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Proteomic Analysis of Human Cervico-Vaginal Fluid

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Human cervico-vaginal fluid (CVF) is a mixture of fluids originating from the vagina, cervix, endometrium, and oviduct. CVF has been shown to play an important role in protecting the vagina from infection. We used "bottom-up" proteomic approaches to characterize the protein repertoire of human CVF. We applied two different sample prefractionation methods, one-dimensional-SDS-PAGE (1D-SDS-PAGE) and strong cation-exchange chromatography, followed by LC−MS/MS and bioinformatic analysis. We identified a total of 685 proteins. Strong cation-exchange chromatography prefractionation resulted in a larger number of proteins identified when compared with 1D-SDS-PAGE. Extracellular or membrane proteins made up 30% of the proteins identified, according to Genome Ontology (GO) classifications. We confirmed the presence of defense-related proteins, such as haptoglobin, defensins, and lactoferrin; and identified new ones such as azurocidin and dermcidin. We also identified many serine and cysteine proteases, including 6 members of the kallikrein family (KLKs 6, 7, 10, 11, 12, and 13). The same KLKs were also confirmed quantitatively by ELISA assays. Knowledge of the CVF proteome will aid in the discovery of potential biomarkers for gynecological malignancies and infections and provide additional clues for its physiological functions.

Keywords: cervico-vaginal fluid • mass spectrometry • proteomes • intravaginal infections • host defense • proteases • protease inhibitors • kallikreins

Introduction

Resolution of human biological fluid proteomes has become feasible with advances in mass spectrometric technologies.[1,2] It is now possible to comprehensively analyze proteins in very complex mixtures.[3] The analysis of human biological fluid proteomes may aid in the discovery of potential biomarkers for pathological conditions.[4] The proteome of human cervico-vaginal fluid (CVF) in nonpregnant women has not as yet been resolved in detail.

CVF is comprised of fluid originating from the vagina, as well as other fluids flowing into the vagina, such as cervical mucus and endometrial and oviductal fluids.[5] CVF plays an important role in innate defense.[6] CVF has been shown to contain antimicrobial substances, such as cationic peptides,[7] lysozyme,[8] lactoferrin,[9,10] secretory leukocyte protease inhibitor (SLPI),[11,12] human neutrophil peptides, and human β-defensins.[13] These substances play a role in host protection against invading microbes and viruses, including the HIV virus.[6]

The CVF proteome has primarily been studied in search for potential markers for pregnancy-associated conditions such as preterm labor and intra-amniotic infections. For example, increased levels of fetal fibronectin in the CVF of pregnant women has been suggested to be predictive of preterm delivery.[14] Decreased levels of inflammatory cytokines measured in CVF have also been shown to be associated with preterm delivery.[15]

Two recent studies by Dasari et al.[16] and Pereira et al.[17] identified 205 proteins in CVF from pregnant women, as well as potential biomarkers for preterm birth in this fluid. A recent study by Di Quinzio et al.[18] used 2D-SDS-PAGE, followed by mass spectrometry, to analyze the proteome of CVF from 5 pregnant women at term. They detected approximately 400 protein spots and chose to identify 15 proteins common to all 5 women. Identified proteins included those associated with blood transport, calcium binding, enzyme inhibition, and enzymes involved in response to oxidative stress.[18]

Until now, the human nonpregnant CVF proteome has not been systematically delineated. We performed proteomic analysis by different prefractionation methods and compiled a list of 685 unique proteins present in human CVF. Some of these proteins were also identified by confirmatory ELISA testing.

Materials and Methods

Collection of CVF from Healthy Volunteers. Pieces of sterile gauze (5 × 5 cm) were provided to healthy, 20−40 year old, female volunteers. Subjects were not pregnant or experiencing any vaginal infection at the time of sample collection. We did not control for subject's time point during the menstrual cycle.

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Subjects were asked to insert the gauze into their vagina for 1 h. The gauze was then removed and stored in 50 mL plastic conical tubes (BD Biosciences), at −20 °C until use. Our protocol has been approved by the Institutional Review Boards of Mount Sinai Hospital and the University of Toronto.

Extraction of CVF. Gauze used to collect CVF was thawed, 10 mL of sterile phosphate-buffered saline (PBS) was added to the tube with the gauze and was mixed by rotation for 6 h. The extract was removed from the gauze using a 20 mL syringe, which was also used to squeeze the fluid out of the gauze.

Five milliliters of each of 4 CVFs were pooled together for the pooled experiments. Two and a half milliliters of an individual CVF sample was used for the gel experiment (see below).

