.0 U/ml (4.0–47.0),  $P < 0.0001$ ; TNF- $\alpha$ : 0.06 pg/ml (0–14.66) versus 0.44 pg/ml (0–4.88),  $P < 0.0001$ , respectively]. In women with moderate–severe endometriosis, plasma levels of IL-6, IL-8, CA-125 and hsCRP were increased, and those of TNF- $\alpha$  were decreased, when compared with controls [IL-6: 0.73 pg/ml (0–228.8) versus 0.34 pg/ml (0–5.48),  $P < 0.0001$ ; IL-8: 1.85 pg/ml

(0–27.32) versus 0.875 pg/ml (0–6.26),  $P = 0.0003$ ; CA-125: 32 U/ml (9–746) versus 13.0 U/ml (4.0–47.0),  $P < 0.0001$ ; hsCRP: 1.35 mg/l (0.23–34.78) versus 0.64 mg/l (0.11–15.03),  $P < 0.0001$ ; TNF- $\alpha$ : 0 pg/ml (0–24.81) versus 0.44 pg/ml (0–4.88),  $P < 0.0001$ , respectively].

#### *Controls versus women with minimal–severe endometriosis (secretory phase only)*

As shown in Fig. 1, in women with endometriosis, plasma levels of IL-8, IL-6, CA-125 and hsCRP were increased and those of TNF- $\alpha$  were decreased when compared with controls [IL-8: 1.528 pg/ml (0–52.12) versus 0.24 pg/ml (0–3.97),  $P < 0.0001$ ; IL-6: 0.73 pg/ml (0–51.72) versus 0.27 (0–1.06) pg/ml,  $P = 0.0003$ ; CA-125: 24.0 U/ml (7.0–190.0) versus 14.0 U/ml (4.0–47.0),  $P < 0.0001$ ; hsCRP: 0.88 mg/l (0.12–27.23) versus 0.56 mg/l (0.11–14.14),  $P = 0.03$ ; TNF- $\alpha$ : 0 pg/ml (0–24.81) versus 0.5 pg/ml (0–1.79),  $P < 0.0001$ , respectively].

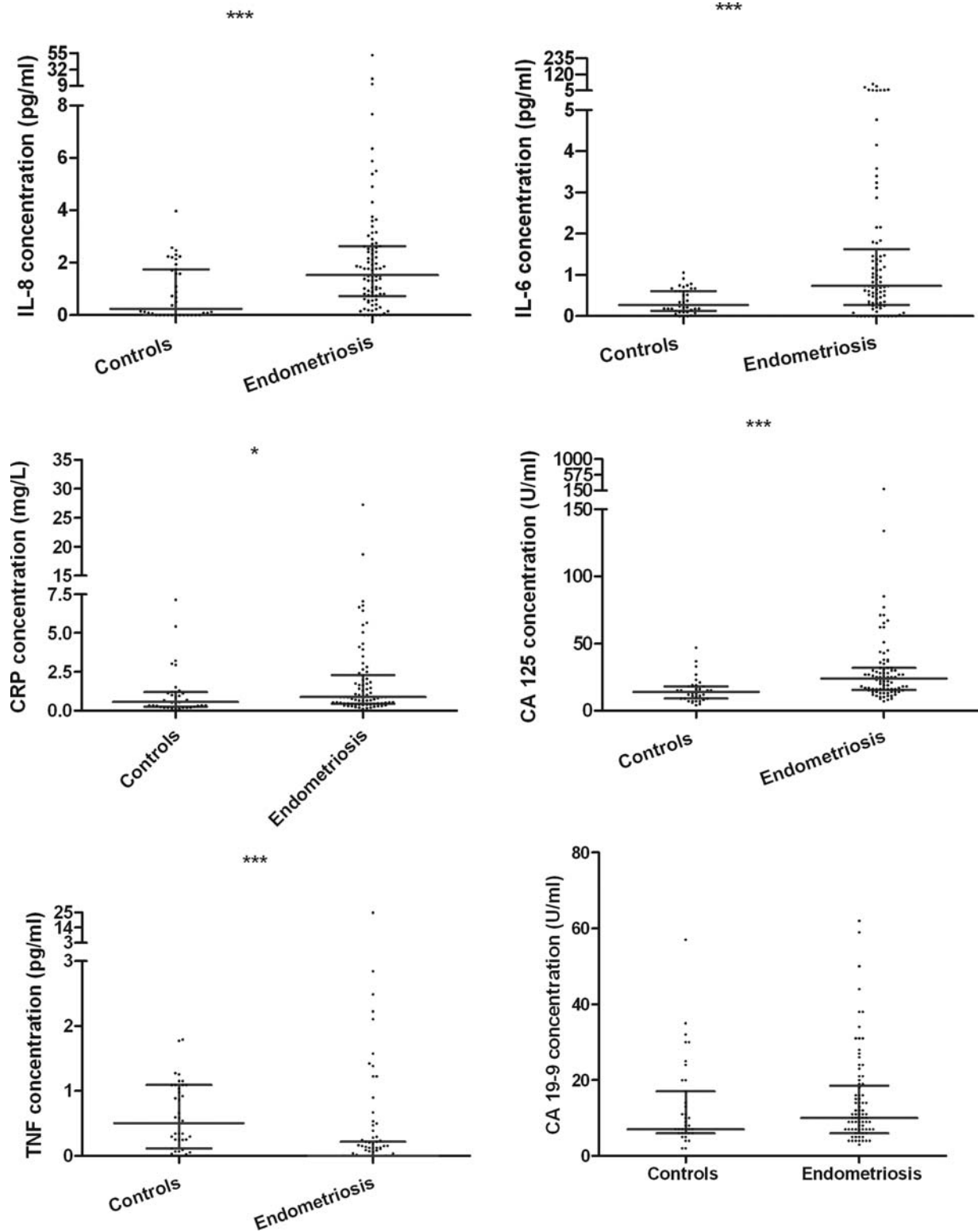
#### *Controls versus women with minimal–mild and moderate–severe endometriosis (secretory phase only)*

