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ARTICLE *in* JOURNAL OF PROTEOME RESEARCH · SEPTEMBER 2009

Impact Factor: 4.25 · DOI: 10.1021/pr9004426 · Source: PubMed

research articles Journal of proteome research

Comprehensive Proteomic Analysis of Human Endometrial Fluid Aspirate

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Received May 18, 2009

The endometrial fluid is a noninvasive sample which contains numerous secreted proteins representative of endometrial function and reflects the state of the endometrium. In this study, we describe, for the first time, a comprehensive catalogue of proteins of the endometrial fluid during the secretory phase of the menstrual cycle. To achieve this objective, three different but complementary strategies were used: First, in-solution digestion followed by reverse phase high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS); second, protein separation by denaturing one-dimensional electrophoresis (SDS-PAGE) followed by HPLC-MS/MS analysis. Finally, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) followed by MALDI-TOF/TOF analysis. The combination of the three strategies led to the successful identification of 803 different proteins in the International Protein Index (IPI) human database (v3.48). An extensive description of the endometrial fluid proteome will help provide the basis for a better understanding of a number of diseases and processes, including endometriosis, endometrial cancer and embryo implantation. We believe that the thorough catalogue of proteins presented here can serve as a valuable reference for the study of embryo implantation and for future biomarker discovery involved in pathologic alterations of endometrial function.

Keywords: Endometrial fluid • biofluids • proteomics • HPLC-MS/MS • mucins • embryo implantation

1. Introduction

The endometrium is the mucosa which covers the uterine cavity. This mucosa is comprised mainly of reticular connective tissue, epithelial cells, proliferating blood vessels and secreted proteins.[1] The endometrial cycle follows a tightly regulated series of physiological events provoked by changes in expression of different genes and proteins linked to the endometrium.[1] Therefore, a comprehensive study of the proteins present in the endometrium at defined stages of the endometrial cycle could help elucidate the mechanisms involved in embryo implantation and also provide insights into pathologic endometrial alterations.[2,3]

The study of endometrial conditions is done by means of the histological sampling (either by means of a blinded biopsy or by a hysteroscopically guided biopsy), or by means of a diagnostic hysteroscopy, although vaginal ultrasound is a useful tool in a number of cases. Currently, the diagnosis of endometrial diseases such as endometriosis is achieved by invasive methods that often include laparoscopic surgery under general anesthesia.

The endometrial fluid is a complex biological fluid which is in direct contact with the endometrial cavity and contains a multitude of proteins and proteolytic enzymes secreted from the endometrium.[4,5] It lubricates the endometrium, acting as a line of defense against pathogens, signals fertility and aids sperm migration and pregnancy. The endometrial fluid can be collected by aspiration in a painless manner using noninvasive methods.[6] Interest in the protein content of endometrial secretions has gained much momentum in recent years and has been suggested to play a key role in the embryo implantation process.[2,7] It has been shown that defects in the expression of these proteins may result in the failure of embryos to implant.[8] A recent differential proteomics study using two-dimensional electrophoresis also demonstrated that the endometrial fluid can be used for the identification of novel biomarkers for endometriosis.[3]

Several studies have recently stated that the analysis of the human cervico−vaginal fluid using two-dimensional electrophoresis or HPLC-MS/MS can be used to detect biomarkers for vaginal infections and sexually transmitted diseases. In a

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10.1021/pr9004426 CCC: $40.75
Published on Web 08/11/2009

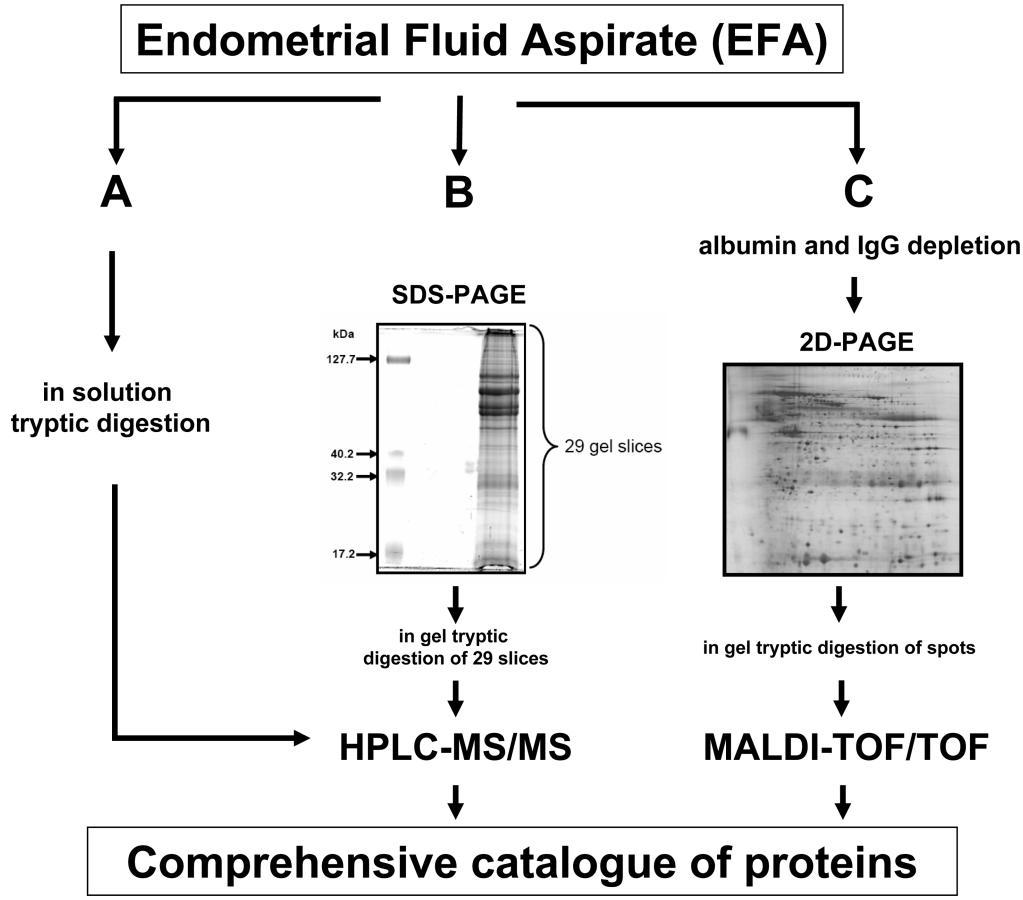

Figure 1. Schematic view of the three strategies (A, B and C) followed for the analysis of endometrial fluid aspirate. (A) In solution tryptic digestion of 1 μg of protein and analysis of peptides by HPLC-MS/MS. (B) 10 μg of EFA proteins resolved by 1D-PAGE and stained by Sypro Ruby. Twenty-nine gel bands were manually excised from the gel for subsequent analysis by HPLC-MS/MS. The molecular weight (Da) of the proteins is indicated. (C) 2D-PAGE separation of 300 μg of proteins, followed by in-gel tryptic digestion of Flamingo stained spots and analysis by MALDI-TOF/TOF.