Preparation of Samples for SDS-PAGE Fractionation. CVF samples were desalted using a disposable PD-10 desalting column (GE Healthcare), according to the manufacturer's recommendations. Briefly, the column was equilibrated with 200 mM ammonium bicarbonate. Two and a half milliliters of vaginal fluid was then applied to the column and allowed to flow through. Three and a half milliliters of 200 mM ammonium bicarbonate were then added to the column, and the eluate collected.

The PD-10 column eluate was lyophilized to dryness and then resuspended in 350 μL of water. A 30 μL aliquot was then resolved by SDS-PAGE and stained using Simply Blue Safe Stain (Invitrogen), according to the manufacturer's protocol. Thirty-four to thirty-six evenly sized bands were then cut from the gel, comprising the entire lane of the stained protein sample.

In-Gel Preparation of Proteins for Mass Spectrometry. Individual gel bands were placed in 1.5 mL microfuge tubes. To shrink the gel band, enough acetonitrile (ACN) was added to cover the band, and incubated at room temperature for 10 min. The ACN was then removed, and 10 mM DTT (Sigma) was added to cover the gel band and reduce the proteins present. Gel bands were incubated with DTT for 10 min at 50 °C and then at room temperature for an additional 20 min.

Following reduction, DTT was discarded and alkylation was performed by adding 100 mM iodoacetamide (Sigma) to cover the gel band. Gel bands were incubated with iodoacetamide for 15 min at room temperature, in the dark. Iodoacetamide was then discarded and 0.5 μg of trypsin (Promega) were added, along with sufficient 50 mM ammonium bicarbonate to cover the gel, and incubated overnight at 37 °C.

Preparation of Samples for SCX Fractionation. CVF was dialyzed using a 3500 molecular weight cutoff tubing for 24 h in 1 mM ammonium bicarbonate, in order to remove salts. Dialysis buffer was changed twice during this 24 h period. The sample was then lyophilized to dryness, denatured with 8 M urea (Fisher), and reduced with 13 mM DTT at 50 °C for 30 min. Alkylation was then performed with iodoacetamide (125 mM) followed by incubation at room temperature for 1 h, in the dark, with constant shaking. The sample was then desalted using a NAP5 column (GE Healthcare). Five-hundred microliters of eluate from the NAP5 column was lyophilized to dryness, followed by typsin digestion (1 μg/100 μg of total protein) overnight at 37 °C. The sample was then lyophilized to dryness.

SCX Liquid Chromatography. The lyophilized sample was reconstituted in 120 μL of mobile phase A (0.26 M formic acid in 10% acetonitrile) and loaded directly onto a polySULFOETHYL A column with a 2.0 μm pore size and 5 μm diameter (The Nest Group Inc.). A 1 h fractionation was performed using high-performance liquid chromatography (HPLC), with an Agilent 1100 system. A linear gradient was used with 0.26 M formic acid in 10% acetonitrile as running buffer and 1 M ammonium formate as elution buffer. Forty 200 μL fractions were collected and pooled into 8 fractions. Each of the 8 fractions was concentrated to 100 μL using a SpeedVac system.

Mass Spectrometry. For samples fractionated by SCX, the peptides in each fraction were purified with a ZipTip$_{C18}$ pipet tip (Millipore), and eluted in 4 μL buffer B (90% ACN, 0.1% formic acid, 10% H_2O, 0.02% TFA). Eighty microliters of buffer A (95% H_2O, 0.1% formic acid, 5% ACN, 0.02% TFA) were added to each sample, and 40 μL of each sample were injected onto a 2 cm C18 trap column (inner diameter 200 μm). For samples fractionated by SDS-PAGE, 40 μL of sample (not zip-tipped) were injected as above. Peptides were eluted from the trap column onto a resolving analytical C18 column (inner diameter 75 μm) with an 8 μm tip (New Objective). This liquid chromatography was setup online to a 2-D linear ion trap (LTQ, Thermo Inc.) mass spectrometer, using a nanoelectrospray ionization source (ESI). Samples prepared by SCX and SDS-PAGE were run for 120 and 30 min gradients, respectively. The eluted peptides were subjected to tandem mass spectrometry (MS/MS). Data files were collected using Mascot Daemon (v2.1.03) and extract_msn, using the following parameters: min. mass 300, max. mass 4000, automatic precursor charge selection, min. peaks 10 per MS/MS scan for acquisition and min. scans per group of 1.

