Effect of endometriosis on the protein expression pattern of follicular fluid from patients submitted to controlled ovarian hyperstimulation for in vitro fertilization

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BACKGROUND: The aim of this study was to evaluate protein expression profile and quantify the proteins present in follicular fluid (FF) samples from women with endometriosis and pregnant women without endometriosis.

METHODS: A prospective case–control study was carried out including women with Stage III or IV endometriosis (Group I) and pregnant women without endometriosis (Group II), both at the maximum age of 35 years. Women were submitted to controlled ovarian stimulation for in vitro fertilization, and FF was collected after ultrasound-guided ovarian aspiration. FF from both ovaries was pooled, and patient samples were pooled according to Group I or II. Pooled protein samples were separated and analyzed by MudPIT (multidimensional protein identification technology followed by ExpressionE and label-free quantification with ProteinLynxGlobalServer 2.4v, IdentityE and ExpressionE software).

RESULTS: A total of 416 proteins or randomic sequence were identified, 62 proteins differentially expressed between Groups I and II. One (1.6%) was expressed at a higher level and 36 (58.1%) were uniquely expressed in Group I, whereas 8 (12.9%) were expressed at a higher level and 17 (27.4%) were uniquely expressed in Group II. Of all these, 15 (24.2%) are related to binding, 1 (1.6%) to immune response, 8 (12.9%) to cell division, 3 (4.8%) to cellular metabolism, 16 (25.8%) to general function and 19 (30.6%) do not yet present an identified function.

CONCLUSIONS: Protein expression profiles of patients with and without endometriosis identified at least 64 proteins differentially expressed, which may be related to the physiopathology of endometriosis. These proteins may additionally be useful in determining potential biomarkers for diagnostics, as well as for therapeutic intervention in women with infertility due to endometriosis.

Key words: biomarkers / endometriosis / IVF / follicular fluid / proteomics

Introduction

During follicular maturation, follicular fluid (FF) provides the microenvironment for the oocyte and contains many substances involved in oocyte maturation, possibly affecting fertilization and embryo development (Schweigert et al., 2006). This microenvironment may be altered by conditions such as endometriosis, resulting in ovulatory dysfunction, poor oocyte quality, reduced fertilization rate, low-grade embryos and reduced implantation rates (Pellicer et al., 2000a; Garrido et al., 2003).
Endometriosis is a chronic gynecological disease characterized by the presence of functional endometrial tissue outside the uterine cavity (Konincx et al., 1991; Liu et al., 2008). This disease affects 10–32% of women at reproductive age and can result in pelvic pain and infertility (Ballard et al., 2006). The most common form of endometriosis, which accounts for ~55% of all cases, is the ovarian endometrioma (Jenkins et al., 1986). Many studies have reported that pregnancy rates are lower in women with endometriosis than in controls, but the mechanism accounting for this difference is poorly understood (D’Hooghe et al., 2003; Gupta et al., 2008; Tatone et al., 2008). Numerous putative mechanisms have been described for decreased fertility, such as altered folliculogenesis (Doody et al., 1988) leading to ovulatory dysfunction and poor oocyte quality, as well as luteal phase defects (Grant, 1966), reduced fertilization rates (Wardle et al., 1985) and abnormal embryogenesis (Garrido et al., 2002).

Recently, the development of high-throughput proteomic technologies (Geromanos et al., 2009) has led to the promise of early diagnosis of endometriosis by comparing the protein composition in affected and normal tissues. Aside from accelerating diagnosis, proteomics may lead to a better understanding of the physiopathology of disease development by identifying proteins involved in its different stages (Taylor, 2004) and, ultimately, present targets for endometriosis-specific therapeutic intervention.

Among high-throughput proteomic approaches, multidimensional protein identification technology (MudPIT) is a recently developed, powerful research tool to separate and identify proteins in body fluids (Gonzalez-Begne et al., 2009). MudPIT has rapidly become a popular approach for shotgun proteomics since it combines high-resolution separation with tandem mass spectrometry (MS/MS; Washburn et al., 2001). In the present study, we therefore used a MudPIT approach via nanoUPLC tandem nanoESI-MS² to identify proteins with high resolution and accuracy. To our knowledge, this is the first of the proteomics of human endometriosis or in FF using the MudPIT nanoESI-MS² technology.

