Proteomics Analysis of Plasma for Early Diagnosis of Endometriosis

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OBJECTIVE: To test the hypothesis that differential surface-enhanced laser desorption/ionization time-of-flight mass spectrometry protein or peptide expression in plasma can be used in infertile women with or without pelvic pain to predict the presence of laparoscopically and histologically confirmed endometriosis, especially in the subpopulation with a normal preoperative gynecologic ultrasound examination.

METHODS: Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry analysis was performed on 254 plasma samples obtained from 89 women without endometriosis and 165 women with endometriosis (histologically confirmed) undergoing laparoscopies for infertility with or without pelvic pain. Data were analyzed using least squares support vector machines and were divided randomly (100 times) into a training data set (70%) and a test data set (30%).

RESULTS: Minimal-to-mild endometriosis was best predicted (sensitivity 75%, 95% confidence interval [CI] 63–89; specificity 86%, 95% CI 71–94; positive predictive value 83.6%, negative predictive value 78.3%) using a model based on five peptide and protein peaks (range 4.898–14.698 m/z) in menstrual phase samples. Moderate-to-severe endometriosis was best predicted (sensitivity 98%, 95% CI 84–100; specificity 81%, 95% CI 67–92; positive predictive value 74.4%, negative predictive value 98.6%) using a model based on five other peptide and protein peaks (range 2.189–7.457 m/z) in luteal phase samples. The peak with the highest intensity (2.189 m/z) was identified as a fibrinogen β-chain peptide. Ultrasonography-negative endometriosis was best predicted (sensitivity 88%, 95% CI 73–100; specificity 84%, 95% CI 71–96) using a model based on five peptide peaks (range 2.058–42.065 m/z) in menstrual phase samples.

CONCLUSION: A noninvasive test using proteomic analysis of plasma samples obtained during the menstrual phase enabled the diagnosis of endometriosis undetectable by ultrasonography with high sensitivity and specificity.

LEVEL OF EVIDENCE: II

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Endometriosis is an enigmatic, benign, estrogen-dependent disease associated with infertility and pain. The most effective approach to manage endometriosis would be through early diagnosis. However, in many cases, endometriosis is not diagnosed and

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treated until the disease has established itself and caused pathological symptoms. At present, the only way to conclusively diagnose endometriosis is through laparoscopic inspection, preferably with histological confirmation.[1] This contributes to the diagnostic delay of endometriosis (between the onset of symptoms and a diagnosis) of 8–11 years.[2,3] Because current evidence suggests that endometriosis can be progressive in 50%,[4] early noninvasive diagnosis has the potential to offer early treatment and prevent progression.

Currently, there are no blood tests for the diagnosis of endometriosis.[1] In peripheral blood, neither a single biomarker nor a panel of biomarkers has been validated as a noninvasive test for endometriosis.[5] In a clinical practice dealing with women with subfertility with or without pain, a noninvasive test of endometriosis with high sensitivity would allow to identify those women with endometriosis who could benefit from laparoscopic surgery reported to improve these symptoms, ie, increase fertility and decrease pain.[1,6] Ideally, decreased levels of such a biomarker during or after treatment would also correlate with decreased pelvic pain and increased fertility. Such a test would especially be useful in women with endometriosis, which cannot be detected during gynecological ultrasonography examination. Transvaginal ultrasonography 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.[1,7]

In peripheral blood, earlier surface-enhanced laser desorption/ionization coupled to time-of-flight mass spectrometry investigations[8–14] have shown differentially expressed protein or peptides in women with and without endometriosis. This research is generally compromised by unclear patient characterization with respect to cycle phase, endometriosis stage, control group, limitations in the number of chip types tested (maximum of one), lack of well-described reproducibility studies, and lack of identification of peptide and protein peaks. In this study, we tested the hypothesis that differential surface-enhanced laser desorption/ionization time-of-flight mass spectrometry protein or peptide expression in plasma can be used in infertile women with or without pelvic pain to predict the presence of laparoscopically and histologically confirmed endometriosis, especially in the subpopulation with a normal gynecologic ultrasonogram preoperatively.

