Published in final edited form as:

Fertil Steril. 2010 May 1; 93(7): 2137–2144. doi:10.1016/j.fertnstert.2008.12.121.

Proteomic analysis of serum yields six candidate proteins that are differentially regulated in a subset of women with endometriosis

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Abstract

Objective—To identify potential novel biomarkers that differ between subjects with and without endometriosis that might aid in developing a non-invasive serum-based diagnostic test.

Design—Case-control evaluation of a diagnostic test

Setting—University medical center

Patients—Consenting women of reproductive age undergoing laparoscopy for indications of pain, infertility, elective tubal ligation, tubal reanastomosis, or other benign indication

Interventions—Diagnostic laparoscopy and peripheral venipuncture

Main outcome measures—Concentrations of low-molecular weight proteins in serum; surgical staging of endometriosis

Results—Six proteins were found that were differentially expressed between those with and without disease and which had good diagnostic properties. Taken together in a two-step diagnostic algorithm, we were able to diagnose 55% of subjects with 99% accuracy as to the status of disease. Further combining this algorithm with that derived by our previous study of serum putative markers (MCP-1, MIF, Leptin, CA-125) improved our diagnostic capability to 73% of subjects with 94% overall accuracy.

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Presented at the annual meeting of the ASRM, October 2006, New Orleans, LA

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Conclusions—This study is the critical first step in the identification of potential novel biomarkers of endometriosis. Future identification of the proteins and further validation in a second population is needed before applying these findings in clinical practice.

Keywords

biomarkers; Endometriosis; non-invasive diagnosis; proteomics

Introduction

Endometriosis is estimated to affect 2–18% of reproductive age women and upwards of 40% of women with infertility (1). The disease is characterized by the presence of viable endometrial tissue (glands and stroma) outside of the uterine cavity resulting in multiple gynecological problems, including pelvic pain, dysmenorrhea, dyspareunia, and infertility.

Researchers have searched for non-invasive methods to diagnose endometriosis, thus far without successfully resulting in a clinically-applicable test. Many of these investigations have examined single markers of the inflammatory process to look for ones that may be up-regulated in women with endometriosis. This avenue of research is based on previous findings of higher concentrations of macrophages and activated macrophages in the peritoneal fluid of women with endometriosis (2). Researchers have reported elevations in the inflammatory cytokines IL-1, IL-6, and TNF α , though sometimes with conflicting results (3–7). Angiogenic factors such as IL-8 and VEGF have also been found in elevated amounts in peritoneal fluid of women with endometriosis (2,8). Fewer studies have focused on concentrations of these markers in serum and these investigations report less definitive results (7,9,10). Another area of study has been the role of tumor markers, specifically CA-125 and CA 19-9, in the diagnosis of endometriosis (11,12). However, neither of these has shown to have adequate sensitivity and specificity to be used as a diagnostic test.

Endometriosis research, like other fields of study, has recently entered the genomic era, with growing interest in the molecular and genetic biology of the condition. The study of proteins may prove more fruitful than that of genes, since protein diversity cannot be fully characterized by gene expression alone. Over the recent years, the study of proteomics has been applied to many fields of medicine, most commonly to tumor marker discovery (13–16). A recent review called for the use of proteomic pattern profiling as an area to be explored for the development of a possible screening test for endometriosis (17). To our knowledge, only two studies have been published applying proteomic techniques to the analysis of serum from women with endometriosis, with some promising preliminary findings of differential protein expression between diseased and non-diseased subjects (18,19). Another two studies have investigated the proteome of other biological material from endometriosis patients, namely peritoneal fluid and endometrium, and found elevated expression of several proteins, most of which have immunological or inflammatory mechanisms of action (20,21).