This study therefore aimed to identify and quantify the protein profiles of FF of women with endometriosis and controls (women without endometriosis who achieved pregnancy), aiming at identifying possible protein targets for diagnostics and therapeutic intervention against endometriosis-derived infertility.

Materials and Methods

Study group
A prospective case–control study was carried out, FF samples obtained from 10 women submitted to in vitro fertilization (IVF). This study received the Institutional Review Board approval from the São Paulo Federal University Research Ethics Committee. Patients were subdivided into two groups. The study group consisted of five patients with endometriosis grade III or IV with the presence of peritoneal lesions and ovarian endometriomas, diagnosed by videolaparoscopy (age 32.12 ± 3.41 years, mean ± SD) referred to the IVF program at the São Paulo Federal University. The control group consisted of five women without endometriosis referred to the IVF program and who achieved pregnancy in that treatment cycle (age 31.88 ± 3.28 years, mean ± SD). Controls underwent IVF due to either a tubal factor for female infertility and/or a mild male infertility factor (at least 3 million sperm ml⁻¹ and over 5% strict morphology; Kruger et al., 1987). Further inclusion criteria for both groups were only couples with a female age of up to 35 years and a serum follicle-stimulating hormone (FSH) level of between 3 and 9 μg/ml on Day 3 of the menstrual cycle previous to the treatment cycle. Finally, only couples who had not been submitted to previous IVF cycles were included in the study.

We only included women who had received a similar ovarian stimulation protocol for IVF. Controlled ovarian stimulation was achieved through the use of exogenous recombinant gonadotrophins (225 IU/day of Gonal-F, Merk-Serono, Darmstadt, Germany) starting on cycle day 2. When the leading follicle reached 14 mm in diameter, endogenous LH release was suppressed by use of a GnRH antagonist analog (Cetrorelix—Cetrotide; Merk-Serono) until the day of hCG administration. When the leading follicle reached 17 mm in diameter, a total dose of 250 μg of hCG was administered. Ultrasound-guided transvaginal oocyte retrieval was performed 36 h after hCG administration. For all patients, FF was obtained from the follicles present in each ovary. Ovarian endometriomas were not aspirated for the study.

Protein identification and quantification
Protein concentration was initially evaluated using a Bradford (1976) assay. FF samples from different patients were pooled according to the group and normalized according to the protein concentration. Two pools were formed: (i) women with endometriosis grades III and IV (study group) and (ii) women without endometriosis who achieved pregnancy (control group).

The FF pools and an internal standard (alcohol dehydrogenase, spiked to 50 fmol, SwissProt accession number P00330) were submitted to tryptic digestion. Briefly, the protein (fluid extract) samples were denatured with 0.1% RapiGestSM SF Protein Digestion surfactant (Waters, Milford, USA), reduced (10 mM dithiothreitol), alkylated (10 mM iodoacetamide) and enzymatically digested with trypsin at 1:50 (w/w) enzyme:protein ratio.