work published by Tang et al.,[9] proteins obtained by vaginal lavage were separated by two-dimensional gel electrophoresis (2D-PAGE) and analyzed by MALDI-TOF/TOF, allowing the identification of 59 different proteins, whereas two studies based on HPLC-MS/MS identified 150[10] and 685[11] unique proteins, respectively, following interrogation of the Swiss-Prot (version 46.6) and IPI (version 3.24) databases. Although some proteins are common to both cervico−vaginal fluid and endometrial fluid, recent cytokine profiling studies using multiplexed immunoassays showed significant differences in the protein content between both type of samples.[12] Therefore, differential proteomics of the endometrial fluid aspirate proteome could give a new insight to understand the mechanisms involved in the onset of endometrial pathologies[13,14] and the process of embryo implantation.[12]

Current proteomic technologies allow the high-throughput screening of the proteins present in biological samples. Mass spectrometry (MS) is ideally suited for the identification and characterization of complex mixtures of proteins in an automated fashion.[15] Proteomes are normally fractionated or resolved either by polyacrylamide gel electrophoresis or using chromatographic methods. Nowadays, there is no preferred or standardized protocol to separate proteins from body fluid proteomes, and both approaches are considered to be complementary.[16−20] With the aim of achieving a thorough description of the proteome of the endometrial fluid aspirate, we used three different methodological strategies (A, B and C) that combine chromatographic and gel separation methods, as depicted in Figure 1. In strategy A, we carried out in-solution digestion of the protein content of endometrial fluid aspirate (EFA) with HPLC-MS/MS analysis of digested peptides. In strategy B, we combined protein separation by molecular weight using SDS-PAGE followed by in-gel digestion of proteins in gel slices and HPLC-MS/MS. In strategy C, proteins were separated by 2D-PAGE and all detected spots processed for protein identification by MALDI-TOF/TOF analysis. The integrated proteomic approaches used here led, for the first time, to a thorough identification of the catalogue of proteins present in EFA.

2. Materials and Methods

2.1. Sample Preparation and Determination of Protein Content. Healthy donor women without endometriosis were enrolled at the Valencian Institute of Infertility (www.ivi.es) and at the Reproductive Unit of Cruces Hospital (University of the Basque Country) (www.hospitalcruces.com). The study was approved by the Ethics Committee of the participating clinical institutions. Informed consent was obtained from each patient included in our study according to the ethical guidelines of the 1975 Declaration of Helsinki.

Endometrial fluid aspirates were collected from women aged between 18 and 45 with no laparoscopic evidence of en-

dometriosis or other endometrial alteration. Endometrial fluid was obtained during the secretory phase of the menstrual cycle by introducing an 'embryo-transfer' cannula (Delphin cannula, Gynetics Medical Products, NV) connected to a 20 mL syringe into the uterine cavity. Sample extraction was performed by manual vacuum application with the syringe. Only those samples showing no presence of blood were considered for analysis, which typically yielded 5−10 μL of EFA. Samples were expelled into standard cryogenic tubes and immediately frozen at −80 °C until processed. To provide enough protein for subsequent analysis by the three strategies (A, B and C), 10 samples from 10 different donors ($n = 10$) were pooled. Protein concentration was determined by measuring the absorbance at 570 nm using the Bradford colorimetric assay (Bio-Rad) and BSA as standard, according to the instructions of the manufacturer.

2.2. Preparative 1D-PAGE Gel. Sample volumes corresponding to 10 μg of EFA protein were resuspended in loading buffer (urea 7 M, thiourea 2 M, CHAPS 2%, SDS 4%, Tris 0.1 M pH 6.8, glycerol 30%, β-mercaptoethanol 12.5%, and bromophenol blue 0.0062%) and run using 10 cm and 12% SDS-PAGE gels for 1 h at 120 V. The gel was stained with Sypro Ruby (Bio-Rad) following the manufacturer's instructions. Gel image was acquired using Typhoon Trio scanner (GE Healthcare). After Sypro Ruby staining gel was sliced in 29 consecutive portions followed by in-gel trypsin digestion. After digestion, the peptides obtained from each gel slice were analyzed by HPLC-MS/MS.

2.3. Reverse Phase Chromatography Coupled with Tandem Mass Spectrometry (HPLC-MS/MS). Protein samples were cleaned by precipitation using the 2-D cleanup kit (GE Healthcare) according to the manufacturer's instructions before analysis by HPLC-MS/MS. After protein precipitation, a sample volume corresponding to 2 μg of protein was resuspended in 50 μL of 25 mM ammonium bicarbonate. Trypsin (Promega) was added at a ratio of 1:50 (w/w) (enzyme/protein) and incubated at 37 °C for 10 h. The supernatants were dried down using a speed-vac and resuspended in 10 μL of 0.1% formic acid (FA) suitable for mass analysis by reverse phase chromatography and mass spectrometry (HPLC-MS/MS). The resulting peptides were processed using two HPLC-MS/MS platforms:

2.3.1. Agilent 1200 nHPLC Coupled with LTQ Orbitrap. Tryptic peptides were preconcentrated and desalted using Zorbax 300SB-C18 cartridges (5 × 0.3 mm and 5 μm particle size) followed by elution on a Zorbax 300SB RP C18 column (75 μm × 150 mm and 3.5 μm particle size; Agilent Technologies) using a linear gradient from 3 to 50% solvent B in 4 h. Solvent A was 0.1% FA and solvent B was 0.1% FA in acetonitrile (ACN). All HPLC runs were performed using an Agilent 1200 HPLC system operated at the 300 nL/min constant flow rate. The peptides eluting from the chromatographic column were scanned and fragmented with a LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with a Proxeon nano-ESI source. An electrospray voltage of 1800 V and a capillary voltage of 50 V at 190 °C were used. Survey scan ranging from 400 to 2000 m/z were detected in the Orbitrap analyzer. MS/MS fragmentation and measurement of the six most intense ions was performed in the LTQ linear ion trap. Normalized collision energy was set to 35%.

2.3.2. Waters UPLC Coupled with Q-ToF Premier. Dried samples were resuspended in 0.1% FA and analyzed using UPLC reverse phase chromatography system (Waters). Tryptic peptides were desalted on a Symmetry C18 trapping cartridge (Waters) and further separated on an analytical column (Atlantis C18, 75 μm × 15 cm; Waters) with an integrated electrospray ionization emitter tip (SilicaTips for Micromass ZSpray NanoFlow, 10 μm diameter; New Objective). Peptides were eluted at a flow rate of 250 nL/min from the analytical column directly to the electrospray ionization emitter tip (SilicaTips for Micromass ZSpray NanoFlow, 10 μm diameter; New Objective) using a 4 h gradient from 3 to 50% solvent B (solvent A, 1% FA; solvent B, 100% ACN, 1% FA). Data were acquired in data-dependent acquisition mode (DDA), in which a full scan mass spectrum (m/z: 300−1500) was followed by MS/MS (m/z: 50−1995) in the eight most abundant multicharged ions (+2 and +3) every 4 s. Collision energies were varied as a function of the m/z and charge state of each peptide. Dynamic exclusion was incorporated for 30 s. A scan of the reference compound (Glu-fibrinopeptide B) was acquired every 10 scans through the whole chromatographic run.