As shown in Fig. 2, increased secretory phase plasma levels of IL-8 and IL-6 and decreased levels of TNF- $\alpha$  were detected in women with minimal–mild endometriosis compared with controls [IL-8: 1.49 pg/ml (0–52.12) versus 0.24 pg/ml (0–3.97),  $P = 0.0003$ ; IL-6: 0.69 pg/ml (0–10.88) versus 0.27 pg/ml (0–1.06),  $P = 0.001$ ; TNF- $\alpha$ : 0.05 pg/ml (0–2.23) versus 0.5 pg/ml (0–1.79),  $P < 0.0001$ , respectively]. In women with moderate-to-severe endometriosis, decreased secretory phase plasma levels of free TNF- $\alpha$  and increased IL-6, IL-8, hsCRP, CA-125 plasma levels were observed compared with controls [TNF- $\alpha$ : 0 pg/ml (0–24.81) versus 0.5 pg/ml (0–1.79),  $P < 0.0001$ ; IL-6: 0.74 pg/ml (0–51.72) versus 0.27 pg/ml (0–1.06),  $P = 0.001$ ; IL-8: 1.85 pg/ml (0–19) versus 0.24 pg/ml (0–3.97),  $P = 0.0003$ ; hsCRP: 1.42 mg/l (0.23–27.23) versus 0.56 mg/l (0.11–14.14),  $P = 0.001$ ; CA-125: 32.0 U/ml (13.0–190.0) versus 14.0 U/ml (4.0–47.0),  $P < 0.0001$ , respectively]. Additionally, hsCRP and CA-125 levels in secretory phase plasma were increased in moderate-to-severe endometriosis compared with women with minimal-to-mild disease [hsCRP: 1.42 mg/l (0.23–27.23) versus 0.64 mg/l (0.12–6.67),  $P = 0.001$ ; CA-125: 32.0 U/ml (13.0–190.0) versus 16.0 U/ml (7.0–77.0), respectively,  $P < 0.0001$ ].