NanoUPLC tandem nanoESI-MS² (MudPIT) conditions
Qualitative and quantitative nanoUPLC tandem nanoESI-MS² experiments were conducted using either a 1.5-h reversed phase gradient from 5% to 40% (v/v) acetonitrile (0.1% v/v formic acid) at 600 nL/min on a nanoACQUITY UPLC core system. A nanoACQUITY UPLC C18 BEH 1.7 μm, 100 μm × 10 cm column was used in conjunction with an SCX 5 μm, 180 μm × 23 mm column. Typical on-column sample loads were 250 ng of total protein digests. For all measurements, the mass spectrometer was operated in the ‘W’ mode with a typical resolving power of at least 20 000. All analyses were performed using electrospray ionization in the positive ion mode ESI+(+) and a NanoLockSpray source. The lock mass channel was sampled every 30 s. The mass spectrometer was calibrated with a GFP solution (100 fmol/μl) delivered through the reference sprayer of the NanoLockSpray source. The doubly-charged ion ([M + 2H]+²) was used for initial single-point calibration (Lref), and MS/MS fragment ions of GFP were used to obtain the final instrument calibration. Data-independent scanning (MS²) experiments were performed with a Synapt HDMS mass spectrometer (Waters, Manchester, UK), which was automatically planned to switch between standard MS (3 eV) and elevated collision energies MS² (12–40 eV) applied to the trap ‘T-wave’ CID (collision-induced dissociation) cell with argon gas; the transfer collision cell was adjusted for 1 eV, using a scan time of 1.0 s, both in low-energy and in high-energy CID orthogonal acceleration time-of-flight (oa-TOF) MS² from m/z 50 to 3000. The RF offset (MS profile) was adjusted such that the LC/MS data were effectively acquired from m/z 300 to 3000, which ensured that any masses observed in the LC/MS²
data less than m/z 300 were known to arise from dissociations in the collision cell.

Database searching

Protein identifications and quantitative data packaging were generated by the use of dedicated algorithms (Silva et al., 2005) and searching against a species-specific database (Kramer-Albers et al., 2007). The utilized databases were randomized ‘on-the-fly’ during the database queries and appended to the original database to access the false-positive rate of identification. For proper spectra processing and database searching conditions, a ProteinLynxGlobalServer v.2.4 (PLGS) with an ExpressionEngineinformatics v.2.4 license installed was used. A UniProtKB/Swiss-Prot Release 57.1 and a UniProtKB/TrEMBL Release 40.1 database were used and the search conditions were based on taxonomy [Homo sapiens (human)], maximum missed cleavages by trypsin allowed up to 1, variable modifications by carbamidomethyl (C), acetyl N-terminal and oxidation (M). Proteins obtained were organized by the PLGS into a list corresponding to a unique protein for both conditions (study or control group), and a logarithmic ratio between the different groups was plotted onto a scatter plot to observe differences between groups. Only proteins in attendance scores and confidence higher than 50% and 99%, respectively, were considered in order to accept these database searches, and when the same protein was identified for different MS/MS fragment ions, those presenting the highest score were considered for comparisons and data presentation (Chambery et al., 2009; Cockman et al., 2009; Levin et al., 2009; Li et al., 2009).

Results

Table I presents clinical results for both groups. Only serum LH was lower in the study group, when compared with the control group. All peptide spectra displaying high-resolution precursor ions were previously de-convoluted into singly charged ions obtained through the nanoLC-MS² acquisition mode and were selected for cluster analysis. Around 1500 MS/MS experiments were performed, and data were divided into protein data sets (Fig. 1).

Only LH levels were lower in patients from the study group. In FF, a total of 416 proteins and/or randomic sequences were identified for different MS/MS fragment ions, those presenting the highest score were considered for comparisons and data presentation (Chambery et al., 2009; Cockman et al., 2009; Levin et al., 2009; Li et al., 2009).

Table I Clinical characteristics of patients from the study (endometriosis stage III or IV) and control (women without endometriosis who achieved pregnancy after a first IVF treatment cycle) groups.