2.4. Two-Dimensional Electrophoresis and MALDI-TOF/TOF Analysis. A fraction of the pooled EFA samples was purified using the Vivapure human albumin and class G immunoglobulin (IgG) immunodepletion kit (Vivascience AG) to specifically deplete albumin and class G immunoglobulins. After immunodepletion, the remaining proteins were precipitated with 15% (w/v) TCA and centrifuged at 13 000g for 30 min. The pellet was washed with 2 mL of chilled acetone and dried for 30 min at room temperature. This pellet was resuspended in rehydration solution (7 M urea, 2 M thiourea and 2% (w/v) 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)) and protein content determined with the Bradford colorimetric assay (Bio-Rad). A volume corresponding to 300 μg of protein with 1.2% DeStreak rehydration solution (GE Healthcare), 0.002% (w/v) bromophenol blue and 0.5% IPG buffer pH 3−10 nonlinear gel gradient (Non Linear) (GE Healthcare) was loaded in a 24 cm pH 3−10 NL Immobiline DryStrip (GE Healthcare) and isoelectric focusing (IEF) was performed in an Ettan IPGphor3 IEF System (GE Healthcare). Conditions for performing IEF were as follows: active rehydration at 50 V for 11 h, 250 V for 15 min followed by a linear gradient increase to 10 000 V, finishing the IEF run when the voltage reached >90 000 VhT. Focused strips were then treated with equilibration solution (6 M urea, 2% (w/v) sodium dodecyl sulfate (SDS), 50 mM Tris-HCl pH 8.8, 30% (v/v) glycerol, 0.002% (w/v) bromophenol blue and 2% (w/v) dithiothreitol) for 15 min and 2.5% (w/v) iodoacetamide for an additional 15 min. Electrophoresis to separate proteins in the second dimension was performed using 12% SDS-PAGE gels and Tris-glycine running buffer at 1.5 W per gel. After fixing with a solution containing 40% ethanol and 10% acetic acid, gels were stained overnight with the fluorescent dye Flamingo (Bio-Rad) following the manufacturer's instructions. Gel images were acquired using a Typhoon Trio scanner (GE Healthcare) and Progenesis PG240 version 2007 software (Nonlinear Dynamics) was used to detect and select spots on the digitized gel image and create a spot picking list. The 607 selected protein spots were recovered from the gel using an Ettan Spot Picker (GE Healthcare). The spots were in-gel digested with trypsin in a Proteineer DP automated digestor (Bruker) following Bruker's standard in-gel digestion protocol, and the resulting peptides were analyzed by MALDI-TOF/TOF. After digestion, each sample was prepared on an AnchorChip 800 target (Bruker), following a thin-layer preparation with α-cyano-4-hydroxycinnamic acid (HCCA) matrix. MS and MS/MS analysis was performed with an Autoflex Smartbeam TOF/TOF (Bruker) spectrometer equipped with a LIFT ion selector and a Reflec-

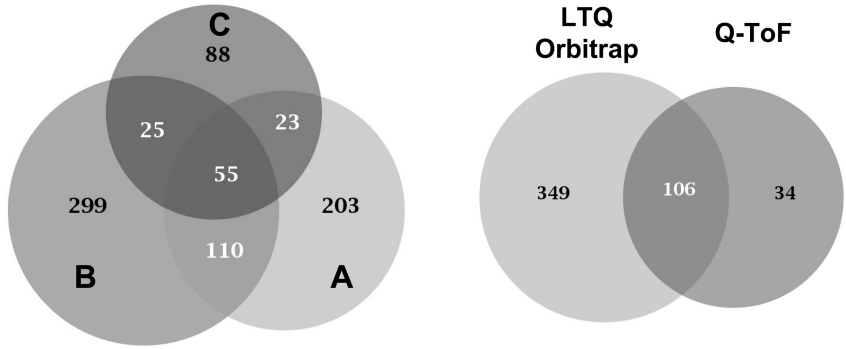

Figure 2. (Left panel) Venn diagram comparing the number of proteins identified after the analysis of endometrial fluid aspirate with the three different strategies (A, B and C). (Right panel) Venn diagram comparing the proteins identified in strategy B (one-dimensional SDS-PAGE) in two technical replicates using LTQ Orbitrap and Q-ToF.

tron ion reflector. Data collection was performed in fully automated, fuzzy-logic mode. Those samples failing identification in automatic acquisition mode were selected and desalted with homemade microcolumns using GEloader tips (Eppendorf) containing 1 mm chromatographic bed consisting of 50:50 (w/w) Poros R2 and R3 (Applied Biosystems). Peptides were eluted with HCCA matrix over a MALDI target (Anchorchip 600−384, Bruker) and allowed to dry at room temperature. The resulting spots were analyzed again with the same equipment, but in manual mode. Typically, 1400 scans for peptide mass fingerprint (PMF) and 2000 scans for MS/MS were collected.

2.5. Database Search and Bioinformatic Tools. Mascot v. 2.2.03 (www.matrixscience.com) was used as search engine to analyze mass spectrometric results. The parameters for the database searches were set as follows: enzyme, trypsin (KR); fixed modifications, carboxyamidomethyl cysteine; variable modifications, oxidation of methionine; number of missed cleavage sites allowed, 2. All fragmentation spectra were searched against human IPI v 3.48 FASTA database containing 71 401 entries (ftp://ftp.ebi.ac.uk/pub/databases/IPI/current/). The following tolerances were allowed for the different mass spectrometers: LTQ Orbitrap (±10 ppm for precursor ions and ±0.5 amu for fragment ions), Q-Tof (±10 ppm for precursor ions and ±0.1 amu for fragment ions), MALDI-TOF/TOF (±100 ppm for precursor ions and ±0.8 amu for fragment ions in automatic mode acquisition; ±50 ppm for precursor ions and ±0.7 amu for fragment ions in manual mode acquisition). For strategies A and B, only those proteins with at least two peptides with a false discovery rate (FDR) ≤1% after decoy database searches[21−23] were accepted as true positives. In the case of MALDI-TOF/TOF, those peptide mass fingerprints with scores ≥100 or a statistically significant score (≥65) and with a MS/MS score ≥20 after MASCOT searches were accepted as true positives. The comparative analysis of the lists of proteins obtained by the different techniques, their classification according to Gene Ontology (GO) and the elaboration of Venn diagrams were carried out using Protein Center (Proxeon Bioinformatics).