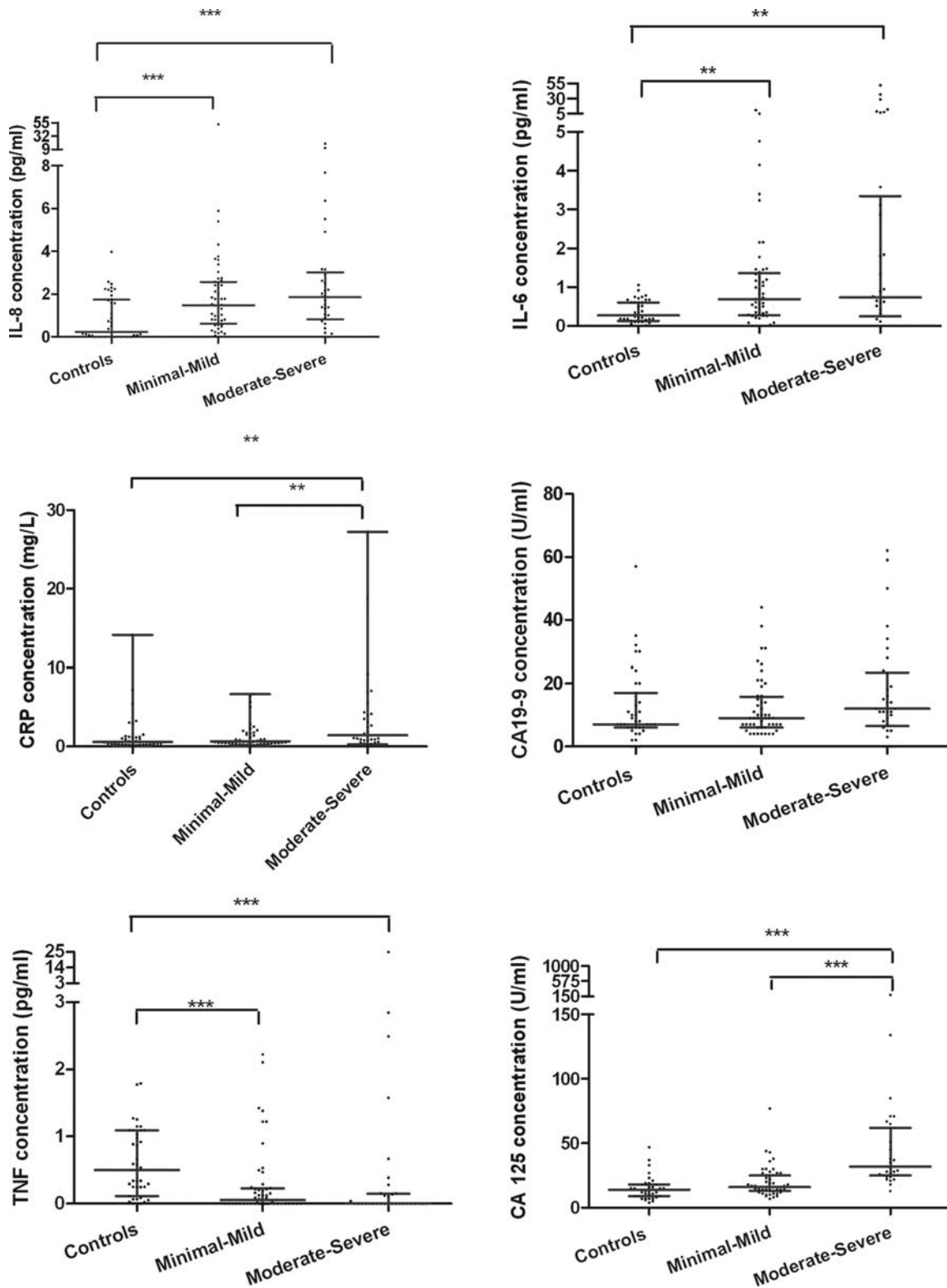
### Stepwise logistic regression models

First, we analysed the complete data set according to disease stage regardless of the cycle phase. This implies that we built a model for all endometriosis patients, for patients with minimal–mild endometriosis only and for patients with moderate–severe endometriosis only, each time compared with controls. The first three rows of Table II show the performance statistics and the selected proteins of these logistic regression models. The logistic regression model for distinguishing between controls and patients with moderate–severe endometriosis had the best performance (AUC of 0.934 and sensitivity of 91.3%), but the model was not good enough to distinguish between controls and women with minimal–mild endometriosis (AUC of 0.736, sensitivity of 95.5%, specificity of 39.8%).

Secondly, we analysed the data set according to disease stage and according to cycle phase (Table II). The protein markers were selected



**Figure 1** Women with endometriosis compared with controls during the secretory phase. Increased plasma levels for IL-8 ( $P < 0.0001$ ), IL-6 ( $P = 0.0003$ ) and CA-125 ( $P < 0.0001$ ), hsCRP ( $P = 0.03$ ) and decreased plasma levels for TNF- $\alpha$  ( $P < 0.0001$ ) were found in women with endometriosis. \* $P < 0.05$ , \*\*\* $P < 0.001$ .



**Figure 2** Women with minimal–mild and moderate–severe endometriosis compared with controls during the secretory phase. When compared with controls, women with minimal–mild endometriosis had increased plasma levels of IL-8 ( $P = 0.0003$ ) and IL-6 ( $P = 0.001$ ) and decreased levels of TNF- $\alpha$  ( $P < 0.0001$ ), and women with moderate–severe endometriosis had decreased plasma levels of TNF- $\alpha$  ( $P < 0.0001$ ) and increased plasma levels of IL-6 ( $P = 0.001$ ), IL-8 ( $P = 0.0003$ ), hsCRP ( $P = 0.001$ ) and CA-125 ( $P < 0.0001$ ). When compared with women with minimal–mild endometriosis, those with moderate–severe disease had increased plasma levels of hsCRP ( $P = 0.001$ ) and CA-125 ( $P < 0.0001$ ). \*\* $P < 0.01$  \*\*\* $P < 0.001$ .