|                      | Control group | Study group | P-value |
|----------------------|---------------|-------------|---------|
| Age (years)          |               |             |         |
| Mean; SD             | 32.2; 2.2     | 31.8; 3.42  | 0.830   |
| 95% CI               | 29.5–34.9     | 27.6–36.1   |         |
| FSH (µg ml⁻¹)        |               |             |         |
| Mean; SD             | 5.5; 1.4      | 5; 2.1      | 0.683   |
| 95% CI               | 3.7–7.2       | 2.4–7.6     |         |
| LH (µg ml⁻¹)         |               |             |         |
| Mean; SD             | 5; 1.4        | 2.6; 1.1    | 0.015   |
| 95% CI               | 3.3–6.8       | 1.2–3.9     |         |
| Follicles (n)        |               |             |         |
| Mean; SD             | 30.8; 6.5     | 29.6; 8.7   | 0.812   |
| 95% CI               | 22.7–38.9     | 18.8–40.5   |         |
| Oocytes (n)          |               |             |         |
| Mean; SD             | 18.6; 6.5     | 12; 5.5     | 0.121   |
| 95% CI               | 10.5–26.7     | 5.2–18.8    |         |
| MII oocytes (%)      |               |             |         |
| Mean; SD             | 84.6; 10.5    | 83.5; 22.2  | 0.920   |
| 95% CI               | 71.5–97.6     | 55.9–100.0  |         |
| Fertilization rate (%)|              |             |         |
| Mean; SD             | 62.5; 22      | 79.9; 15.6  | 0.187   |
| 95% CI               | 35.2–89.8     | 60.5–99.3   |         |

Bold indicates statistical significance.

15 proteins were related to binding or apoptosis, 1 to immune response, 8 to cell division, 3 to cellular metabolism, 16 to general function and 19 do not yet present an identified function (Tables II and III, Fig. 4).

To verify interactions between identified proteins and current interactome databases, proteins from our study were submitted by further interactome analysis using Cytoscape (www.cytoscape.org). Figures 5–8 show interaction maps where proteins overexpressed or exclusively expressed in patients with endometriosis are shaded in red, whereas those overexpressed or exclusively expressed in controls are shaded in green. ***Interacting proteins not differentially expressed in our study are shaded in grey.

Endometriosis patients’ interactomes demonstrated proteins participating in inflammation and in apoptosis mainly, whereas controls presented an interactome containing transcription factors, oncogenes and proteins participating in cell signaling.

Discussion

FF is comprised of a variety of chemicals, such as metal ions, enzymes, steroids, proteoglycans, hormones and prostaglandins (Yen, 1986). Currently, over 200 different proteins have been identified in FF (Von Wald et al., 2009). FF provides a substrate for oocyte growth,
and because endometriosis may alter the follicular microenvironment, oocyte quality and embryo development are compromised in this disease (Pellicer et al., 2000b). Most studies report that pregnancy rates are lower in women with endometriosis. Nonetheless, specific mechanisms that may account for this decrease are poorly understood (Garrido et al., 2003).

A few studies have been performed aimed at determining proteomics of FF. A recent study evaluating FF from the leading follicle...
Proteomics in follicular fluid

Figure 3 FF expression plot indicating 102 proteins and randomic sequences which were expressed differently in the two groups.

during hormonal stimulation for IVF in women up to 32 years old demonstrated increased expression of haptoglobin, predominantly fetal expressed T1 domain, mitochondrial genome integrity gene, apolipoprotein H, dihydrolipoyl dehydrogenase, lysozyme C, fibrinogen α-chain and Ig heavy-chain V-III region BRO and decreased expression of antithrombin, vitamin D-binding protein and complement 3 in women with successful outcome (live birth). These authors carried out two-dimensional electrophoresis (2D SDS–PAGE) followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) of FF samples (Estes et al., 2009).

Another study evaluating FF from patients with recurrent spontaneous miscarriage including six women (three study and three controls) utilizing 2D SDS–PAGE followed by MALDI-TOF and LC-MS/MS identified that women with recurrent spontaneous miscarriage presented expression of complement component C3c chain E, fibrinogen γ, antithrombin, angiotensin and hemopexin precursor, not observed in controls. This result indicated that these proteins may participate in determining alterations to oocytes and embryos (Kim et al., 2006).

Other studies also reported thioredoxin peroxidase-1, transthyretin, retinol-binding protein (Anahory et al., 2002), hormone sensitive lipase, apolipoprotein IV (Lee et al., 2005), haptoglobin α-1 and α-2 chains and haptoglobin 1 (Schweigert et al., 2006). Moreover, a recent study utilizing different proteomic approaches determined proteins specifically accumulated into or depleted from the FF, when compared with blood plasma, which demonstrated that different approaches may lead to increased capacity to observe protein profiles (Jarkovska et al., 2010). To observe proteins present in lower concentrations, the authors depleted FF and blood plasma from some of the more abundant proteins.