MATERIALS AND METHODS

A total of 254 plasma samples collected previously (2001–2009) from women at the time when they received laparoscopy for infertility with or without pelvic pain and that had been frozen at −80°C and stored in our biobank were selected for this study (Table 1). All patients had signed a written informed

Table 1. Clinical Characteristics of the Study Population

	All Study Population (n=254)	Control Group (n=89)	Endometriosis (n=165)	Ultrasound-Negative Endometriosis (n=113)	Stage I–II (n=89)	Stage III–IV (n=76)
Age (y)						
Mean±SD	31.74±4.59	32.32±5.19	31.44±4.24	31.28±4.13	31.5±4.07	31.7±4.47
Median (range)	31 (23–44)	32 (23–44)	31 (23–44)	31 (23.5–40)	31 (23.6–44)	31 (23–41)
Subfertility	240	82	158	109	86	72
Pain symptoms						
Dysmenorrhea	177	56	121	80	59	62
Dyspareunia	67	20	47	30	25	22
Chronic pelvic pain	30	9	21	12	10	11
Dyschezia	17	4	13	7	4	9
Cycle phase						
Menstrual	68	22	45	29	23	22
Follicular	98	33	65	45	33	32
Luteal	88	33	55	38	33	22
Cycle information						
Regular cycle	198	67	131	89	69	62
Irregular cycle	40	17	23	17	15	8
Other pelvic pathology						
Myoma	16	8	8	5	4	4

SD, standard deviation.
Data are n unless otherwise specified.

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consent before recruitment and the study protocol was approved by the institutional ethical and review board of University Hospital Gasthuisberg.

Samples were selected specifically to have an equal representation according to cycle phase of the menstrual cycle and according to the presence or absence of endometriosis (Table 1). Plasma samples from patients using hormonal medication (combined oral contraceptive pill or progestins or gonadotropin-releasing hormone analogs) and from patients operated within 6 months before the time of sample collection were excluded. Endometriosis (n=165) was classified according to the most recent classification by the American Society of Reproductive Medicine[15] and had been histologically confirmed in all patients. A subset analysis was done on samples collected from 113 women with laparoscopically confirmed endometriosis without evidence of endometriosis on preoperative gynecological ultrasonography (minimally to severe, n=113; minimal to mild, n=81; moderate to severe, n=32) obtained during menstrual (n=52), follicular (n=76), or luteal (n=69) cycle phases. The absence of endometriosis was documented by laparoscopy in 89 control patients.[16]

Peripheral blood samples were collected before anesthesia using 4×4-mL ethylenediamine tetraacetic acid Vacutainer tubes through venipuncture or central venous line. These blood samples were centrifuged at 3,000 rpm for 10 minutes at 4°C, and the plasma was aliquoted and stored at −80°C until analysis. The median time interval between sample collection and storage at −80°C was 50 minutes (25–60 minutes). Before the actual study, we investigated the most appropriate method to deplete plasma from highly abundant proteins and identified the best performing surface-enhanced laser desorption ionization chip surfaces in preliminary experiments in two plasma samples from patients with endometriosis who were not included in the actual study.

Plasma depletion is important to allow detection of low abundant proteins or peptides that cannot be observed in native plasma as a result of the presence of highly abundant proteins (Box 1). After depletion, the remaining plasma proteins or peptides can be loaded onto a proteinchip array (surface-enhanced laser desorption ionization target plate) in more concentrated levels, improving their detection. We compared a Proteominer depletion kit from Bio-Rad with an ultrafiltration method using two Microcon filters allowing the filtration of proteins or peptides with molecular weight lower than, respectively, 30 kDa and 50 kDa in the collection tube and used untreated plasma and plasma treated with U9 buffer as control samples. The Proteominer kit was selected because separation and elution were nonreproducible using ultrafiltration methods (plasma tended to block the filters) and because better enriched spectra (more low abundant proteins or peptides peaks) were observed when compared with ultrafiltration methods. Subsequently, we selected the CM10, Q10, and H50 surface-enhanced laser desorption ionization chip surfaces because they rendered more enriched spectra (more low abundant proteins or peptides peaks) than the IMAC chip surface.

Box 1. Description of the 22 Most Abundant Proteins Representing Approximately 99% of the Total Protein Mass in Human Plasma

Protein		
Albumin	90%	99%
IgGs		
Transferrin		
Fibrinogen		
IgAs		
Alpha-2-macroglobulin		
IgMs		
Alpha-1-antitrypsin		
Complement C3		
Haptoglobin		
Apolipoprotein A1		
Apolipoprotein B		
Acid-1-glycoprotein		
Ceruloplasmin		
Complement C4		
Complement C1q		
Prealbumin		
Plasminogen		
Lipoprotein(a)		
Complement factor H		
Complement factor B		
Complement C9		

(90%: Albumin through Haptoglobin; 99%: all 22 proteins)

Ig, immunoglobulin.
Data from Tirumalai RS, Chan KC, Prieto DA, Issaq HJ, Conrads TP, Veenstra TD. Characterization of the low molecular weight human serum proteome. Mol Cell Proteomics 2003;2:1096–103.