3. Results and Discussion

3.1. Protein Identification Following Different Strategies. The three different strategies followed for EFA analysis (A, B, and C) led to the identification of 391, 489, and 191 proteins, respectively (Figure 2). Combining the three strategies, we successfully identified 803 proteins having different IPI accession numbers (Supplementary Table 1). The MASCOT output files containing the proteins identified with each strategy are available in the following link: http://ftp.cicbiogune.es/sproteomics.

Interestingly, a significant number of proteins were exclusively identified in each strategy: 203 proteins were exclusively identified in strategy A, 299 found in strategy B and 88 were identified in strategy C. Only 56 proteins were identified by the three approaches, which represent 6.8% of all the identifications. This is indicative of the complementarity of the strategies proposed, which led to a comprehensive catalogue of the proteins present in the endometrial fluid aspirate.

When the three strategies were compared, it became evident that the SDS-PAGE strategy (strategy B) led to the identification of the greatest number of proteins. From the 489 proteins identified in this strategy after two technical replicates with two different MS platforms, 21.7% were identified in both of them, 71.4% were identified using LTQ-Orbitrap and 7.0% were exclusively identified after the analysis with Q-ToF (Figure 2). Focusing on the sensitivity of each of the two gel-based approaches (strategies B and C), the SDS-PAGE separation of 10 μg of proteins followed by HPLC-MS/MS analysis led to the identification of more than 2.5-fold the amount of proteins identified by 2D-PAGE, where 300 μg of protein was analyzed.

The 2D-PAGE approach (strategy C) led to the successful identification of 378 spots (Supplementary Figure 1) (191 proteins), representing more than 62% of all the spots analyzed (Supplementary Table 2). One of the advantages of the 2D-PAGE is the ability to distinguish different isoforms among the proteins resolved (see Supplementary Table 1 for isoform information). An example of this is the identification of S100-A9 calcium binding protein, detected in five different spots in the low molecular weight region of the 2D-PAGE gel (Figure 3).

Comparing the proteins identified in our study with previous proteomic analyses of cervico−vaginal fluid[9−11,24] and endometrial tissue,[25] we observed that there is a different degree of overlapping identifications going from 48 (out of 59),[9] to 194 proteins (out of 685),[11] (see Supplementary Table 3). Taking into account this last study, which is the most comprehensive proteomic catalogue to date for the cervico−vaginal fluid, the protein overlapping with the endometrial fluid aspirate was less than 30%. When comparing our data to the proteomic catalogue from endometrial tissue obtained by DeSouza et al.,[25]

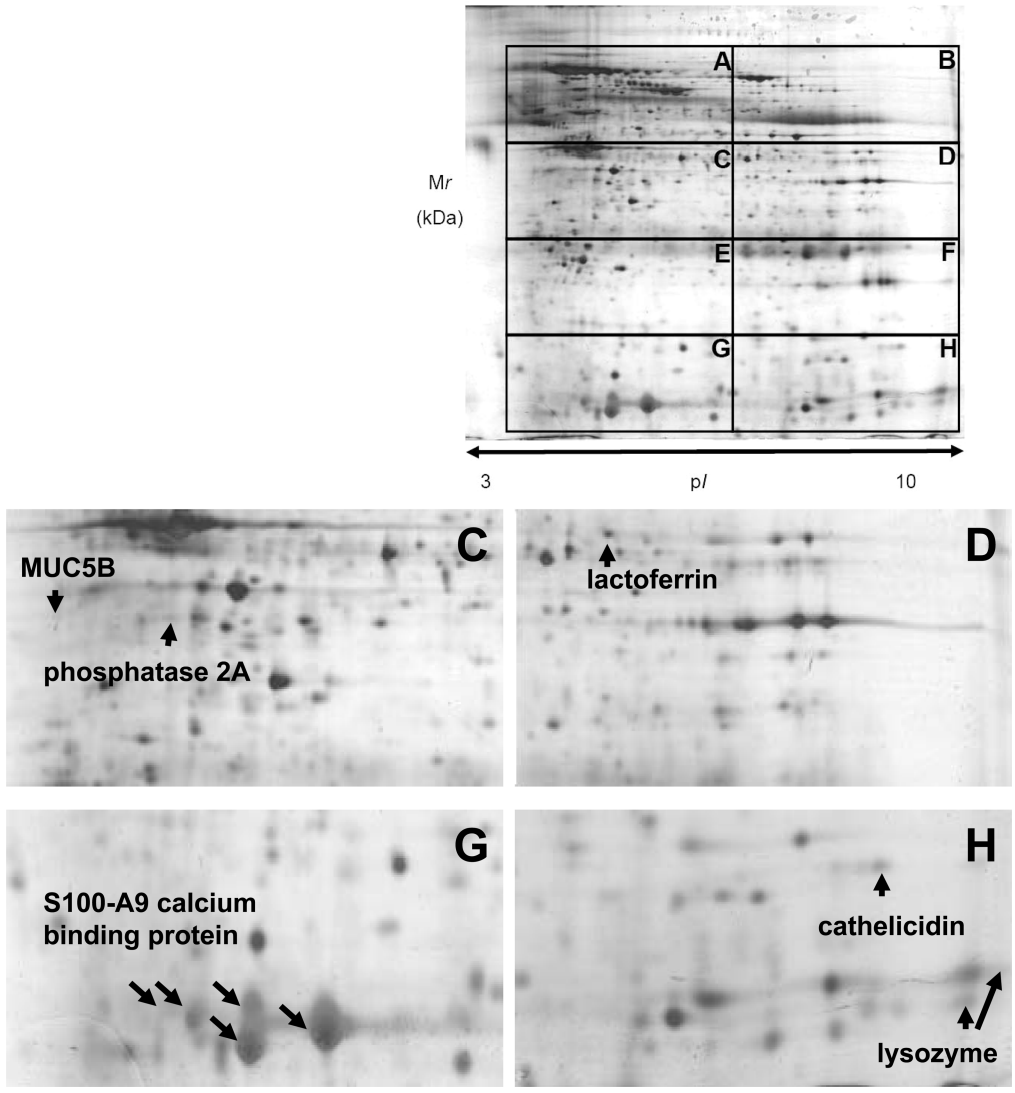

Figure 3. 2D-PAGE image after Flamingo fluorescent staining corresponding to the separation of 300 μg of proteins extracted from the endometrial fluid aspirate. A nonlinear 3−10 pH gradient was used for the isoelectrofocusing in the first dimension and 12% SDS-PAGE gel for the second dimension. Panels C, D, H and G show close-up views of the 2D-PAGE gel showing the proteins of interest identified by MALDI-TOF/TOF analysis (see Supplementary Table 2). (C) The presence of Mucin 5B (MUC5B) and phosphatase 2A, that intervenes in the secretion of mucins, is shown. (D, G and H) The identification of several defense proteins is indicated, including lactoferrin, S100, cathelicidin and lysozyme. S100-A9 calcium binding protein was identified in five intense spots in the low molecular weight acidic region of the 2D-PAGE (panel G).

we observed 57 proteins in common out of the 89 human proteins described in their analysis.