In our study, we utilized the MudPIT achieving higher accuracy than other related protocols. When combined with bioinformatics, a larger number of proteins are potentially identified by MudPIT when compared with other proteomic approaches (Steel et al., 2005). We were able to statistically identify 416 proteins and random sequences in human FF samples, of which 62 were differentially expressed between women with endometriosis group III or IV (study group) and women without endometriosis who achieved pregnancy (control group).

Proteins identified in this study may represent potential biomarker targets for tailored diagnostic and therapeutics according to the oocyte requirements made specific in women with endometriosis. Of the differentially expressed proteins, 24.19% are related to binding and apoptosis, 1.61% to immune response, 12.9% to cell division, 4.48% to cellular metabolism, 25.81% to general function and 30.65% do not yet present an identified function (Fig. 4).

Binding and apoptosis
Twenty proteins identified may be related to binding to DNA, RNA, pro- and anti-apoptotic factors and other proteins. In our study, PBX3 post-miotic segregation factor, exclusively expressed in controls, may act by increasing expression levels of BMI1 (overexpressed in controls) and may therefore be related to pre-transcriptional control through binding to DNA, ultimately inducing gene expression or silencing. In turn, post-miotic segregation increased 2-like protein 3, exclusively expressed in the study group, interacts selectively and non-covalently with ATP, a universally important co-enzyme and enzyme regulator (Somervaille and Cleary, 2006; Faber et al., 2009).

We also observed 2 proteins overexpressed and 12 only expressed in the study group and 3 only present and 2 overexpressed in controls. Our findings indicate that endometriosis alters substantially the follicular microenvironment, by inducing overexpression of some proteins that have binding as a main function, whereas also partially suppressing expression of proteins participating in regulation of apoptosis. Figure 5 shows an interaction map for FAN (exclusively expressed in endometriosis), a protein participating in the apoptosis cascade. A better overall comprehension of apoptosis pathways in endometriosis may assist in understanding pathogenesis during initial development of the disease.

Immunologic function
Numerous proteins identified in the present study have some relationship with immunologic functions. In samples from the study group, IGL@, IGLC1 and serotransferrin participate in a large immune response cycle (Fig. 6). On the other hand, controls presented expression of IL-2 (Fig. 7).

IL-2 (score: 713.11) is produced by activated T cells and has an immunoregulatory effect on a variety of immune cells (Paul and Seder, 1994). This protein is probably inhibited in endometriosis, an effect that, added to overexpression of the serotransferrin-IGL@ cycle, induces an unbalanced inflammatory response in the follicular microenvironment.

Cell division
Cell division is orchestrated by interaction of numerous proteins. We observed five proteins that have some relation with cell division. Two of these proteins showed a high score: cell division cycle-associated protein 2 (CDCA2; score: 201.52) and TGF-β-activated kinase 1 (TAK-1; score: 31.75).

CDCA2 up-regulation may involve activators of cell cycle progression, DNA replication and repair (Trinkle-Mulcahy et al., 2006).
Table II  Proteins exclusively or overexpressed in women with endometriosis stage III or IV who did not achieve pregnancy after a first IVF treatment cycle when compared with the control group (women without endometriosis who achieved pregnancy after a first IVF treatment cycle).