First, the frozen plasma was thawed on ice. The depletion kit from Bio-Rad was used to deplete the most abundant proteins (Box 1). According to the manufacturer's instructions, we added 800 microliters to 1 mL of plasma sample to the column. The elution contained the protein or peptide of interest and was stored at −80°C until the experiment.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry was used to analyze 254 samples for the obtained albumin depletion (focus at approximately 66 kDa). The protein concentration of each sample was measured with the aid of BCA Protein assay kit. The eluted fraction was screened

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using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry on three different surfaces (CM10, Q10, H50).

To enhance reproducibility across the different surfaces of the project, a reference sample was spotted in duplicates on each surface to calculate experimental intra and intercoefficient of variations and to optimize array reading parameters (such as laser intensity, focus mass, and mass range). The reference sample was taken from a pool (5 mL) of randomly selected plasma samples (500 microliters per patient) from five women with endometriosis and from five control participants without endometriosis.

Surface-enhanced laser desorption ionization time-of-flight mass spectrometry, employs a 8–16 spot chip and each spot contains a solid-phase chromatographic surface for binding proteins at a particular binding condition.[17] There are several types of surface-enhanced laser desorption ionization target plate arrays with different chromatographic properties, including hydrophobic, hydrophilic, anion and cation exchange, and metal affinity. These properties enable them to capture different subsets of proteins according to their physicochemical properties.[17]

To increase the number of detectable proteins, three different chip surfaces (CM10, Q10, and H50) with distinct chromatographic properties and binding affinities were used (Table 2). Briefly, proteinchip array spots of H50 first were preactivated by applying 5 microliters of 50% acetonitrile and incubated in a humidity chamber for 5 minutes. Subsequently, the spots were equilibrated twice with 150 microliters of corresponding binding buffer while shaking for 5 minutes at room temperature. Proteinchip array spots of CM10 and Q10 were equilibrated directly with 150 microliters of corresponding binding buffer while shaking for 5 minutes at room temperature to preactivate binding surfaces.

For all three surfaces (CM10, Q10, and H50), the equilibration buffer was removed and 10 microliters of sample (15 micrograms per spot) diluted with surface-type-dependent binding buffer (Table 2) was loaded onto each spot in duplicate and incubated for 30 minutes at room temperature while being shaken. The unbound proteins or peptides on the proteinchip array surfaces were washed away with appropriate buffer (see Table 2) three times for 5 minutes while being shaken and rinsed twice in 150 microliters of Milli-Q water. The water was removed and the surface was centrifuged upside down lying on Whatman paper at 1,000 g for 2 minutes. Mass spectra of the bound proteins were obtained by ionizing the proteins using two types of energy-absorbing molecules: alpha-cyano-4-hydroxy cinnamic acid, for small molecules (less than 15 kDa) and sinapinic acid for larger molecules. One microliter of 20% alpha-cyano-4-hydroxy cinnamic acid was applied twice onto the retained proteins on the spots. Fifty percent saturated solution of sinapinic acid was applied in two consecutive steps in volumes of 1 microliter. Analyses of the retained peptides and proteins were performed on a Protein Chip System Series 4000 surface-enhanced laser desorption ionization time-of-flight mass spectrometry instrument. Calibration was performed using all-in-one peptide molecular mass standard for the mass range of 1.6–20 kDa and all-in-one protein molecular mass standard for the mass range of 8–150 kDa.

Table 2. Different Surface-Enhanced Laser Desorption/Ionization Target Plate Surfaces With Their Respective Binding Buffer Used in the Study

Surface-Enhanced Laser Desorption/Ionization Target Plate Surfaces	Binding Buffers
Weak cation exchange surface (CM10)	Low stringency binding buffer (50 mM NaOAC, pH 4.0)
Hydrophobic surface (H50)	10% acetonitrile, 0.1% triflouroacetic acid
Strong anion exchange surface (Q10)	50 mM Tris-HCl, pH 8.0

Matrix-assisted laser desorption/ionization time-of-flight and time-of-flight mass spectrometry was used for identification of the resulting plasma peaks using the Ultraflex II MS equipped with a 200-Hz Smartbeam laser. The surface-enhanced laser desorption ionization time-of-flight mass spectra were baseline corrected and normalized on the basis of total ion current using the ProteinChip data manager software 3.5 and smoothed using a least squares polynomial filter in Matlab 7.

Differentially expressed mass peaks with $P>.15$ were removed from the analysis. Data were analyzed using custom scripts written in Matlab. The data analysis was performed first on all the samples and second only on the samples from women with a normal preoperative gynecological ultrasonographic examination.