Data Analysis. The resulting raw mass spectra were analyzed using Mascot (V 2.1.03, Matrix Science) and X!Tandem (V 2006.04.01.2, Proteome Software) search engines and searched against the nonredundant IPI Human database (Version 3.24). Up to one missed cleavage was allowed and searches were performed with fixed carbamidomethylation of cysteines and variable oxidation of methionine residues. A fragment tolerance of 0.4 Da and parent tolerance of 3.0 Da were used in searches with each search engine, and trypsin was chosen as the digesting enzyme. The resulting files were loaded into Scaffold (Proteome Software), a bioinformatics program which validates each MS/MS by assigning protein and peptide probabilities based on chosen parameters. We chose 80% probability of protein identification and 95% probability of peptide identification, based on the peptide prophet algorithm.[19] Each protein was assigned a cellular localization based on information from Swiss-Prot and Genome Ontology (GO) databases.

To calculate the false-positive rate, identical searches to the ones performed above were performed using the "sequence-reversed" IPI human database (Version 3.24). The false positive rate was calculated as: Number of peptides identified by the reverse database/(Number of peptides identified by the forward database + reverse database)*100.

Functional and Pathway Analysis of Identified Proteins. Functional and pathway analysis was performed using Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com) and identified functions and/or diseases that were most significant to the set of proteins identified. For analysis in which the Fischer's exact test was used to calculate a p-value determining the probability that each function or interaction assigned to the set of proteins is due to chance alone, 394/685 genes were eligible.

KLK-Specific ELISA Immunoassays. All ELISA immunoassays used in this study were "sandwich" type, with one antibody used for capture, and another used for detection. The ELISA methods for all KLKs have been recently described in detail.[20]

Table 1. Unique Proteins Identified in Each Sample and Unique Peptides Associated with These Proteins[a]

SDS-PAGE # of peptides identified	SDS-PAGE single sample	SCX pooled sample	pooled sample
1	102	51	249
2	56	29	93
3	35	18	39
4	13	13	23
5	11	14	11
>5	65	56	27
total	282	181	442

[a] Overlap of proteins in each sample is presented in Figure 1.

Table 2. Kallikrein Proteins Identified in Vaginal Fluid by Mass Spectrometry and ELISA

Kallikrein	concentration[a] (μg/L)	# of peptides
6	599	7
7	247	2
10	155	2
11	1690	5
12	3188	1
13	1750	7

[a] Median values by ELISA based on 5 samples.

Results

Identification of Proteins by Mass Spectrometry: SDS-PAGE Gel Fractionation. A CVF sample from one individual (2.5 mL) and a pooled sample from four individuals (performed in duplicate, 5 mL per duplicate) were analyzed. Two-hundred eighty-two proteins were identified from the single CVF sample and 181 proteins were identified from the pooled sample, after combining the two duplicates. Complete lists of the proteins identified in these two experiments are presented in Supplementary Table 1 (see Supporting Information).

Identification of Proteins by Mass Spectrometry: SCX Fractionation. A pooled sample was used, and this experiment was performed in duplicate. Four-hundred forty-two proteins were identified using this method (from both duplicates). The numbers of proteins identified with this experiment and their breakdown by number of detected peptides are shown in Table 1. A complete list of proteins identified with this experiment can be found in Supplementary Table 1.

Identification of Selected Marker Proteins by ELISA. Analysis of the same five CVF samples used in this study by ELISA identified the following members of the kallikrein family: KLKs 1, 5, 6, 7, 8, 10, 11, 12, 13, and 14. KLKs 6, 11, 12 and 13 were the most abundant, as shown in Table 2. We were able to identify KLKs 6, 7, 10, 11, 12, and 13 by mass spectrometry. The number of peptides identified and sequence coverage are shown in Table 2 and Supplementary Figure 1 (see Supporting Information).

Reproducibility between Duplicates. Analyses of the pooled CVF samples prepared by SDS-PAGE and SCX were performed in duplicate. Venn diagrams illustrating the number of proteins found in each replicate and the overlap between duplicates are found in Figure 1A and B. For the samples prepared by SCX, there was 54% reproducibility between the proteins identified; 36% reproducibility was found with the samples prepared by SDS-PAGE.