The aim of the present study was to analyze patterns of protein expression in serum of women with and without endometriosis to potentially identify new proteins that may serve as candidate markers of endometriosis. We have previously analyzed the serum from this study population for the concentration of several suspected markers of endometriosis, including CA-125 and inflammatory cytokines, and wanted to compare the diagnostic ability of the “old” and “new” markers (22). Because serum is a complex fluid with an abundance of proteins (including the large-sized and plentiful albumin and immunoglobulins that may mask the smaller-sized, less abundant proteins), we decided to focus our efforts on the fraction containing proteins of small molecular mass where we felt potential biomarkers may be found. We chose surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) as the

platform for performing the protein profiling experiments because this technology is well-suited for analysis of proteins in the small-molecular weight range, requires relatively small amounts of sample for analysis, has high throughput capability, and allows for the facile analysis of multiple variables with computer-supported data collection and analysis. A recent study testing the feasibility of using SELDI for proteomics research in endometriosis concluded that it is, indeed, a viable approach for studying this disease (23).

Materials and Methods

General Protocol

This study was approved by the Institutional Review Board of the University of Pennsylvania. The protocol for sample collection has been previously described in detail (22). Briefly, serum was obtained from consenting women of reproductive age (18–48 years old) already scheduled for laparoscopic surgery (for whom the gold standard diagnosis would be known). The subjects underwent surgery for the indications of infertility, pelvic pain, tubal sterilization or tubal reversal, or other benign etiology. During the laparoscopy, evidence of endometriosis was recorded and staged according to the published revised ASRM scoring system (24). Subjects were allocated to groups based on their post-surgical diagnosis. To avoid the potential for outcome misclassification, only those subjects who had at least stage II endometriosis diagnosed during surgery were included in the diseased group. Women with stage I disease were excluded and considered neither a case nor a control. Protein profiling of serum was accomplished using SELDI-TOF-MS.

Serum and ProteinChip Preparation

Large molecular weight proteins were precipitated from the raw serum samples by the addition of two parts acetonitrile with 0.1% trifluoroacetic acid (ACN with 0.1% TFA) to one part serum. The mixture was vortex-mixed at room temperature for 30 seconds and allowed to sit for 15 minutes at room temperature. The resultant mixture was then centrifuged at 13,200 rpm at 4°C for 15 minutes. The supernatant was removed and diluted 1:10 with binding buffer (50 mM sodium acetate, pH 4.0) to make the final solution used for spotting the chips, while the precipitated pellet was discarded.

Various chip chemistries (hydrophobic, ionic, cationic) were initially evaluated to determine which affinity chemistry provided the best serum profiles in terms of number and resolution of proteins (data not shown). The cation-exchange chips (CM10) were found to give the best results and were used for the remainder of the analyses. Eight-spot CM10 chip arrays (CM10) were assembled on a 96-well bioprocessor (CIPHERGEN Biosystems, Inc. Fremont, USA), a device that holds 12 chips and allows for the application of uniform volumes of sample to each chip spot. The application of serum samples and washing solutions was automated by using a Biomek 2000 liquid handling robot (Beckman-Coulter, Inc. Fullerton, USA).

The arrays were equilibrated three times with 100 µl of binding buffer (50 mM sodium acetate, pH 4.0) on a platform shaker for 5 minutes each time, followed by two quick rinses with 100 µl of distilled water. 100 µl of the prepared diluted serum solution was then spotted onto each CM10 array spot. Each serum specimen was spotted in duplicate in a random location on the 96-well bioprocessor. Each ProteinChip was also spotted in a random location with a previously prepared pooled-serum sample to assess chip-to-chip reproducibility. Four specimen-free spots were purposely left on each bioprocessor to test for cross-spot contamination. After a 60-minute incubation period on a shaker, the arrays were washed three times with 100 µl of the binding buffer for 5 minutes each, followed by one quick rinse with 100 µl of distilled water, and then air-dried. An energy absorbing molecule (matrix) in the form of 1.0 µl of 30% CHCA (alpha-

cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.5% trifluoroacetic acid) was applied to each spot.

ProteinChip analysis

MS analysis was performed using a PBS-II ProteinChip reader (Ciphergen Biosystems, Inc.). In this system, the prepared chip array is introduced into the reader, where a laser beam is directed at the sample on each spot. Proteins that are coated with the energy absorbing molecule desorb and ionize. Released ions then experience an accelerating electrical field which causes them to fly through a vacuum tube towards a detector. These ionized proteins are detected and their accurate mass is determined based on their time of flight. The typical output of the ProteinChip reader is a spectrum represented graphically as peaks, with each peak representing the molecular mass and relative abundance of a protein or protein fragment.