For each of the different conditions (namely chip–matrix–cycle phase), three different setups were examined: control compared with minimal to mild, control compared with moderate to severe, and control compared with minimal to severe. For each of the three different setups, a "combined" spectrum was calculated combining relevant mass over charge (m/z)

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values from each of the two comparing conditions. Peak picking was then performed on this combined spectrum. The peaks were quantified using the peak height. Peaks below 2 kDa were excluded from data analysis as recommended by the manufacturing company and previous investigators.[18,19]

To attain robust results, the analysis was performed using repeated random subsampling crossvalidation.[20] First, the data set was randomly split into two stratified parts, the "training data set" (70% of the total data set) and the "test data set" (30% of the total data set). The "training data set" was used to identify a pattern that discriminates between the presence and absence of disease. Selected potential biomarkers were evaluated on a "test data set" (30%) of samples to determine sensitivity and specificity. For our repeated random subsampling, this split was repeated 100 times, because this method has been reported to produce replicable results.[20] The final model performance was then averaged over these 100 splits.

The presented algorithm is a feature selection algorithm, which means that it will search for those features, in our study the mass spectrometry peaks that best discern between the disease and control groups. These are the peaks with the highest interest for further research and the best peaks for classification.

Because the number of peaks that resulted from the peak picking was relatively high, on average 130 peaks per condition, feeding these peaks directly into the feature selection algorithm would have two major disadvantages: very long calculation times resulting from the high number of repeated subsamplings and model trainings and increased risk to select a possibly good but nonoptimal set of peaks. Therefore, we chose to eliminate those peaks with a *P* value higher than .15 that would not contribute to the selection of the optimal set of peaks. The *P* value was determined using the Wilcoxon rank-sum test, testing whether peaks in the diseased samples were differentially expressed when compared with the control samples. Using this method, we were able to decrease the number of peaks fed to the algorithm by a factor 7 to, on average, approximately 18 peaks per condition. These remaining peaks were then used to construct a least squares support vector machine (linear kernel, $\lambda=0.001$) model using leave-one-out crossvalidation. Least squares support vector machines are supervised machine learning algorithms.[21] In this model, each of the input peaks was ranked in terms of decision power. For each of the 100 constructed models, the five highest ranked peaks were stored. Of this list of 500 peptide peaks, the 20 most frequently observed peaks were selected. A least squares support vector machine model was constructed for each of the training data sets using only these 20 best performing peaks. Of this list of 20 best performing peaks, the five most frequently observed peaks were selected and used to construct the final least squares support vector machine model in the training set. This model was then validated using the independent "test data set" (30% of samples) to determine sensitivity and specificity in each of the 100 splits. Finally, the average performance of the model was calculated over the 100 splits.

RESULTS

The overall results including sensitivity and specificity are given in Table 3. In this plasma surface-enhanced laser desorption/ionization time-of-flight mass spectrometry study, the range of differentially expressed peaks varied between 0 and 92 (depending on chip type, matrix, and stages of endometriosis and phases of the cycle). Minimal-to-mild endometriosis was best predicted (sensitivity 75%, 95% confidence interval [CI] 63–89; specificity 86%, 95% CI 71–94; positive predictive value 83.6%, negative predictive value 78.3%) using a model based on five peptide and protein peaks (4,898 m/z, P=.034; 5,715 m/z, P=.035; 8,328 m/z, P=.040; 9,926 m/z, P=.037; 14.698 m/z, P=.039) in menstrual phase samples. Moderate-to-severe endometriosis was best predicted (sensitivity 98%, 95% CI 84–100; specificity 81%, 95% CI 67–92; positive predictive value 74.4%, negative predictive value 98.6%) using a model based on five other peptide and protein peaks (3,192 m/z, P=.018; 4,519 m/z, P=.027; 2.189 m/z, P=.030; 4,373 m/z, P=.040; 7,457 m/z, P=.002) in luteal phase samples. The peak with the highest intensity (2.189 m/z) was decreased in women with moderate-to-severe endometriosis (103.5±66.35; 87.61 [42.3–151]) when compared with those in the control group (158.9±86.02; 146.7 [94.43–207.7]; P=.035) and was identified as fibrinogen β-chain peptide.