3.2. Gene Ontology Classification of Proteins. Clustering of proteins identified with the three strategies described above was performed according to the Gene Ontology (GO) hierarchy.[26] This protein classification is based on three different categories (cellular component, molecular function and biological process). Each category comprises several subsets.[26] Cellular component, molecular function and biological process include 18, 16, and 19 subsets, respectively. Some proteins can be included in more than one subset at the same time. As an example, annexin 1 (IPI00218918.5) is included in five different subsets within the cellular component category (i.e., nucleus, cytoplasm, cytoskeleton, membrane and extracellular). The classification for cellular component (Supplementary Figure 2A) revealed three major categories of proteins including those from the cytoplasm (65.0%), the membrane (56.3%) and also extracellular proteins (37.9%). The annotation of the molecular function (Supplementary Figure 2B) revealed that most of the proteins identified possessed protein binding capabilities (71.6%) or showed catalytic activity (54.2%). The classification according to the biological process (Figure 4) showed that the highest percentage corresponded to proteins involved in metabolism (68.5%), whereas 4.3% of them were related to reproduction.

A number of the proteins identified corresponded to proteins frequently found in the analysis of human body fluids[27] and also proteins present in blood. Several of the 22 most abundant proteins found in blood were identified in EFA regardless of the strategy followed, including albumin, IgGs, transferrin, fribrinogen, antitrypsin, complement C3, haptoglobin, apolipoprotein, ceruloplasmin and complement factor B. Even if immunodepletion was performed in strategy C, Albumin and IgG were also detected in several spots of the 2D-PAGE gel (Supplementary Table 2). The origin of these abundant proteins could probably be due to the abundant proliferating blood

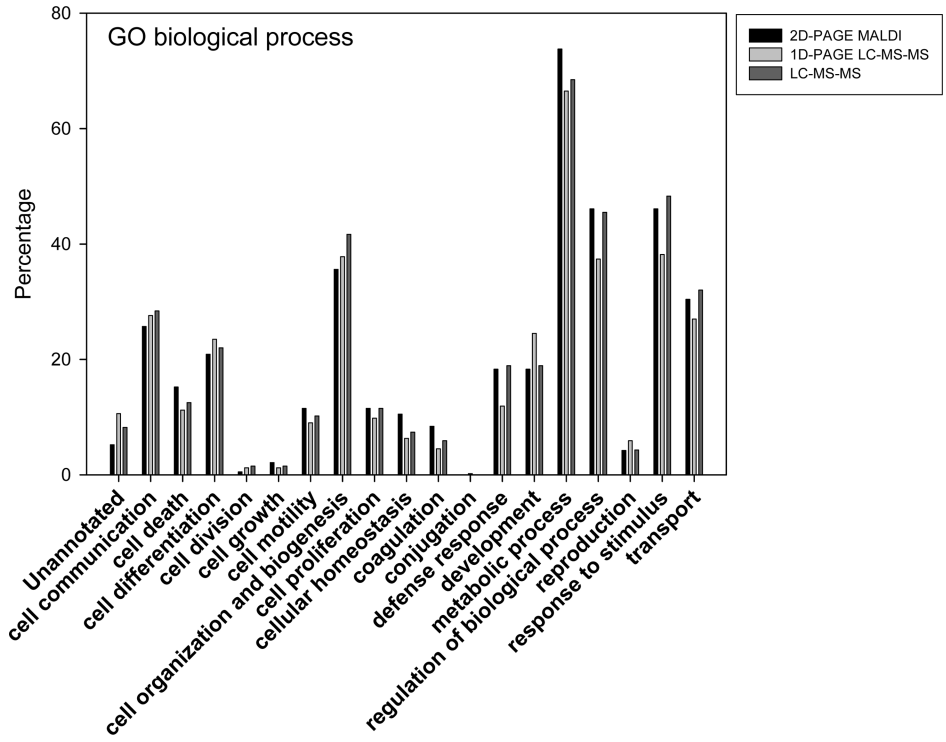

Figure 4. Clustering of all the proteins identified according to the Gene Ontology (GO) hierarchy based on biological processes. Bars indicate the percentage of proteins corresponding to each GO category. The three experimental approaches (1D-PAGE, light gray; HPLC-MS/MS, dark gray; 2D-PAGE, solid black) are displayed.

vessels within the mucosa.[1] These abundant proteins are beyond the scope of this study and here we focus on the discussion of those proteins and protein families that, according to the literature, may serve as biomarkers representative of an abnormal endometrium in several pathologies.

3.3. Proteins Related to Embryo Implantation, Endometriosis and Endometrial Cancer. The most relevant proteins identified in EFA and involved in endometrial alterations or in embryo implantation according to the literature are shown in Table I. There is strong evidence suggesting that both embryo-implantation and endometriosis are linked to the expression of growth factors and interleukins.[12,28−30] The levels of specific interleukins and other cytokines in the endometrium change significantly during the receptivity window and pregnancy.[28,30] As an example, pregnancy is associated with lower secretion levels of Interleukin-1β.[28] Recent studies by Boomsma et al. have described the presence of several interleukins in endometrial secretions using immunoassays.[28] In the same study, they also showed that IL-18 was the most abundant interleukin detected in the endometrial secretion, showing expression levels ranging from 2.3 to 63.5 pg/mg of the total protein content.[28] This agrees with the identification of IL-18 in EFA using mass spectrometry (strategy B). IL-18 was also found to be down-regulated both at the mRNA level and at the protein level in women suffering from endometriosis, and as a consequence, IL-18 has been proposed to play a pathogenic role in the onset of this pathology.[29]

Several proteins have been suggested to be involved in the etiology and pathology of endometriosis, including matrix metalloproteinases (MMPs), tissue inhibitor of MMPs (TIMPs) and cytochrome P450 19A1, also called aromatase (IPI 00878829).[31] It has been shown that MMP-9 and TIMP-1 metalloproteinases are strongly deregulated in the serum and uterine endometrial tissue of women with endometriosis.[32] In the present study, we show that both MMP-9 (IPI00027509) and TIMP-1 (IPI00032292 and IPI00552339) are present in the EFA, opening the possibility of future validations of these biomarkers by noninvasive means in endometrial fluid. Interestingly, aromatase was not detected in any of the strategies followed in our study, which agrees with the analysis of samples from healthy donor women.