Spectra were collected using an average of 165 laser shots with a laser intensity of 170 and detector sensitivity of 8, molecular mass range 1,000–10,000 Da. Mass accuracy was calibrated externally using the all-in-one peptide molecular mass standard (Ciphergen Biosystems, Inc.). Reproducibility was verified using pooled serum samples spotted on each chip.

The raw spectra obtained from the mass spectrometer were processed using Ciphergen ProteinChip Biomarker Wizard software version 3.1 for baseline subtraction, normalization and peak detection. Spectra were normalized according to total ion current. Biomarker Wizard peak detection localized areas of the mass spectrum as peaks by comparing the signal to noise ratio. Autodetection settings were used for peak determination, with settings of signal to noise ratio of at least 5:1 on first pass, and presence in at least 20% of spectra to be considered a peak. On second-pass, the signal to noise ratio was set at 2:1 and peaks were clustered if within 0.3% of mass.

Statistical Analyses

Sensitivity and specificity for each single protein peak localized were assessed with receiver operating characteristic (ROC) curves (25). Mean concentrations of the proteins, and the differences in means between the case and control groups, were purposely not computed as these would not be of clinical usefulness and could not be used for diagnostic purposes. Even if statistically significantly different from each other, differences in means (i.e. evidence of association) do not reflect possibly extensive overlap in the distributions between the two groups. To pare down the number of markers for further consideration, only those protein peaks with the combination of 90% sensitivity or better with at least 20% specificity, or 90% specificity with at least 20% sensitivity were chosen.

The diagnostic performance of the remaining protein markers was evaluated jointly using classification tree analysis (CART, Salford Systems, San Diego, USA). Classification and Regression Tree (CART) is a nonparametric statistical procedure which ultimately classified subjects based on the dependent variable endometriosis or control (26). It does so by examining all possible dichotomous splits on each protein peak, choosing the variable and cut-off value which classify the subjects most accurately (Figure 1). CART creates two child nodes from each parent node, and continues growing the tree, assessing subsequent variables for their ability to classify subjects. Ultimately, the algorithm reaches terminal nodes, at which point all study subjects are classified as either endometriosis or control. To avoid model-overfitting (a tree with so many branches that it is unwieldy and not clinically useful), the investigator needs to prune back the tree and choose one that has good diagnostic properties, yet is parsimonious.

Automatic self-validation procedures employed by CART were carried out as part of the tree-building methodology as a form of internal validation. Using 10-fold cross-validation, 10 different trees were grown, each from a different 10 percent of the total sample, yielding a reliable determination of the optimal tree size. CART identified a set of candidate predictive trees with their associated costs and standard errors using the Gini single-variable standard splitting rules, and we accepted the simplest trees with fewest nodes yet acceptable error rates.

Baseline characteristics of the two groups were compared using t-test, chi-square, and Fisher's exact test, where appropriate.

Results

A total of 197 patients were enrolled in the study. Sixty-three (63) patients had Stage II through IV endometriosis and were assigned to the disease group (22 (35%) Stage II, 17 (27%) Stage III and 24 (38%) Stage IV); 78 were endometriosis-free and were assigned to the control group. The remaining 56 women were diagnosed with Stage I endometriosis and were excluded from the analyses because of concern for misclassification bias due to equivocal or erroneous diagnosis. Of the women with endometriosis, 40 (64%) had pelvic pain and 22 (35%) infertility compared to 21 (27%) and 25 (32%) of the control group, respectively. The endometriosis group rated the pain as more severe and had twice the rate of dyspareunia as the control group (35% vs. 17%). More detailed demographic and clinical data of the subjects in the two groups have been previously published (22).

The serum proteomics spectra for two of the 63 subjects in the disease group were excluded from the proteomics analysis due to poor quality results on the duplicate spectra (paucity of proteins), consistent with an error in sample processing. No cross-contamination from adjacent spots was seen in any of the blank specimen-free spots on the bioprocessors.