Ultrasonography-negative laparoscopy confirmed endometriosis could be diagnosed by proteomic analysis (least squares support vector machine model for CM10 SPA data) of plasma samples obtained during the menstrual phase based on five peptide and protein peaks (2.058 m/z, P=.009; 2,456 m/z, P=.045; 3.883 m/z, P=.039; 14.694 m/z, P=.010; 42.065 m/z, P=.049) in a statistical model developed in the training data set. Data analysis of the test data set confirmed that this diagnosis was made with high accuracy (86.6%, 95% CI 73.3–100), sensitivity (88%, 95% CI 73–100), and specificity (84%, 95% CI 71–96; positive predictive value 75%, 95% CI

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Table 3. Diagnostic Performance (Sensitivity, Specificity) of Peptide and Protein Peaks for Endometriosis (Minimal to Mild, Moderate to Severe, Minimal to Severe) in the Test Set (30% of Samples) Based on the Least Squares Support Vector Machine Model Developed in the Training Set (70% of Samples)

	Cycle Phase					
	Follicular	Luteal	Menstrual	Follicular	Luteal	Menstrual
Minimal to Mild						
Surface	CM10 CHCA	CM10 CHCA	CM10 CHCA	CM10 SPA	CM10 SPA	CM10 SPA
	4,335.6	4,715.19	3,095.97	2,620.77	3,106.24	14,698.5
	5,997.28	4,368.76	2,304.12	9,774.43	5,720.3	8,328.23
	2,930.44	3,916.91	2,177.68	5,886.81	4,442.09	9,926.31
	3,728.68	7,889.62	4,683.69	7,246.31	10,070.7	5,715.95
	2,867.8	2,178.35	2,394.6	9,927.73	4,075.38	4,898.41
Sensitivity	51 (35–70)	51 (40–66)	58 (39–77)	77 (62–93)	61 (55–86)	75 (63–89)
Specificity	57 (46–89)	69 (52–85)	62 (44–81)	64 (47–79)	71 (63–93)	86 (71–94)
Surface	H50 CHCA	H50 CHCA	H50 CHCA	H50 SPA	H50 SPA	H50 SPA
	2,875.75	17,285.3	4,571.2	7,782.84	3,031.42	3,033.83
	3,768.48	8,573.92	5,757.11	4,887.49	9,293.26	3,322.1
	5,758.19	8,208.83	3,319.37	28,258.8	4,865.31	3,403.1
	6,683.93	4,349.64	14,069.4	3,398.86	9,515.35	2,822.84
	4,463.06	5,758.19	2,875.75	17,040.1	17,040.1	6,261.67
Sensitivity	52 (40–68)	49 (28–64)	57 (40–76)	57 (42–69)	49 (33–66)	62 (42–75)
Specificity	70 (53–86)	57 (38–82)	76 (52–91)	57 (41–72)	63 (43–72)	74 (57–89)
Surface	Q10 CHCA	Q10 CHCA	Q10 CHCA	Q10 SPA	Q10 SPA	Q10 SPA
	3,769.53	3,769.53	3,769.53	4,556.09	12,862.4	10,820.2
	6,440.76	6,439.62	6,638.94	3,438.97	17,467.5	8,621.14
	6,636.62	6,638.94	—	8,690.35	6,166.84	4,870.55
	—	12,625	—	6,291.44	4,129.41	12,860.7
	—	—	—	8,806.75	10,069.9	3,519.27
Sensitivity	60 (42–72)	53 (30–63)	43 (21–54)	57 (44–69)	46 (26–70)	61 (41–72)
Specificity	66 (53–77)	59 (34–73)	67 (50–86)	65 (48–79)	67 (53–76)	65 (51–82)
Moderate to Severe						
Surface	CM10 CHCA	CM10 CHCA	CM10 CHCA	CM10 SPA	CM10 SPA	CM10 SPA
	2,209.29	5,160.12	3,943.92	7,049.85	3,192.73	2,488.51
	3,887.3	2,984.33	4,335.6	7,929.17	4,519.4	10,505.7
	3,662.33	4,785.47	2,209.29	7,554.66	2,189.47	3,194.37
	2,930.44	4,736.91	2,304.12	3,140.37	4,373.07	2,057.34
	7,957.37	2,930.44	7,090.7	2,084.8	7,457.78	6,968.18
Sensitivity	61 (46–74)	89 (71–100)	58 (38–75)	72 (56–84)	98 (84–100)	72 (63–96)
Specificity	55 (35–75)	81 (69–96)	80 (64–93)	55 (40–75)	81 (67–92)	77 (68–95)
Surface	H50 CHCA	H50 CHCA	H50 CHCA	H50 SPA	H50 SPA	H50 SPA
	3,768.48	8,771.43	3,319.37	12,582.2	6,544.64	17,606.6
	2,875.75	4,349.64	3,220.02	17,140.8	16,231.2	3,267.78
	4,572.16	5,756.03	3,768.48	12,875.4	17,397.4	3,033.83
	6,968.55	9,378.85	17,405.3	3,168.62	6,419.66	6,508.64
	2,758.57	3,766.73	14,069.4	10,432.4	6,262.81	3,169.44
Sensitivity	50 (38–71)	78 (64–93)	56 (37–74)	71 (54–91)	86 (68–100)	68 (55–85)
Specificity	59 (45–76)	30 (0–55)	76 (57–82)	63 (43–82)	56 (38–73)	76 (62–92)
Surface	Q10 CHCA	Q10 CHCA	Q10 CHCA	Q10 SPA	Q10 SPA	Q10 SPA
	12,621.8	3,768.66	3,769.53	2,459.29	3,411.83	13,980.8
	18,048.4	6,438.47	6,311.07	4,555.11	3,647.5	8,982.1
	6,638.94	6,440.76	6,439.62	5,181.71	6,931.51	17,386.6
	6,440.76	6,637.78	6,637.78	7,147.98	4,718.97	2,536.63
	3,267.78	6,638.94	12,615.4	3,339.42	4,129.41	4,868.54
Sensitivity	51 (32–68)	78 (62–94)	40 (32–60)	70 (58–85)	85 (68–100)	71 (57–84)
Specificity	47 (34–68)	20 (0–45)	74 (57–88)	77 (62–92)	79 (62–98)	65 (45–86)