Previous studies by DeSouza et al. have reported and validated a small range of proteins that can act as potential biomarkers for endometrial cancer.[25,33] According to their findings, the proteins achieving the best predictive values in terms of sensitivity and specificity include pyruvate kinase-M1/M2, alpha 1-antitrypsin and chaperonin-10. The first two proteins (i.e., pyruvate kinase-M1/M2 and alpha 1-antitrypsin) were also detected in EFA from healthy women in our study (Supplementary Table 2). Recently, DeSouza et al. quantified the levels of pyruvate kinase-M1/M2 extracted from endometrial tissues using two synthetic tryptic peptides (GVNLPGAAVDLPAVSEK and LDIDSPPITAR) by multiple reaction monitoring (MRM) mass spectrometry.[34] In their study, the authors found discrepancies in the relative quantitative values obtained for pyruvate kinase-M1/M2 protein using the first peptide, which was attributed to the potential occurrence of a population of peptides containing an N-terminal Lys due to a missed cleavage of trypsin. The list of tryptic peptides identified from pyruvate kinase-M1/M2 identified in our study (Supplementary Table 2) includes the identification of the peptide KGVNLPGAAVDLPAVSEK, including one missed cleavage. We did not observe GVNLPGAAVDLPAVSEK, which agrees with the hypothesis of DeSouza that the missed cleavage at the N-terminus of this peptide from pyruvate kinase-M1/M2 is a common occurrence.[34] Therefore, we believe that the information obtained in comprehensive catalogues, like in this work, including lists of proteins identified, different isoforms detected

Table I. Proteins Identified in Endometrial Fluid Aspirate (EFA) Involved in Endometrial Alterations or in Embryo Implantation[a]

protein description (including synonyms)	endometriosis/ectopic endometrium	endometrial cancer/ vulvar cancer	embryo implantation	references
Matrix metalloproteinase 9	X			31
Tissue inhibitor of matrix metalloproteinase	X			31
Annexin A1	X			3, 54
Mucin 16/CA125	X	X		45, 55−57
Cyclophilin A/peptidyl-prolyl *cis*−*trans* isomerase A		X		58
Interleukin-18	X		X	29
Pyruvate kinase-M1/M2		X		25, 33, 34
Alpha 1-antitrypsin/Alpha-1 protease inhibitor/ Serpin 1		X		25, 33, 59
Fatty acid-binding protein		X		60
Vimentin; Heat shock protein 70	X			61
Vitamin D binding protein	X			62
Carbonic anhydrase 1	X			63
Heat shock protein 90; Annexin A2; Peroxiredoxin 2; Apolipoprotein A1	X			64
Mucin 1			X	65
Protein S100-A8; Apolipoprotein A-1; Alpha 1-antitrypsin	X			66
Moesin; Beta-actin; Tubulin beta chain; F-actin; capping protein subunit beta; WD repeat protein 1; Heat-shock protein beta-1; Rho GDP-dissociation; inhibitor 1 and 2; 14-3-3 sigma and gamma; Glycodelin; Beta-2-glycoprotein 1; Sialic acid synthase; Alcohol dehydrogenase [NADP+]; Glutathione transferase omega-1; Ribose-phosphate pyrophosphokinase II; Poly(rC)-binding protein 1; Ferritin heavy chain	X			3

[a] Protein description: protein most used synonyms are separated by "/" symbols. Alterations where the proteins identified in our study have been shown to have relevance: Endometriosis/ectopic endometrium, endometrial cancer/vulvar cancer, embryo implantation and the reference of the publication.

and/or gene products using 2-DE, and the list of peptides identified with high confidence using shotgun proteomic approaches, are of great value for further hypothesis-driven and clinical validation studies.

3.4. The Mucin Family and Mucin-Related Proteins. Mucins (MUCs) are the main structural proteins of the mucus layer produced by epithelial tissues found in the gastrointestinal tract, lungs and urogenital tract (see refs 35 and 36 for review). MUCs are responsible for the characteristic viscoelastic properties of the mucus layer and play a role in the maintenance of mucosal homeostasis, being responsible for the regulatory responses against a plethora of microorganisms.[35] The MUC protein family comprises high molecular weight, highly glycosylated proteins. To date, 21 different MUC genes have been identified in humans, encoding both membrane-bound (MUCs 1, 3A, 3B, 4, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20 and 21) and secreted proteins (MUCs 2, 5AC, 5B, 6, 7, 8, 9 and 19).[35,36] Six different MUCIN proteins were identified after the analysis of the endometrial fluid aspirate. MUCIN 1, MUCIN 2, MUCIN 5B, MUCIN 5AC, MUCIN 6 and MUCIN 16. MUCs 1, 5B, 5AC and 16 were observed in strategy B and MUCs 1, 2, 5B, 5A, 6 in strategy A.

MUC1 is an abundant component in the human endometrium and plays a crucial role in embryo implantation.[37] It has been suggested that alterations in the expression levels of several epithelial cell surface proteins such as MUC1 may affect the receptivity of the endometrium. In our study, five tryptic peptides were attributed to MUC1 isoform 1 (IPI 00013955) after database searches: NYGQLDIFPAR, EGTINVHDVETQFNQYK, QGGFLGLSNIK, DISEMFLQIYK and YVPPSSTDR, indicating that this protein could also be studied by noninvasive methods using the endometrial fluid as a source of proteins. After the alignment of the 10 isoforms described for MUC1 (Supplementary Figure 3), we showed that those peptides are found in isoforms 1−4, 7, and 8. Therefore, the assignment MUC1 isoform 1 after database searching is incomplete, as no proteotypic peptides identifying a single isoform were detected.

Supplementary Figure 4 shows the amino acid sequence alignment of MUC5AC and 5B and all the peptides identified after protein analysis through strategies A and B. These two proteins, although sharing a high degree of homology in their amino acid sequences, could be distinguished due to the successful identification of proteotypic tryptic peptides exclusively found in each of the two proteins. Mucin 5B has been described as a new potential marker of endometrial cancer.[33] Interestingly, only MUC5B was identified in strategy C (Figure 3). MUC5B is characterized by a very high theoretical molecular weight (590.5 kDa), which is above the maximum molecular weight resolved by 2D-PAGE. This protein was detected as a spot (approximately 45 kDa). Eight peaks with different m/z ratios from this spot (spot 378) were attributed to eight different peptides from MUC 5B, with a score of 77 after database searching (Supplementary Figure 5). Two of these peptides were successfully attributed to MUC 5B after fragmentation and analyzed by MALDI-TOF/TOF (Supplementary Figure 5A,B). As shown in the figure, seven out of eight tryptic peptides identified in 2D gel spot number 378 are located near the

A

Trefoil factor 3 (accession number IPI00018909; TFF3_HUMAN)

M[1]AARALCMLGLVLALLSSSSA[21]***E***[22]***EYV***[25]***GLSANQCAVPAK***[37]DRV[40]D
CGYPHVTPK[50]ECNNRGCCFDSRI[63]**PGVPWCFKPLQEAECTF**[80]

Average weight of the precursor protein (80 amino acids): 8641.07 Da
Average weight of the mature protein (59 amino acids): 6580.49 Da