| Gene name | Description | Score | Ratio endometriosis:control | Aminoacids |
|-----------|-------------|-------|-----------------------------|------------|
| **Binding** |             |       |                             |            |
| PMS2L3    | Post-meiotic segregation increased 2-like protein 3 | 60.04 | Only in endometriosis | 264 |
| BAE0T2    | cDNA FLJ56404, highly similar to peroxisomal targeting signal 1 receptor | 69.38 | Only in endometriosis | 660 |
| B4DZ45    | cDNA FLJ52372, highly similar to peroxisomal targeting signal 1 receptor | 70.02 | Only in endometriosis | 654 |
| PEX5      | Peroxisomal targeting signal 1 receptor | 71.66 | Only in endometriosis | 639 |
| APBA3     | Amyloid-β (A4) protein-binding, family A, member 3 variant | 82.7 | Only in endometriosis | 337 |
| B4DVW0    | cDNA FLJ50721, highly similar to peroxisomal targeting signal 1 receptor | 112.07 | Only in endometriosis | 519 |
| Q5HYG5    | Putative uncharacterized protein DCFZp686N0152 | 147.94 | Only in endometriosis | 110 |
| RIPP2     | Protein rippl2 | 312.04 | Only in endometriosis | 128 |
| A6NBZ8    | Putative uncharacterized protein ALB | 500.83 | Only in endometriosis | 627 |
| A8K891    | cDNA FLJ78290, highly similar to peroxisomal C-terminal targeting signal import receptor | 71.66 | Only in endometriosis | 639 |
| **Without specific function** |             |       |                             |            |
| Q6DHV4    | Putative uncharacterized protein | 165.23 | Only in endometriosis | 237 |
| Q6PS3     | Putative uncharacterized protein | 165.93 | Only in endometriosis | 236 |
| Q6GMW6    | Putative uncharacterized protein | 166.64 | Only in endometriosis | 235 |
| IGL@      | IGL@ protein | 166.64 | Only in endometriosis | 235 |
| Q5CZ94    | Putative uncharacterized protein DCFZp781M0386 | 167.35 | Only in endometriosis | 234 |
| IGL@      | IGL@ protein | 167.35 | Only in endometriosis | 234 |
| IGL@      | IGL@ protein | 168.07 | Only in endometriosis | 233 |
| Q6ZIP19   | cDNA FLJ26714 fis, clone PNC01043 | 252.49 | Only in endometriosis | 200 |
| C1 segment| C1 segment protein | 372.95 | Only in endometriosis | 105 |
| Q96E61    | Putative uncharacterized protein | 165.93 | Only in endometriosis | 236 |
| Q6GMV7    | Putative uncharacterized protein | 165.93 | Only in endometriosis | 236 |
| Q6P2J1    | Putative uncharacterized protein | 166.64 | Only in endometriosis | 235 |
| P01842    | Ig lambda chain C regions | 168.07 | Only in endometriosis | 105 |
| QBN355    | IGL@ protein | 168.07 | Only in endometriosis | 234 |
| **Metabolic function** |             |       |                             |            |
| PPP1R3B   | Protein phosphatase 1 regulatory subunit 3B | 66.61 | Only in endometriosis | 285 |
| **General function** |             |       |                             |            |
| Q56G89    | Serum albumin | 237.26 | 1.04 | 609 |
| B4DP96    | cDNA FLJ54371, highly similar to serum albumin | 251.11 | 1.04 | 618 |
| B2RB58    | cDNA, FLJ95666, highly similar to Homo sapiens albumin (ALB), mRNA | 296.77 | 1.01 | 609 |
| A8K9P0    | cDNA FLJ78413, highly similar to Homo sapiens albumin, mRNA | 297.26 | 1.04 | 608 |
| Q8LUK7    | Albumin protein | 397.62 | 1.04 | 396 |
| Q53H26    | Transferrin variant | 83.75 | Only in endometriosis | 698 |
| B4DI57    | cDNA FLJ53691, highly similar to serotransferrin | 95.56 | Only in endometriosis | 571 |
| Q1HBA5    | Transferrin | 116.71 | Only in endometriosis | 698 |
| Q6AH7     | Transferrin | 120.91 | Only in endometriosis | 698 |
| ALBU      | Serum albumin | 296.77 | 1.04 | 609 |
| B4DF80    | cDNA FLJ58357, highly similar to protein FAN | 66.13 | Only in endometriosis | 948 |
| A8K9G4    | cDNA FLJ77745, highly similar to sphingomyelinase (N-SMase) | 69.29 | Only in endometriosis | 917 |
| B2RA06    | cDNA, FLJ94643, highly similar to sphingomyelinase (N-SMase) | 69.29 | Only in endometriosis | 917 |
| P02787    | Serotransferrin | 120.91 | Only in endometriosis | 698 |

**Continued**
Ryu et al. (2007) observed this gene expression in patients with melanoma. We observed CDCA2 expression only in women with endometriosis (B3KRS8), which seems to indicate that this disease leads to changes in cell cycle progression factors (Gupta et al., 2006). Gupta et al. (2006) have also suggested that CDCA2 expression may lead to follicular oxidative stress, probably from erythrocytes and apoptotic endometrioma cells (Gupta et al., 2006).