(continued)

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Table 3. Diagnostic Performance (Sensitivity, Specificity) of Peptide Peaks for Endometriosis (Minimal to Mild, Moderate to Severe, Minimal to Severe) in the Test Set (30% of Samples) Based on the Least Squares Support Vector Machine Model Developed in the Training Set (70% of Samples) (*continued*)

	Cycle Phase					
	Follicular	Luteal	Menstrual	Follicular	Luteal	Menstrual
Minimal to Severe						
Surface	CM10 CHCA	CM10 CHCA	CM10 CHCA	CM10 SPA	CM10 SPA	CM10 SPA
	7,908.76	4,736.91	3,477.01	2,831.02	11,366.3	9,926.31
	3,662.33	2,930.44	2,281.42	7,554.66	5,712.69	10,072.2
	2,930.44	5,160.12	7,090.7	4,241.29	10,070.7	6,753.04
	4,336.54	3,916.91	2,304.12	2,953.25	3,017.68	4,302.67
	7,959.93	2,011.42	2,393.9	9,927.73	3,824.44	9,328.49
Sensitivity	27 (12–47)	26 (15–44)	30 (14–66)	39 (24–56)	52 (36–72)	40 (29–57)
Specificity	88 (77–98)	83 (73–97)	87 (68–93)	84 (68–98)	82 (62–96)	84 (66–100)
Surface	H50 CHCA	H50 CHCA	H50 CHCA	H50 SPA	H50 SPA	H50 SPA
	4,572.16	8,772.77	6,635.14	6,262.81	3,399.71	6,419.66
	6,485.24	6,438.32	3,768.48	17,040.1	4,538.47	3,033.83
	7,336.93	2,875.75	2,875.75	9,518.14	9,834.85	6,469.28
	3,768.48	4,349.64	3,320.2	28,299.4	10,433.8	3,271.11
	6,769.16	3,766.73	14,069.4	12,873.8	6,262.81	3,168.62
Sensitivity	27 (0–46)	10 (0–27)	15 (0–20)	9 (0–25)	23 (10–41)	23 (0–46)
Specificity	85 (62–100)	88 (71–100)	88 (76–100)	93 (77–100)	80 (61–96)	88 (73–100)
Surface	Q10 CHCA	Q10 CHCA	Q10 CHCA	Q10 SPA	Q10 SPA	Q10 SPA
	12,621.8	3,769.53	3,769.53	4,005.09	17,467.5	4,868.54
	17,397.9	6,440.76	6,311.07	6,078.11	4,831.43	3,338.59
	3,033.47	6,636.62	6,439.62	17,257.3	10,069.9	8,980.75
	3,168.36	—	6,637.78	8,690.35	2,886.9	12,860.7
	6,440.76	—	12,615.4	4,556.09	4,129.41	3,806.42
Sensitivity	15 (0–34)	9 (0–30)	18 (0–42)	31 (21–47)	46 (25–66)	20 (4–36)
Specificity	88 (68–98)	88 (68–100)	87 (66–97)	90 (69–100)	82 (63–96)	92 (78–100)

CHCA, alpha-cyano-4-hydroxy cinnamic acid; SPA, sinapinic acid.
Data are peptide and protein peaks mass over charge (m/z) or % (95% confidence interval).
— shows that no peaks were found.