**Table II** Logistic regression model performance: AUC, sensitivity, specificity, accuracy, PPV and NPV for logistic regression models according to cycle phase and disease stage

Cycle phase	Stage	Selected proteins	AUC	Sensitivity*	Specificity*	Accuracy	PPV	NPV	LR+	LR-
All	Ctrl versus All	IL-8, CA-125	0.790	71.3	71.0	71.2	84.2	53.2	2.46	0.40
All	Ctrl versus I, II	IL-8, CA-125	0.736	95.5	39.8	72.6	69.4	86.0	1.59	0.11
All	Ctrl versus III, IV	IL-6, TNF- $\alpha$ , CA-125	0.934	91.3	86.0	88.3	82.9	93.0	6.52	0.10
Menstrual	Ctrl versus All	CA-125	0.817	80.5	73.7	78.3	86.8	63.6	3.06	0.26
Menstrual	Ctrl versus I, II	IL-6, TNF- $\alpha$	0.814	88.5	63.2	77.8	76.7	80.0	2.40	0.18
Menstrual	Ctrl versus III, IV	CA-125	0.951	100.0	73.7	85.3	75.0	100.0	3.80	0.00
Proliferative	Ctrl versus All	CA-125	0.731	65.1	72.2	67.2	84.4	47.3	2.34	0.48
Proliferative	Ctrl versus I, II	CA-125	0.679	58.3	72.2	63.5	77.8	51.0	2.10	0.58
Proliferative	Ctrl versus III, IV	CA-125	0.867	82.6	72.2	76.3	65.5	86.7	2.97	0.24
Secretory	Ctrl versus All	IL-8, TNF- $\alpha$ , CA-125	0.852	89.7	71.1	83.6	86.4	77.1	3.10	0.14
Secretory	Ctrl versus I, II	IL-6, TNF- $\alpha$	0.845	87.2	71.1	80.0	78.8	81.8	3.02	0.18
Secretory	Ctrl versus III, IV	IL-6, TNF- $\alpha$ , CA-125	0.966	100.0	84.2	91.3	83.8	100.0	6.33	0.00

AUC, area under the ROC curve; PPV, positive predictive value; NPV, negative predictive value; LR+, positive likelihood ratio; LR-, negative likelihood ratio.

\*The operating point on the ROC was chosen by maximizing the sum of the sensitivity and specificity with the following constraints: sensitivity >90% or specificity >60%.

using a stepwise logistic regression, meaning that iteratively the best marker is added to the model based on *P*-value statistics of the inserted marker and the markers already in the model. After adding the best marker, each marker is again tested to see if it is still significant; if it is not, it is removed from the model. The procedure stops when the marker that is added to the model is the same as the one that is removed from the model. The stepwise procedure is thus a forward selection (which involves starting with no variables in the model, trying out the variables one by one and including them if they are 'statistically significant') and a backward elimination (which involves starting with all candidate variables and testing them one by one for statistical significance, deleting any that are not significant). This entails that other combinations of markers were tested. The final model is chosen based on statistical significance of all of the markers in the model.

Overall, the best results were obtained in the secretory phase and the worst results in the proliferative phase, regardless of disease stage. However, the performance of the reported models was not significantly different due to small data set sizes in each subgroup (unpaired ROC curve comparison). The logistic regression model for distinguishing between controls and patients with moderate-severe endometriosis had an AUC of 0.966, a sensitivity of 100% and a specificity of 84.2%, whereas the model for distinguishing between controls and women with minimal-mild endometriosis had an AUC of 0.845, a sensitivity of 87.2% and a specificity of 71.1%. By building models for each cycle phase separately, it was possible that different proteins were selected for each cycle phase. This was observed in our results: in all but one case, CA-125 was the only variable in the models for proliferative and menstrual phase while the logistic regression models for secretory phase were based on IL-6, IL-8, TNF- $\alpha$  or CA-125 (Table II).

Thirdly, we compared the univariate logistic regression model for all six proteins with the multivariate logistic regression model during the secretory phase (Table III). In all but three comparisons, the multivariate model was statistically better to distinguish between women

with endometriosis and controls. In two of these three comparisons, the multivariate model was borderline but not significantly better than the univariate TNF- $\alpha$  model to distinguish between controls and all endometriosis patients ( $P = 0.057$ ) or between controls and patients with minimal-mild endometriosis ( $P = 0.050$ ). In the other case, the multivariate model was not significantly better than the univariate CA-125 model to distinguish between controls and patients with moderate-severe endometriosis ( $P$ -value 0.088, paired ROC curve comparison).