Overlap of Proteins between Experiments. A total of 685 unique proteins were identified when combining the results of all experiments; the identified proteins are listed in Supplementary Table 1. Three-hundred forty-one (49.8%) of the proteins were identified by 2 or more peptides (Table 1). Figure

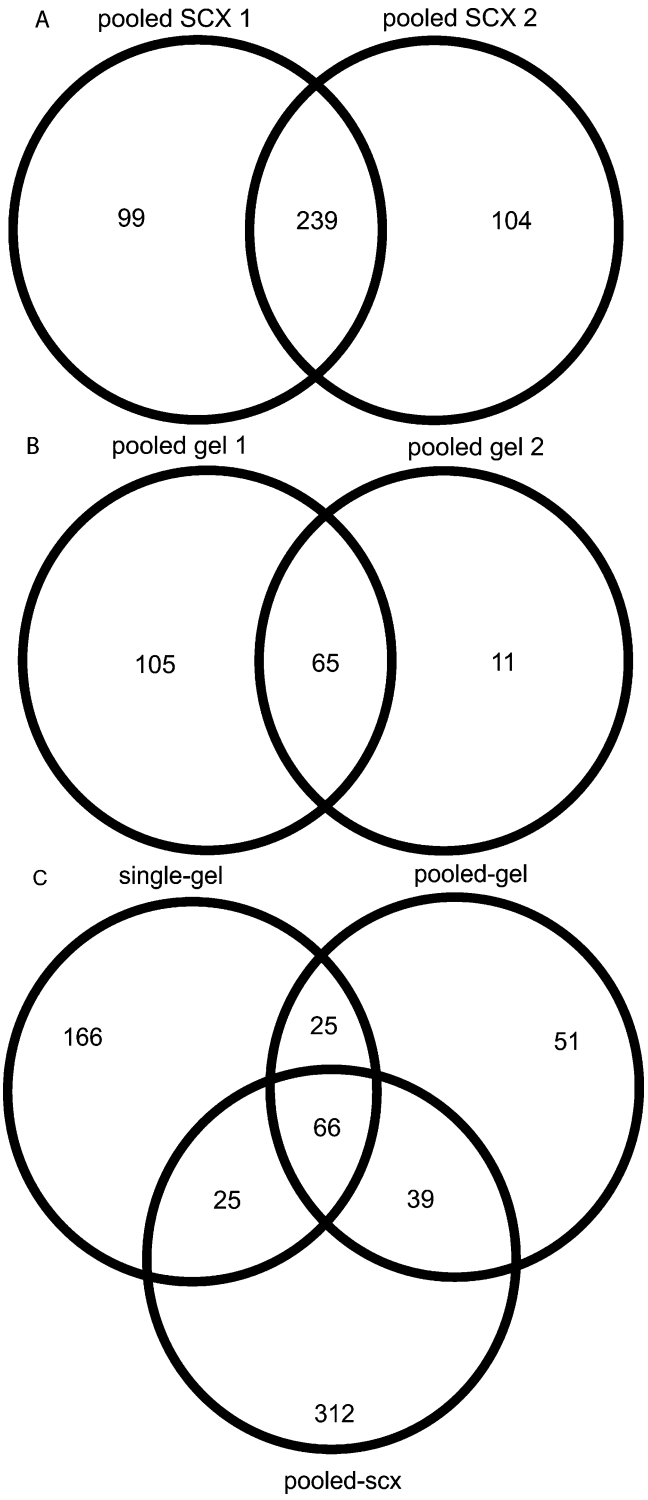

Figure 1. Venn diagrams illustrating the number of proteins identified by each of the experimental approaches used and for each replicate. (A) Number of proteins identified in each replicate and overlap for SCX-fractionated samples, (B) number of proteins identified in each replicate and overlap for SDS-PAGE-fractionated samples, and (C) number of proteins identified and overlap between all experiments. Numbers refer to the number of proteins identified.

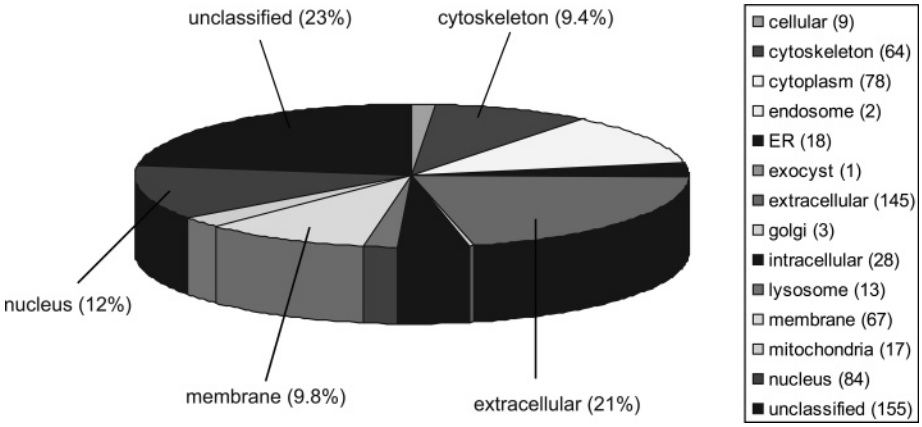

Figure 2. Proteins identified in this study with respect to genome ontology (GO) classifications. In brackets are the percent of proteins per location.