63–84, negative predictive value 92%, 95% CI 78–98). Acceptable intra (10%) and interassay variations (9%) were observed using the reference sample spotted on the CM10 sinapinic acid surface-enhanced laser desorption/ionization surface.

DISCUSSION

In this study, we confirmed the hypothesis that differential protein or peptide expression could be used for the diagnosis of endometriosis because five protein or peptide peaks (4–14 kDa) selected from menstrual phase plasma samples of the training data set allowed a noninvasive diagnosis of minimal-to-mild endometriosis in the test data set and five other selected protein or peptide peaks (2–7 kDa) selected from luteal phase plasma samples of the training data set allowed a noninvasive diagnosis of moderate-to-severe endometriosis in the test data set. Furthermore, we developed a noninvasive test for the patient population with the highest clinical need (ultrasonography-negative endometriosis, laparoscopically classified as minimal to severe endometriosis) with high sensitivity and specificity. When compared with previous endometriosis biomarker research using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry, the present study is marked by the following strengths, as explained in the Materials and Methods section: 1) a larger sample size and better characterized patient population (cycle phase, endometriosis stage, control group); 2) depletion of highly abundant plasma proteins; 3) a higher number of chip types; 4) better assessment of reproducibility; and 5) peptide peak identification.

In our study, the total number of patients included was much higher (n=254) than in previous reports (median 87, range 32–141; Table 4). Our study population was also well characterized with respect to menstrual cycle phase, whereas previous studies did not include any cycle phase description[8,9,11,12,14] or did not confirm cycle phase descrip-

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Table 4. Summary of Peripheral Blood Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry Analysis as a Diagnostic Test for Endometriosis

Reference	Sample No. (n)	Cycle Phase (n)	Surface	Results	Sensitivity (%)	Specificity (%)	Validation
Current study Fassbender et al, 2011	Plasma: 254 Minimal to mild: 89 Moderate to severe: 76 Control group: 89	Menstrual: 68 Follicular: 98 Luteal: 88	Q10 H50 CM10	Minimal to mild compared with control group: 4,898 m/z; 5,715 m/z; 8,328 m/z; 9,926 m/z; 14,698 m/z Moderate to severe compared with control group: 3,192 m/z; 4,519 m/z; 2,189 m/z; 4,373 m/z; 7,457 m/z	75 98	86 81	No
Seeber et al, 2009	Serum: 141 Mild: 22 Moderate to severe: 41 Control group: 78	Cycle day less than 14: follicular (n=91); Cycle phase greater than 14 (n=25); Unknown cycle phase (n=25)	CM10	Mild to severe compared with control group: 1,629 m/z; 3,047 m/z; 3,526 m/z; 3,774 m/z; 5,046 m/z; 5,086 m/z Minimal to mild; moderate to severe compared with control group: not mentioned	66	99	No
Jing et al, 2009	Serum: 120 Minimal-to-mild: 29 Moderate-to-severe: 30 Control group: 61	Not mentioned	Immobilized metallic affinity capture 30	Minimal to severe compared with control group: 5,830 m/z, 8,865 m/z Minimal to mild; moderate to severe compared with control group: not mentioned	89.66 89.67 (after blinded test)	96.67 96.77(after blinded test)	Yes A blinded test was performed on 30 endometriosis cases and 31 controls
Wolfler et al, 2009	Serum: 90 Minimal to mild: 19 Moderate to severe: 32 Control group: 39	Luteal: 39 Follicular: 51	Q10	Minimal to severe compared with control group: 4,159 m/z; 5,264 m/z; 5,603 m/z; 9,861 m/z; 10,533 m/z Minimal to mild compared with control group: 4,161 m/z; 4,597 m/z; 6,895 m/z; 6,955 m/z; 7,034 m/z Moderate to severe compared with control group: 4,157 m/z; 6,239 m/z; 6,318 m/z; 7,029 m/z; 12,449 m/z	81.3 89 56.9	60.3 66.7 48.5	No
Zhang et al, 2009	Serum: 80 Endometriosis [stages not mentioned]: 48 Control group: 32	Not mentioned	CM10	Endometriosis compared with control group: 4,974 m/z; 5,813 m/z; 4,290 m/z Minimal to mild; moderate-to-severe compared with control group: not mentioned	91.7 (training test) 91.7 (after blinded test)	95.8 (training test) 75 (after blinded test)	Yes; a blinded test was performed on endometriosis cases [stages not mentioned] (n=12) and controls (n=8)
Wang et al, 2008	Serum: 66 Minimal to mild: 22 Moderate to severe: 14 Control group: 30	Not mentioned	H4	Minimal to severe compared with control group: 8,142 m/z; 5,640 m/z; 5,847 m/z; 8,940 m/z; 3,269 m/z Minimal to mild; moderate to severe compared with control group: not mentioned	91.7	90	No
Liu et al, 2007	Plasma: 87 Endometriosis (stages not mentioned): 52 Control group: 46	Not mentioned	CM 10	Endometriosis compared with control group: 3,956 m/z; 11,710 m/z; 6,986 m/z Minimal to mild; moderate to severe compared with control group: not mentioned	87.5	85.7	No
Wang et al, 2007	Serum: 32 Minimal to mild: 10 Moderate to severe: 6 Control group: 16	Not mentioned	H4	Minimal to severe compared with control group: 3,269 m/z; 6,096 m/z; 5,894 m/z; 8,141 m/z Minimal to mild; moderate to severe compared with control group: not mentioned	—	—	No