Finally, we present the logistic regression model for the secretory phase. The logistic regression model provides the estimated probability of endometriosis for a particular patient. This probability is equal to  $y = 1/(1 + e^{-z})$ , where *e* is a mathematical constant, called Euler's number and where *z* is  $-1.3053 + 0.6010 (1) + 0.0918 (2) - 1.4517 (3)$  for the control versus all diseased patients model, *z* is  $-0.3953 - 2.0883 (3) + 2.8778 (4)$  for the control versus early stage model and *z* is  $-4.4511 + 0.1447 (2) - 3.6299 (3) + 4.3599 (4)$  for the control versus advanced stage disease model with (1) IL-8 (pg/ml), (2) CA-125 (kU/l), (3) TNF- $\alpha$  (pg/ml) and (4) IL-6 (pg/ml). These parameters are the log odds ratios of their corresponding proteins and can be interpreted as the log unit increase (or decrease depending on the sign) of the odds of having endometriosis. For example, in the last model, the odds of having endometriosis increase more than 78-fold for every unit increase of IL-6 and drops almost 38-fold for every unit increase of TNF- $\alpha$ .

### LSSVM modelling

Table IV shows the results of the LSSVMs on all data and selected for cycle phase or disease stage. The performance of LSSVM models was similar during the secretory phase and during the menstrual phase of the cycle (unpaired ROC curve comparison, data not shown) and appeared overall to be comparable to their corresponding multivariate logistic regression models (unpaired ROC curve comparison, data not shown).

**Table III** Univariate logistic regression model performance for secretory phase and their comparison with corresponding multivariate logistic regression models

Protein	Disease stage	AUC	Sensitivity	Specificity	Accuracy	PPV	NPV	P-value*	LR+	LR-
CA-125	Ctrl versus All	0.772	67.9	71.1	69.0	82.8	51.9	0.039	2.35	0.45
CA-125	Ctrl versus I, II	0.676	48.9	71.1	58.8	67.6	52.9	0.016	1.69	0.72
CA-125	Ctrl versus III, IV	0.917	96.8	71.1	82.6	73.2	96.4	0.088	3.35	0.05
CA-19-9	Ctrl versus All	0.567	35.9	71.1	47.4	71.8	35.1	<0.001	1.24	0.90
CA-19-9	Ctrl versus I, II	0.552	31.9	71.1	49.4	57.7	45.8	<0.001	1.10	0.96
CA-19-9	Ctrl versus III, IV	0.590	41.9	71.1	58.0	54.2	60.0	<0.001	1.45	0.82
hsCRP	Ctrl versus All	0.625	41.0	71.1	50.9	74.4	37.0	0.001	1.42	0.83
hsCRP	Ctrl versus I, II	0.447	17.0	73.7	42.4	44.4	41.8	<0.001	0.65	1.13
hsCRP	Ctrl versus III, IV	0.734	54.8	71.1	63.8	60.7	65.9	<0.001	1.90	0.64
IL-6	Ctrl versus All	0.705	59.0	76.3	64.7	83.6	47.5	0.018	2.49	0.54
IL-6	Ctrl versus I, II	0.710	55.3	76.3	64.7	74.3	58.0	0.017	2.33	0.59
IL-6	Ctrl versus III, IV	0.697	64.5	76.3	71.0	69.0	72.5	<0.001	2.72	0.47
IL-8	Ctrl versus All	0.716	48.7	71.1	56.0	77.6	40.3	0.004	1.69	0.72
IL-8	Ctrl versus I, II	0.693	42.6	71.1	55.3	64.5	50.0	0.021	1.47	0.81
IL-8	Ctrl versus III, IV	0.750	58.1	71.1	65.2	62.1	67.5	<0.001	2.01	0.59
TNF- $\alpha$	Ctrl versus All	0.758	79.5	73.7	77.6	86.1	63.6	0.057	3.02	0.28
TNF- $\alpha$	Ctrl versus I, II	0.740	78.7	73.7	76.5	78.7	73.7	0.050	2.99	0.29
TNF- $\alpha$	Ctrl versus III, IV	0.787	80.6	73.7	76.8	71.4	82.4	0.003	3.06	0.26

AUC, area under the ROC curve; PPV, positive predictive value; NPV, negative predictive value; LR+, positive likelihood ratio; LR-, negative likelihood ratio.

\*P-value when comparing the ROC curve of the single variable model with its corresponding multivariate model from Table II.