TAK-1 is involved in regulation of the TGF-beta-activated kinase function by alternative splicing of its mRNA and by TAK-1-like (TAKL) proteins. We identified TAK-1 as a mitogen-activated protein kinase-like protein activated by TGF-β and BMP signaling (Yamaguchi et al., 1995).

TAK-1 may be activated by interleukin-1 (IL-1) and TNF-α (Takii et al., 1999; Holtmann et al., 2001). Ectopic expression of TAK-1 in

### Table III  Proteins exclusively or overexpressed in the control group (women without endometriosis who achieved pregnancy after a first IVF treatment cycle) when compared with women with endometriosis stage III or IV who did not achieve pregnancy after a first IVF treatment cycle.

| Gene Name | Description | Score | Ratio endometriosis:control | Aminoacids |
|-----------|-------------|-------|-----------------------------|------------|
| **Binding** | | | | |
| PBX3 | Pre-B-cell leukemia homeobox 3 (PBX3) | 56.42 | Only in controls | 367 |
| PBX3 | Pre-B-cell leukemia homeobox 3 (PBX3) | 117.64 | Only in controls | 176 |
| BMI1 | BMI1 polycomb ring finger oncogene | 173.48 | 0.68 | 184 |
| CN143 | Chromosome 14 open reading frame 143, isoform CRA_a | 207.51 | Only in controls | 163 |
| CN143 | EF-hand domain-containing protein C14orf143 | 262.01 | Only in controls | 163 |
| **Without specific function** | | | | |
| Q8TCL9 | Putative uncharacterized protein DKFZp564A247 | 191 | Only in controls | 142 |
| A6NIZ7 | Putative uncharacterized protein TMEM141 | 201.51 | Only in controls | 157 |
| B4DW36 | cDNA FLJ8862, highly similar to THUMP domain-containing protein 2 | 246.88 | Only in controls | 290 |
| Q53TTB | Putative uncharacterized protein C2orf8 | 268.14 | Only in controls | 267 |
| BSMD15 | Putative uncharacterized protein THUMPD2 | 295.85 | Only in controls | 242 |
| **Metabolic function** | | | | |
| PLGLA | Plasminogen-related protein A | 390.66 | Only in controls | 68 |
| PLGLA | Plasminogen-related protein A | 183.99 | Only in controls | 96 |
| **General function** | | | | |
| B4E1B2 | cDNA FLJ53691, highly similar to serotransferrin | 91.64 | 0.88 | 678 |
| A8K717 | cDNA FLJ36261 fs, clone THYMU2002695 | 236.29 | Only in controls | 303 |
| **Cell division** | | | | |
| THUMPD2 | THUMP domain-containing protein 2 | 77.05 | Only in controls | 473 |
| B0EVZ7 | TAK-1-like protein | 311.75 | Only in controls | 87 |
| B0EVZ8 | cDNA FLJ31777 fs, clone NT2R12008204, highly similar to TAK-1-like protein | 191 | Only in controls | 142 |
| B4DFW0 | cDNA FLJ60482, highly similar to TAK-1-like protein | 199.43 | Only in controls | 136 |
| **Immunologic function** | | | | |
| O95974 | IL-2 protein | 713.11 | Only in controls | 41 |
Xenopus embryos causes cell death or ventralization of embryos when apoptosis is blocked by Bcl-2 expression (Shibuya et al., 1998). In the present study, we observed TAK-1 only in controls, indicating a likely physiological process.