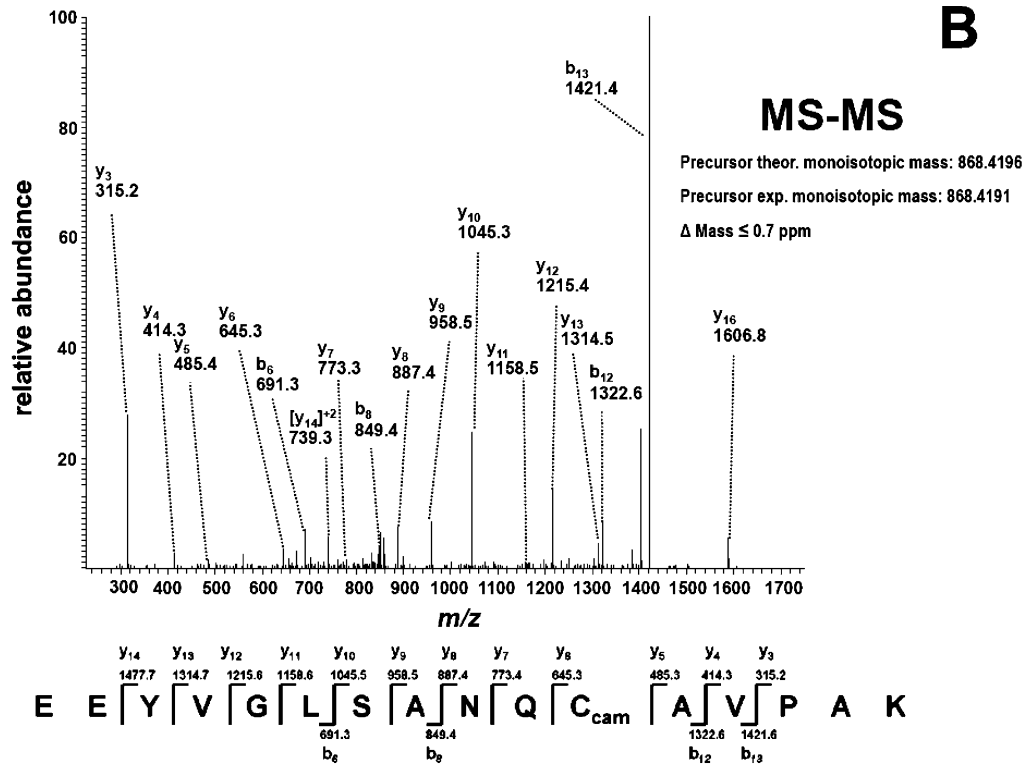

Figure 5. Amino acid sequence of the trefoil factor 3 protein (TFF3). Underlined residues correspond to the first 21 amino acids (Met[1]−Ala[21]) predicted as potential secretion signaling peptide. (A) Peptides identified after database search selecting trypsin (bold residues) or 'no enzyme' (bold italics) as endoprotease, are shown. (B) Fragmentation spectrum of the carboxiamidomethylated (cam) peptide with sequence E[22]EYVGLSANQC$_{cam}$AVPAK[37] corresponding to the N-terminus of the mature TFF3 is shown.

N-terminus of MUC 5B. All together, the findings indicate that the spot detected in the 2D gel corresponds to a truncated form of MUC 5B. The peptide from MUC 5B corresponding to sequence V[4223]LTSPATTPTATSSKATSSSSPR[4245] was identified with a higher mass error deviation (19.2 ppm) compared to the rest of the peptides identified on this protein (Supplementary Figure 5C) and is located far from the N-terminus, which led us to suspect that this peptide was wrongly attributed to MUC 5B by the search engine.

Several other proteins involved in the mucin secretory pathway, as proposed by Li et al. for human epithelial cells,[38] were identified in this study. In the model proposed, the myristoylated alanine-rich C kinase substrate (MARKS) is a key protein involved in mucin granule attachment and mucin secretion. MARKS activity is regulated upon dephosphorylation by protein phosphatase 2A. MARKS also interacts with actin and myosin, thereby linking mucin granules to the cytoskeletal machinery for subsequent movement and exocytotic release.[38] Once secreted, mucins interact with other proteins that aid in the formation of the mucus protecting gel layer. That is the case of trefoil factor 3 (TFF3), an epithelial secreted protein that has only been described to be expressed in small and large intestinal epithelia and in the uterine epithelium.[35,39] TFF3, with an average molecular weight of 8641.07 Da (Figure 5A), was uniquely identified after in-solution digestion followed by HPLC-MS/MS, and not identified in the gel-based strategies (B and C). The first 21 amino acids of TFF3 (M[1]AARALC-MLGLVLALLSSSSA[21]) are predicted to be a potential secretion signaling peptide that is subsequently cleaved from the mature protein.[40] The mature TFF3 protein is characterized by an

average molecular weight of 6580.49 Da (Figure 5A), and is therefore too small to have been readily resolved by the SDS-PAGE and 2D-PAGE gels used in our experiments (see Materials and Methods). Previous reports have described that LTQ ion trap results searched against a small FASTA databases containing the proteins of interest and using SEQUEST engine can lead to the unambiguous identification of nontryptic peptides.[41−43] The last is performed by selecting 'no enzyme' as a variable for database search. Here we have used this methodology in an attempt to unravel whether the processing of the signal peptide was taking place on TFF3. We constructed a FASTA database containing only the sequence of TFF3 protein (IPI00018909) and repeated the search using SEQUEST search engine, the same precursor and fragment ions tolerances used for the LTQ Orbitrap described above (±10 ppm for precursor ions and ±0.5 amu for fragment ions) and selecting 'no enzyme' as endoprotease. This led to the identification of two peptides (E^{22}EYVGLSANQCAVPAK^{37} and V^{25}GLSANQCAVPAK^{37}), showing a differential processing of the N-terminus of TFF3. The fragmentation spectra of the peptide with sequence E^{22}EYVGLSANQCAVPAK^{37} is shown in Figure 5B. Other N-terminal peptides were not identified, which would agree with the predicted cleavage of the N-terminal signaling peptide (Met^{1}−Ala^{21}) of TFF3.

In our analysis of the EFA, we identified three different entries (IPI00103552.4, IPI00646572.1, B3KY81) corresponding to MUC16, also called cancer antigen-125 (CA-125). These three entries differ in their sequence length (22 152, 14 495, and 1647 amino acid residues, respectively) and could indicate the occurrence of, at least, three species of CA-125 in the EFA. Currently, CA-125 is the only clinically useful biomarker for the follow-up of ovarian and endometrial carcinomas in serum.[33,44,45] CA-125 is also elevated in women suffering from endometriosis; however, its clinical value as a biomarker is questionable due to its low specificity.