**Table IV** LSSVM model performance: AUC, sensitivity, specificity, accuracy, PPV and NPV for LSSVM models according to cycle phase and disease stage

Protein	Disease stage	AUC	Sensitivity*	Specificity*	Accuracy	PPV	NPV	LR+	LR-
All	Ctrl versus All	0.783	90.1	52.7	78.3	80.5	71.0	1.90	0.19
All	Ctrl versus I, II	0.753	80.5	60.2	72.1	74.1	67.5	2.02	0.32
All	Ctrl versus III, IV	0.910	91.3	80.6	85.2	77.8	82.6	4.71	0.11
Menstrual	Ctrl versus All	0.851	90.2	73.7	85.0	88.1	77.8	3.43	0.13
Menstrual	Ctrl versus I, II	0.852	92.3	63.2	80.0	77.4	85.7	2.51	0.12
Menstrual	Ctrl versus III, IV	0.972	93.3	89.5	91.2	87.5	94.4	8.89	0.07
Proliferative	Ctrl versus All	0.718	57.8	72.2	62.2	82.8	42.6	2.08	0.58
Proliferative	Ctrl versus I, II	0.702	65.0	63.9	64.6	75.0	52.3	1.80	0.55
Proliferative	Ctrl versus III, IV	0.880	87.0	72.2	78.0	66.7	89.7	3.13	0.18
Secretory	Ctrl versus All	0.834	85.9	71.1	81.0	85.9	71.1	2.97	0.20
Secretory	Ctrl versus I, II	0.808	93.6	60.5	78.8	74.6	88.5	2.37	0.11
Secretory	Ctrl versus III, IV	0.947	96.6	84.2	89.9	83.3	97.0	6.11	0.04

AUC, area under the ROC curve; PPV, positive predictive value; NPV, negative predictive value; LR+, positive likelihood ratio; LR-, negative likelihood ratio.

\*The operating point on the ROC was chosen by maximizing the sum of the sensitivity and specificity with the following constraints: sensitivity >90% or specificity >60%.

When compared with the multivariate logistic regression model, the diagnosis of minimal–mild endometriosis could be made during the secretory phase with somewhat higher sensitivity (93.6% versus 87.2%) at the cost of a somewhat lower specificity (60.5% versus 71.1%) by using the LSSVM model. Interestingly, the LSSVM model also appeared superior to the multivariate logistic regression model in the diagnosis of minimal–mild endometriosis during the menstrual

phase with respect to the sensitivity (92.3% versus 88.5%) and specificity (63.2% versus 63.2%).

## Discussion

The data of our study show that it is possible to diagnose minimal–mild endometriosis using plasma analysis of multiple biomarkers

combined with advanced statistical analysis with a high sensitivity (87–92%) and an acceptable specificity (60–71%) during the secretory phase and the menstrual phase. This observation is very relevant for clinical practice, especially for women of reproductive age with the active or passive desire to become pregnant later in life. Early non-invasive diagnosis of minimal–mild endometriosis (ASRM, 1997) in women who try to conceive should enable gynaecologists to select these women for laparoscopic excision of endometriosis which improves fertility (Kennedy *et al.*, 2005) and may prevent progression of endometriosis to a moderate-to-severe stage. In the presence of subfertility with a history of cyclic or chronic pelvic pain, combined with a clinical examination which is positive for pain, and/or an ultrasound positive for ovarian endometriotic cysts or deep endometriotic nodules, the probability of endometriosis is so high that most gynaecologists will offer the patient a laparoscopy combined with excision of all visible endometriotic lesions, without the need for a non-invasive diagnostic test (D'Hooghe *et al.*, 2006). However, if women have a regular cycle, a partner with a normal sperm examination, and if they have been trying unsuccessfully to conceive for more than 1 year with or without significant pelvic pain combined with a normal clinical examination and a normal pelvic ultrasound, most gynaecologists are not sure if endometriosis is present and whether it is useful to do a diagnostic laparoscopy. From a clinical perspective, it is unlikely that these women will have moderate-to-severe endometriosis, but up to 50% of them (Meuleman *et al.*, 2009) may have extensive peritoneal endometriosis with or without adhesions associated with subfertility and possibly pain. For this population, a non-invasive diagnostic test would be useful to rule out those without endometriosis and those with endometriosis, most likely minimal-to-mild disease, who are known to benefit from surgical therapy for both subfertility and pain and from controlled ovarian stimulation in combination with intrauterine insemination for subfertility (D'Hooghe *et al.*, 2003, 2006; Kennedy *et al.*, 2005). It would not be a problem if the test would also be diagnostic for women with other fertility reducing pelvic pathology such as pelvic adhesions or chronic PID since these women would also benefit from laparoscopic diagnosis and possibly surgical treatment (D'Hooghe *et al.*, 2006). The most important goal of the test is that no women with endometriosis or other significant pelvic pathology, who might benefit from laparoscopic surgery, are missed. To achieve this, a test with a high sensitivity is needed, with a low number of false negative results, i.e. a low number of patients who have a negative test and who do have endometriosis or other significant pelvic pathology justifying surgery. A high specificity implies a low number of false positive results, i.e. a low number of patients who have a positive test but who do not have endometriosis or other pelvic pathology requiring surgery. This is less important in daily clinical practice, since a laparoscopy in this subset of women with subfertility would not only be useful to diagnose and treat endometriosis, but also to assess tubal patency, to rule out other pelvic pathology associated with infertility or pain and to document uterine/endometrial morphology via hysteroscopy during the same surgery session. Taking into account this clinical perspective, a diagnostic test with a sensitivity as high as 100% would be ideal, even if the specificity would be only 50% (D'Hooghe *et al.*, 2006). The results of our study (sensitivity ~90%; specificity 60–71%) come close to this ideal.