Metabolic function
The present study observed two proteins that may be related to metabolic functions, plasminogen-related protein A (PLGLA; score: 390.65) and protein phosphatase 1 regulatory subunit 3B (PPR3B; score: 66.61; Fig. 5).

PLGLA may be involved in the regulation of fibrinolysis and thrombosis, and its sequence displays high homology with plasminogen, the precursor of plasmin, which is a fibrinolytic and pericellular proteolytic enzyme. This protein is considered a secreted extracellular protease that degrades extracellular or cell surface components (Judex and Mueller, 2005). Because only controls presented the expression of PLGLA, it may be suggested that endometriosis alters coagulation mechanisms within the follicle (Krikun et al., 2008).

PPR3B, which was only observed in endometriotic samples, acts as a glycogen-targeting subunit for phosphatase (PP1) that is a member of the Ser/Thr phosphatases and widely distributed in many organisms. PP1 enzyme regulates many important physiological processes, including gene transcription, translation, metabolism, cell growth and division (Wang et al., 2008).

PPR3B facilitates interaction of the PP1 with enzymes of the glycogen metabolism and regulates its activity. It also suppresses the rate at which PP1 dephosphorylates (inactivates) glycogen phosphorylase and enhances the rate at which it activates glycogen synthase and therefore limits glycogen breakdown (Montori-Grau et al., 2007). Thus, endometriosis may determine alteration in metabolic pathways as well, limiting follicular glucose uptake by follicular and/or germ cells.

General function
In the study group, we observed 14 proteins with general function, 7 overexpressed and 7 exclusively expressed in this group. In the control group, we observed one protein overexpressed and another one exclusively expressed in pregnant woman without endometriosis. Most of these proteins have cellular transport as the primary function.

Proteins participating in ferric iron transmembrane transport are exclusively found or overexpressed in patients with endometriosis. Endometriosis may be involved with iron influx to the cell interior,
Figure 6 Interactome maps demonstrating principal interactions for Serotransferrin, IGL@ and IGLC1 Protein, proteins with inflammatory or general function exclusively expressed in women with endometriosis stage III or IV who did not achieve pregnancy after a first IVF treatment cycle when compared with the control group (women without endometriosis who achieved pregnancy after a first IVF treatment cycle).

Figure 7 Interactome maps demonstrating principal interactions for IL-2, an inflammatory protein exclusively expressed in the control group (women without endometriosis who achieved pregnancy after a first IVF treatment cycle) when compared with women with endometriosis stage III or IV who did not achieve pregnancy after a first IVF treatment cycle.
resulting in the oxidative modification of lipids and proteins, which leads to cell and DNA damage, and subsequently fibrosis development (Kobayashi et al., 2009).

A number of different proteins and pathways were observed in this study which may contribute to elucidating endometriosis since this disease determines alterations to oocyte competence and an overall decrease in fertility. Although only five patients were included in each group, we utilized inclusion and exclusion criteria to select samples appropriate to represent each group. Although the sample size is still very small, MudPIT associated with MS² is a robust and proven technique which helps to identify even small protein differences that may effect alterations under different biological conditions. Because MudPIT-MS² does not require separation of proteins in 2D gels, a lot of experimental variation is removed. Owing to the dynamic range and selectivity, techniques for depletion of albumin and other abundant proteins were not employed, which also greatly decreases variability. Currently, MudPIT utilizes pooling of samples in groups prior to trypsin digestion. We wish to build larger pools in the future and to also be able to do individual paired analyses, but our results in this study are encouraging in showing that MudPIT-MS² assists in determining a great number of proteins associated with endometriosis.

Conclusion

Protein profiles of women with endometriosis and pregnant women without endometriosis have been obtained by MudPIT-MS² analysis. Patients with endometriosis expressed at least 62 proteins that may be related to endometriosis physiopathology. The identification of these potential biomarker proteins may assist in understanding the mechanisms of endometriosis and in determining potential target biomarkers for diagnosis and prevention of endometriosis. This information may also be useful for the search of effective therapies in women with infertility due to endometriosis.

Supplementary data

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

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