3.5. Other Defense Enzymes. Under normal conditions, the endometrial cavity is sterile; however, it can be invaded by microorganisms potentially leading to embryo implantation failure.[46] A variety of endometrial responses are required to mediate protection against invasion by pathogens and to achieve sterility. It has been suggested that antimicrobial proteins and peptides are present in several body fluids (see Levy for review)[47] and several of these proteins were found in EFA, including lactotransferrin, lysozyme, defensins, bactericidal permeability increasing protein (BPI), cathelicidins and dermcidin[48] among others.

S100 proteins are involved in the regulation of a variety of cellular processes[49] and possess antimicrobial activity against bacteria and fungi.[50,51] The human S100 protein family comprises, at least, 25 proteins.[52] These are abundant proteins in oral and vaginal epithelial cells.[50,51] Several proteins from this family were identified in EFA, including S100-A7 (IPI00219806.7), S100-A7A (IPI00328396.3), S100-A8 (IPI00007047.1), S100-A9 (IPI00027462.1), S100-A11 (IPI00013895.1) and S100-A14 (IPI00010214.1). Only S100-A9 (IPI00027462) was identified using strategy C (2D-PAGE; Figure 3). As an indicator of the relative abundance of proteins in the endometrial fluid aspirate, we used the Protein Abundance Index (emPAI),[53] which is described as a useful indicator of the protein abundance when complex mixtures of peptides are analyzed by HPLC-MS/MS.[53] The emPAI values obtained for S100-A9 after the analysis through strategy A were 6.93 for the LTQ Orbitrap and 3.95 for the Q-ToF, respectively. The high emPAI values obtained agrees with the identification of S100-A9 as intense spots in the 2D-PAGE gel (Figure 2). These results indicate that the S100-A9 protein is a relatively abundant protein in EFA, and suggests its potential role in endometrial defense against pathogens.

4. Conclusions

The EFA is a biofluid that serves to monitor the *status quo* of the endometrial environment in women. We show here, for the first time, an in-depth analysis of the protein content of the EFA using proteomic techniques.

We used an integrated experimental approach, which combines gel-based and chromatographic-based methods coupled with MS. In agreement with previous reports combining different proteomic techniques,[21] the triple strategy proposed here led to complementary results. Nowadays, it is generally accepted that HPLC-MS/MS after one-dimensional SDS-PAGE separation of complex mixtures is the best suited strategy to identify the highest number of proteins, which agrees with our results on the analysis of EFA. The use of 2D-PAGE showed an added value since it was able to separate and differentiate protein isoforms. In-solution digestion followed by HPLC-MS/MS offered and advantage for high-throughput screening of the protein content of EFA, leading to the successful identification of mucins, which cannot be resolved by gel-based approaches. Overall, the 803 proteins identified encompasses a comprehensive list of proteins identified in a endometrial related biofluid, showing some but little overlap with other previously described related biofluids, such as cervico−vaginal fluid.

This catalogue of proteins derived from our experiments, corresponding to the secretory phase of the endometrial cycle, could be representative of the receptivity of the endometrium and serves as a first step for future studies on proteome changes during the endometrial cycle and for the identification of novel protein biomarkers specific for different endometrial alterations. The list of peptides identified using HPLC-MS/MS platforms may serve as a first stone for the design of targeted validation analysis and hypothesis-driven studies using selected reaction monitoring and multiple reaction monitoring MS methods. To make these data available for future studies, all our raw data (accession numbers 9738, 9739, 9740, and 9741) have been uploaded to the public data repository PRIDE (http://www.ebi.ac.uk/pride/). Moreover, the information achieved in the 2D-PAGE mapping of EFA may be of help as the information of isoforms of several proteins is provided.

It is important also to underline that the study of human endometrial−embryonic interactions has traditionally been complicated due to the disruptive impact of endometrial biopsy collection on the process of implantation itself.[28] Conversely, we believe that our analysis of EFA obtained by noninvasive methods should not impact the process of embryo implantation and could help to predict implantation in women undergoing *in vitro* fertilization techniques.

Data Availability. The raw data from HPLC-MS/MS platforms (accession numbers 9738, 9739, 9740, and 9741) have been uploaded to the public data repository PRIDE (http://www.ebi.ac.uk/pride/). MASCOT output files from HPLC-MS/MS and MALDI-TOF/TOF platforms are provided at http://ftp.cicbiogune.es/sproteomics.

Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; BPI, bactericidal permeability increasing protein; ACN, acetonitrile; CA-125, cancer antigen 125; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; DDA, data dependent acquisition mode; HCCA,

α-cyano-hydroxycinnamic acid; EFA, endometrial fluid aspirate; emPAI, exponentially modified Protein Abundance Index; FA, formic acid; FDR, false discovery rate; HPLC-MS/MS, reverse phase high-performance liquid chromatography coupled to tandem mass spectrometry; GO, gene ontology; IgG, class G immunoglobulin; IL, interleukin; IPI, International Protein Index; MARKS, myristoylated alanine-rich C kinase substrate; MMPs, matrix metalloproteinases; MRM, multiple reaction monitoring; MUC, mucin; NL, nonlinear gel gradient; PMF, peptide mass fingerprint; RP, reverse phase; SDS-PAGE, denaturing one-dimensional gel electrophoresis; TFF3, trefoil factor 3; TIMPs, tissue inhibitor of metalloproteinases; X_{corr}, cross correlation value.

Acknowledgment. We thank Dr. Simon Santa Cruz for critical reading of the manuscript and scientific discussions. We also thank Juan Antonio Vizcaino and Florian Reisinger from the EMBL European Bioinformatics Institute for their support with PRIDE converter v1.17 and PRIDE data repository.

Supporting Information Available: Supplementary Figure 1, spots identified by MALDI-TOF/TOF in the 2D-PAGE after strategy C. Supplementary Figure 2, clustering of the total pool of proteins identified according to the Gene Ontology (GO) hierarchy based on cellular components and molecular functions. Bars indicate the percentage of proteins corresponding to each GO category. The three experimental approaches (Strategy A, dark gray; Strategy B, gray; Strategy C, black) are displayed. Supplementary Figure 3, amino acid sequence alignment of 10 isoforms described for mucin 1 proteins. Supplementary Figure 4, amino acid sequence alignment of mucin 5AC (P98088) and mucin 5B (Q9HC84) proteins using ClustalW 2.0.10. Supplementary Figure 5, MALDI-TOF/TOF analysis of the 2D-PAGE spot corresponding to MUC 5B. Supplementary Table 1, list of spots numbers and proteins identified after strategy C. Supplementary Table 2, list of all proteins identified after strategies A, B, and C. International Protein Index (IPI) accession numbers, gene name and description of the proteins identified are indicated where available. Proteins identified after strategies A, B and/or C are indicated with (X) symbols. Supplementary Table 3, comparison among the proteins identified in Endometrial Fluid Aspirate and other proteomics analyses of cervico−vaginal fluid and endometrial tissue. This material is available free of charge via the Internet at http://pubs.acs.org.

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PR9004426