The results of our study are new and unique due to the high sensitivity (90%) of our test for the diagnosis of minimal–mild endometriosis, based on the combined analysis of six biomarkers, the application of advanced statistics, the large and well-defined patient population, and the differential analysis according to the phases of the menstrual cycle (menstrual, follicular and luteal). The only two other groups of investigators (Gagne *et al.*, 2003; Martinez *et al.*, 2007) who have addressed these issues reported lower sensitivities for the diagnosis of minimal–mild endometriosis (Table V). In one study, a serum IL-6 threshold of 25.75 pg/ml afforded a sensitivity of only 75% and specificity of 83% in the diagnosis of minimal–mild endometriosis (Martinez *et al.*, 2007) (Table V), but the combination of serum IL-6 and CA-125 did not offer any additional value. In the other study, a predictive model based on combined serum (CA-125), endometrium (leukocyte subtypes) and clinical (length of menses) assessment achieved a sensitivity of only 61% and specificity of 95% in the diagnosis of minimal–mild endometriosis (Gagne *et al.*, 2003) (Table V). The diagnostic potential of various panels of combined biomarkers presented in four other reports (Bedaiwy *et al.*, 2002; Agic *et al.*, 2008; Othman *et al.*, 2008; Seeber *et al.*, 2008) was not analysed separately for women with minimal–mild endometriosis and for those with moderate–severe disease (Table V).

Our results show that multivariate methods such as logistic regression and LSSVMs in general perform better than single protein models, suggesting that more than one protein is necessary to predict the presence of endometriosis. Moreover, the performance of these models depends heavily on cycle phase. The logistic regression models were better for predicting moderate–severe disease, whereas the LSSVM models had a higher sensitivity, at the cost of lower specificity, for predicting minimal–mild disease. More data should be gathered to assess which model strategy is superior since the performance of both model strategies was not significantly different. The logistic regression models, however, have an advantage since they are based on a selection of biomarkers and can easily be interpreted using the odds ratios of the participating biomarkers. For all multivariate models and during all cycle phases, it was easier to diagnose women with moderate–severe disease than those with minimal–mild endometriosis. According to the rule of thumb that the sensitivity and specificity of a good test should add up to  $\geq 1.5$ , and those of a very good test should add up to  $\geq 1.8$  (Griffith and Grimes, 1990), our secretory phase test was very good for diagnosing moderate–severe endometriosis (1.84 using stepwise logistic regression analysis), and was good for diagnosing minimal–mild endometriosis (1.59 using stepwise logistic regression; 1.55 using LSSVM analysis).

A possible limitation of our study is that stress factors directly before surgery might have affected plasma biomarker levels, as blood was drawn just prior to anaesthesia. For a general diagnostic test, it would be preferential to perform the blood drawing independently of the surgery. 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.

Prospective testing of the reported models is needed to determine their generalization performance and to test which cycle phase