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tion with endometrial histology.[10,13] This is clinically relevant because it is well documented that the up- or downregulation of plasma or serum proteins or peptides can be dependent on the phase of the menstrual cycle.[5,22,23]

In our study, plasma was depleted from highly abundant proteins before surface-enhanced laser desorption/ionization time-of-flight mass spectrometry analysis, because it contains a large proportion of highly abundant proteins like albumin (Box 1) in a wide dynamic range.[24–26] Based on our pilot study, we chose Proteominer kit to deplete highly abundant proteins from plasma and to enrich the surface-enhanced laser desorption/ionization time-of-flight mass spectrometry spectra. Other investigators depleted plasma or serum samples using a U9 buffer solution (9 mm/L urea; 2% CHAPS; 50 mM Tris HCl, pH 9.0)[8,9,14] or CHAPS combined with Cibacron Blue 3GA.[11,12] However, they never reported if these methods could enrich the peak spectra after surface-enhanced laser desorption/ionization time-of-flight mass spectrometry analysis.[8–14] We analyzed the reproducibility of plasma surface-enhanced laser desorption/ionization time-of-flight mass spectrometry analysis by calculating intra and interassay coefficients of variances in a reference sample. Other investigators did not mention a reference sample to calculate coefficient of variation[10,13] or included a reference sample but did not report clearly how coefficient of variation was calculated[8,9,11,12,14] In the present study, we report an acceptable intra and intercoefficient of variation of 9% and 10%, respectively. Importantly, we used a statistical model constructed based on the training data set and validated in the test data set by repeated random subsampling crossvalidation in that test data set. This method strongly increases the chance of finding the presence of biomarkers that are likely to be increasingly expressed in new data sets other than the selected training test data set.[27] By averaging the performance of the model over the 100 splits, we obtained a more robust estimate of the true performance of the found biomarkers.

The plasma peptide peak with the highest intensity (2.189 m/z), downregulated in women with moderate-to-severe endometriosis when compared with control participants, was identified in this study as fibrinogen β-chain peptide. The relevance of this compound in the pathogenesis of endometriosis is unclear and merits further discussion. Human fibrinogen is a large soluble plasma protein that plays a critical role in protecting the vascular network against the loss of blood after tissue injury.[28] Fibrinogen and fibrin play important, overlapping roles in blood clotting, fibrinolysis, cellular and matrix interactions, inflammation, wound healing, and neoplasia.[29] Because endometriosis is characterized by subclinical inflammation fibrinogen, which is an acute-phase protein in plasma, it could be potentially a biomarker for endometriosis but this has not yet been investigated. Low levels of fibrinogen β-chain in peripheral blood are probably the result of the increased consumption of fibrinogen β-chain, which can be hypothesized to lead to increased formation of fibrin in the peritoneal fluid, facilitating adhesion and attachment of endometrial fragments. Decreased fibrinogen β-chain levels also have been observed in uterine flushings from baboons with induced endometriosis when compared with those in a control group, leading to the hypothesis that endometrial pockets of deposited endometrial β-subunit fragments may lead to the development of a persistent fibrinogen matrix in the endometrium thereby preventing efficient fibrinolysis and facilitating endometrial–peritoneal attachment (Asgerally Fazleabas, www.patentstorm.us/patents/7794958). Decreased fibrinolysis is also a risk factor for uterine bleeding and heavy menstrual bleeding is a known risk factor for endometriosis.

In conclusion, in this study, we confirmed the hypothesis that differential surface-enhanced laser desorption/ionization time-of-flight mass spectrometry peptide and protein expression in plasma can be used in infertile women with or without pelvic pain to predict the presence of laparoscopically and histologically confirmed endometriosis and also in the subpopulation with a normal gynecological ultrasonography preoperatively, which has the highest need for an endometriosis blood test.

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