Biomarker pattern software identified 169 different peaks or proteins in the combined spectra from all subjects, ranging from 1030 to 9426 daltons (Da), according to the detection parameters previously described. These were pared down to 57 peaks that met the aforementioned criteria of demonstrating >90% sensitivity with >20% specificity or >90% specificity with >20% sensitivity for the diagnosis of endometriosis. None of the peaks taken alone demonstrated both high sensitivity and high specificity. CART was then used to evaluate the joint performance of the proteins.

CART developed classification trees using the best performing independent variables. As in the sample example in Figure 1, all subjects were considered together in the parent node 1, and then split off into Terminal nodes where they were assigned a class or diagnosis (1=Endometriosis, 0=Control), or into a child node which became a parent node for subsequent splits. Splitting variables or proteins were named according to their molecular weight in daltons (i.e. M3047 is protein with molecular weight of 3047 Da). The number at which the variable was split is a relative intensity of the protein after rescaling to get at the signal above the noise.

Ideally, a single classification tree of selected proteins would have maximized both sensitivity and specificity. This was not the case in our analyses. Thus, we set the cost of misclassification to first maximize sensitivity in classifying the subjects, yielding a tree that used 5 proteins for classification, with the following molecular weights: 1629 Da, 3047 Da, 3526 Da, 3774 Da, and 5046 Da. Together they had a sensitivity of 100% (95% CI: 95–100%) with a specificity of 45% (95% CI 34–57%). The 10-fold cross validation estimated these to be 72% and 34%, respectively. Then, we set the parameters of CART to maximize specificity and the program created a second tree, with 5 proteins and splitting cut-offs. It happens that 4/5 of the latter group (1629 Da, 3047 Da, 3526 Da, 3774 Da) had already been represented in the first tree that maximized sensitivity. The additional protein was one of weight 5068 Da. The second tree

had a specificity of 99% (95% CI 93–100%) with 66% (95% CI 52–77%) sensitivity for identifying cases with endometriosis (Figure 3). The ten-fold cross validation estimated these at 69% and 62%, respectively. Thus, a total of 6 proteins were identified as potential markers by CART.

To be assigned a diagnosis, a subject needed to be classified as having endometriosis (or being disease-free) consistently by the two classification trees. In other words, subjects who were classified as ‘Endometriosis’ by both trees were assigned the disease diagnosis, those who were classified as ‘No Endometriosis’ were assigned the no disease diagnosis, and those whose classifications did not match remained unassigned.

Using this rule in this population of patients allowed for the definitive diagnosis of 40/61 (66%) subjects with endometriosis with 100% accuracy, and 36/78 (45%) of those who were disease-free with 97% accuracy. Only one subject would have received an erroneous diagnosis of endometriosis despite being disease-free (false-positive), yielding an overall accuracy of 99%. A total of 76/139 (55%) subjects could be diagnosed by applying this algorithm, with the remaining subjects receiving no diagnosis because the classifications by both trees did not match.

We then combined these best-performing protein markers with specific putative markers (MCP-1, Leptin, MIF, CA-125) that we had previously investigated for their diagnostic performance using CART analysis (22). The analysis consistently favored the newly-identified proteins. In fact, the proteins we have identified were variables of greatest importance with the best differentiating power and the tree structures of reasonable branching size were identical whether we included or excluded the putative markers. Next, we separately examined the diagnoses obtained for individual patients using the newly-identified protein algorithm and the putative marker algorithm. A diagnosis was assigned based on the results of the proteins, and for those with equivocal results (N=63), the putative algorithm classification tree was *subsequently* applied. The overall diagnostic performance was much improved with this sequential method. In our study population, an additional 9 patients for a total of 49/63 (78%) subjects with endometriosis would be accurately diagnosed without error. Of the subjects in the control group, an additional 18 subjects or 54/78 (69%) would be given a diagnosis, with 3 of those false positive results. The remaining 14 subjects with endometriosis and 24 subjects without disease would still go undiagnosed. Overall, 103/141 (73%) subjects would be diagnosed using the two-step approach with 94% overall accuracy.

Discussion

Endometriosis remains a disease whose pathophysiology is not completely understood and one that still requires invasive methods for definitive diagnosis. Using a non-biased approach through the application of proteomic analysis, we have successfully identified protein peaks potentially corresponding to biomarkers of disease that appear to be differentially expressed in the serum of women with and without endometriosis.