**Table V** Performance of predictive models in the diagnosis of endometriosis

Combination of tested biomarkers	Predictive model	Control	Endometriosis	Phase of menstrual cycle	Sensitivity/specificity	PPV/NPV	AUC	Authors
IL-1 $\beta$ , IL-6, IL-8, IL-12, IL-13, TNF- $\alpha$	IL-6 (cut-off 2 pg/ml)	27	56 (I–IV), 34 (I–II), 22 (III–IV)	Follicular and luteal	90%/67%	Information not available	87%	Bedaiwy <i>et al.</i> (2002)
Serum CA-125 level, proportion of endometrial leukocytes: CD3 <sup>+</sup> , CD16 <sup>+</sup> , CD3 <sup>-</sup> HLADR <sup>-</sup> , CD3 <sup>-</sup> CD45RA <sup>-</sup> , CD3 <sup>+</sup> CD16 <sup>-</sup> , CD3 <sup>+</sup> CD56 <sup>-</sup> , CD56 <sup>-</sup> CD16 <sup>+</sup> , CD16b <sup>+</sup>	Serum CA-125 level, proportion of endometrial leukocytes CD3 <sup>+</sup> , CD16 <sup>+</sup> , CD3 <sup>-</sup> HLADR <sup>-</sup> , CD3 <sup>-</sup> CD45RA <sup>-</sup> CD3 <sup>+</sup> CD16 <sup>-</sup> , CD3 <sup>+</sup> CD56 <sup>-</sup> , CD56 <sup>-</sup> CD16 <sup>+</sup> , CD16b <sup>+</sup> and length of menses	195	173 (I–IV), Stages: I–II	Luteal	61%/95%	91%/75%	0.819	Gagne <i>et al.</i> (2003)
CA-125, CA 19-9, IL-6	CA-125, CA 19-9, IL-6	35	Stages: III–IV 45 (I–IV), 14 (I–II), 31 (III–IV)	All phases	61%/95% 42%/71%	91%/75% 66%	0.896 Information not available	Somigliana <i>et al.</i> (2004)
CCR1 mRNA, CA-125, MCP-1	CCR1 mRNA, CA-125, MCP-1	28	66 (no information given regarding stage of endometriosis)	Information not available	95.4%/82.1%	92.6%/88.5%	Information not available	Agic <i>et al.</i> (2008)
IL-6, CA-125	IL-6 (cut-off 25.75 pg/ml)	38	47 (I–IV), 11 (I–II), 36 (III–IV), 11 (I–II) ONLY	Follicular	75.0%/83.3%	65.8%/88.6%	0.829	Martinez <i>et al.</i> (2007)
IL-6, CA-125	CA-125 (cut-off 35 IU/L)	38	36 (III–IV) ONLY	Follicular	47.2%/97.5%	89.0%/81.1%	0.812	Martinez <i>et al.</i> (2007)
IL-6, TNF- $\alpha$ , MIF, MCP-1, IFN- $\gamma$ , Leptin, CA-125	CA-125, MCP-1	78	63 (II–IV)	Follicular, non-follicular, unknown	95%/44%	Information not available	Information not available	Seeber <i>et al.</i> (2008)
IL-6, TNF- $\alpha$ , MIF, MCP-1, IFN- $\gamma$ , Leptin, CA-125	CA-125, MCP-1, Leptin	78	63 (II–IV)	Follicular, non-follicular, unknown	49%/94%	Information not available	Information not available	Seeber <i>et al.</i> (2008)
IL-6, TNF- $\alpha$ , MIF, MCP-1, IFN- $\gamma$ , Leptin, CA-125	CA-125, MCP-1, Leptin, MIF	78	63 (II–IV)	Follicular, non-follicular, unknown	100%/40%	Information not available	Information not available	Seeber <i>et al.</i> (2008)
IL-2, IL-6, IL-8, IL-15, MCP-1, IFN- $\gamma$ , VEGF, TNF- $\alpha$ , GM-CSF	IL-6 (cut-off 1.03 pg/ml)	70	68 (I–IV), 32 (I–II), 36 (III–IV)	Follicular, luteal	81%/51%	Information not available	Information not available	Othman <i>et al.</i> (2008)
IL-2, IL-6, IL-8, IL-15, MCP-1, IFN- $\gamma$ , VEGF, TNF- $\alpha$ , GM-CSF	IL-6 (cut-off 1.9 pg/ml)	70	68 (I–IV), 32 (I–II), 36 (III–IV)	Follicular, luteal	71%/66%	Information not available	Information not available	Othman <i>et al.</i> (2008)

AUC, area under the ROC curve; PPV, positive predictive value; NPV, negative predictive value; IL, interleukin; TNF- $\alpha$ , tumour necrosis factor-alpha; CA-125, cancer antigen; CCR1 mRNA, cognate chemokine receptor 1 messenger ribonucleic acid; MIF, macrophage migration inhibitory factor; MCP-1, macrophage chemotactic protein-1; IFN- $\gamma$ , interferon-gamma; VEGF, vascular endothelial growth factor; GM-CSF, granulocyte macrophage colony stimulating factor.

significantly outperforms the other cycle phases. A validation study using an independent patient population is needed and has been planned for the next phase of our research programme.

## Authors' role

Study concept and design: A.M., F.D.S., P.S., C.M.K., T.M.D.

Acquisition of data: A.M., P.S., C.M.K., N.B., J.B.

Analysis and interpretation of data: A.M., P.S., N.P., A.V., V.F., O.G., C.M.K., T.M.D., F.D.S.

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## Funding

This work was supported by grants from the Leuven University Council (Dienst Onderzoekcoördinatie, KU Leuven), the Flemish Fund for Scientific Research (FWO), and Toegepast Biomedisch onderzoek met een primair Maatschappelijke finaliteit (TBM), Leuven, Belgium.

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Submitted on July 6, 2009; resubmitted on October 20, 2009; accepted on November 6, 2009