Using SELDI-TOF we were able to identify 6 proteins in the serum that differed in concentration between subjects with and without endometriosis. By applying a two-step diagnostic algorithm, with nearly perfect accuracy of 99%, we were able to diagnose nearly two-thirds of patients with endometriosis and exclude the disease in almost half of those who were endometriosis-free. When we subsequently used the diagnostic algorithm previously obtained from putative markers studied to assign a disease-status to those subjects who remained undiagnosed, nearly three-fourths of subjects would now receive a diagnosis with a minimal drop in accuracy to 94%. Subjects who could not be diagnosed by these algorithms would have to undergo standard diagnostic methods (i.e. laparoscopy).

Previously, studies examining gene expression in ectopic versus eutopic endometrium, as well as endometrium from diseased and disease-free subjects, have found differences in gene expression (27–32). Since protein diversity cannot be fully characterized by gene expression analysis alone, proteomics can serve as a useful tool for characterizing fluids such as serum for differential protein expression and for potential diagnostic purposes.

In one study applying proteomic techniques to the analysis of serum in women with endometriosis, the investigators used 2-DE to analyze the serum of 12 women, 6 (50%) of whom had endometriosis, to find that eleven proteins in the serum were differentially expressed between women with and without endometriosis (14). The authors went on to preliminarily identify some of these proteins as cytoskeletons and regulatory proteins of the cell cycle by searching an established protein database for potential matches. While promising, such findings need validation in future investigations and in a much larger sample of subjects.

Our present study has several important strengths, most important of which is its large sample size. All disease was confirmed with the present gold standard procedure for diagnosing endometriosis, namely laparoscopy. By including in the disease group only those patients who had Stage II–IV endometriosis, we excluded subjects with questionable endometriosis or non-definitive findings on laparoscopy. A further strength of our study was the heterogeneous control group, composed of healthy subjects as well as those who had other pathologies, including pain and infertility. This lends further credence to our proposal that the diagnostic algorithms we have developed, and the protein markers we have identified, are specific to the disease process of endometriosis and not just identifiers of infertility, inflammation or non-specific pain.

The greatest limitation of our work is that our findings come from a single population of women at a single institution, and all work was performed at a single laboratory. Since we purposefully excluded women with Stage I disease in this study, future validation of the current work will need to include the application of these diagnostic rules to a more heterogeneous population. Thus while the proteins discussed have good diagnostic potential in our study population, their performance and the overall generalizability of our findings need to be further evaluated in a new study population.

Nonetheless, this study is the critical first step in the identification of novel potential serum biomarkers of endometriosis. The next important work that we are undertaking is the purification and identification of the proteins that we have described. Identification of these proteins and their functions may aid in improving our understanding of the pathophysiology of the disease. It would also open the possibility of developing direct immunological assays for these proteins in serum, allowing direct evaluation in the clinical laboratory. In future work, we plan to further validate our results in a new study population which we have begun to recruit and to confirm the diagnostic potential of the protein makers that we have described here.

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Figure 1.

Sample CART output. This non-parametric statistical procedure creates two child nodes from each parent node by splitting on the independent variable that best classifies subjects into binary groups. In this hypothetical example, all 100 subjects are classified using 2 independent variables, with 30 subjects deemed disease positive.

Figure 2.

Protein Marker Classification Tree Optimizing Sensitivity: Sensitivity=100% (95% CI: 95–100%) and specificity=45% (95% CI 34–57%). The Class assignment of subjects in each node is shown under the node number. Class CONT is the control group and Class ENDO is the Endometriosis group. CART initially categorizes all study subjects in Node 1 as ENDO. Colored bars give a graphical representation of the proportion of subjects from each group assigned to that child node, red=control, blue=endometriosis. Splitting variables are shown in the parent node (by protein size in Da), with the cut-off for the split shown above the child node in grey. N=number of subjects. Protein sizes are indicated in each node as the mass preceded by “M” (e.g. M3774 is a protein with a molecular mass of 3774 Da).

Figure 3.
Protein Classification Tree Optimizing Specificity: Specificity=99% (95% CI 93–100%) and sensitivity=66% (95% CI 52–77%).