1C shows the overlap between experiments. Sixty-six proteins were identified in CVF by all three experiments (Supplementary Table 1).

Cellular Localization of Identified Proteins. The identified proteins were classified according to cellular localization using Genome Ontology (GO) classifications. These results are shown graphically in Figure 2. Twenty-three percent of the proteins identified are not classified according to GO. Twenty-one percent of identified proteins are classified as extracellular, 12% as nuclear, and 9.8% as membrane-associated proteins. GO classifications of all identified proteins are listed in Supplementary Table 3 (see Supporting Information).

Calculation of False-Positive Error Rate. When raw mass spectra were searched against the "sequence reversed" IPI human database, 5 peptides were identified. We calculated the false-positive rate to be approximately 0.2%.

Analysis of Biological Function. The potential molecular functions of identified genes/proteins were analyzed using Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com). There were 394/685 genes eligible for molecular function and pathway analysis. The top 15 molecular functions are shown in Figure 3. Major categories include cellular movement, immune response, cell death, cell-to-cell signaling, and cellular assembly and organization.

Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com) was also used to assign the identified proteins to biological processes and to construct biological networks. A total of 394 proteins were known components of existing biological networks. Thirty-nine networks were constructed; the one with the highest score is shown in Figure 4. This network contains 35 genes, whose encoded proteins were identified in our CVF proteome. This network contains 2 members of the kallikrein family, KLK6 and KLK7. Top functions of this network include cell−cell signaling, and hematopoetic, immune, and lymphatic system development and function.

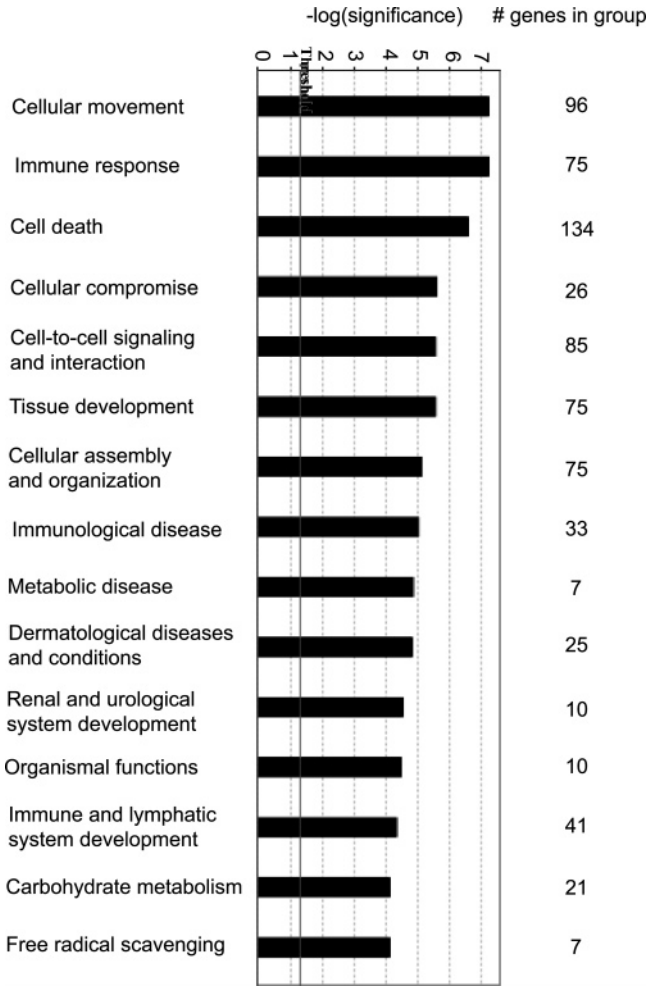

Figure 3. Top 15 functions of gene products identified in CVF, as well as the number of genes identified in each functional group.

Discussion

We identified 685 unique proteins in human CVF from healthy individuals using two different sample preparation methods, followed by mass spectrometry and bioinformatic analysis. A complete list of the proteins identified is presented in Supplementary Table 1. Two recent studies by Dasari et al.[16] and Pereira et al.[17] identified 205 proteins in CVF from pregnant women, 132 of which were also identified by us in this study (Supplementary Table 1B). Another study, utilizing 2D-SDS-PAGE and mass spectrometry, identified approximately 400 protein spots. This study analyzed the CVF proteome of pregnant women at term,[18] and only positively identified 15 proteins which were common to all 5 women studied. Ten of the 15 proteins identified by this group were also identified with our study (Supplementary Table 1B). Differences between

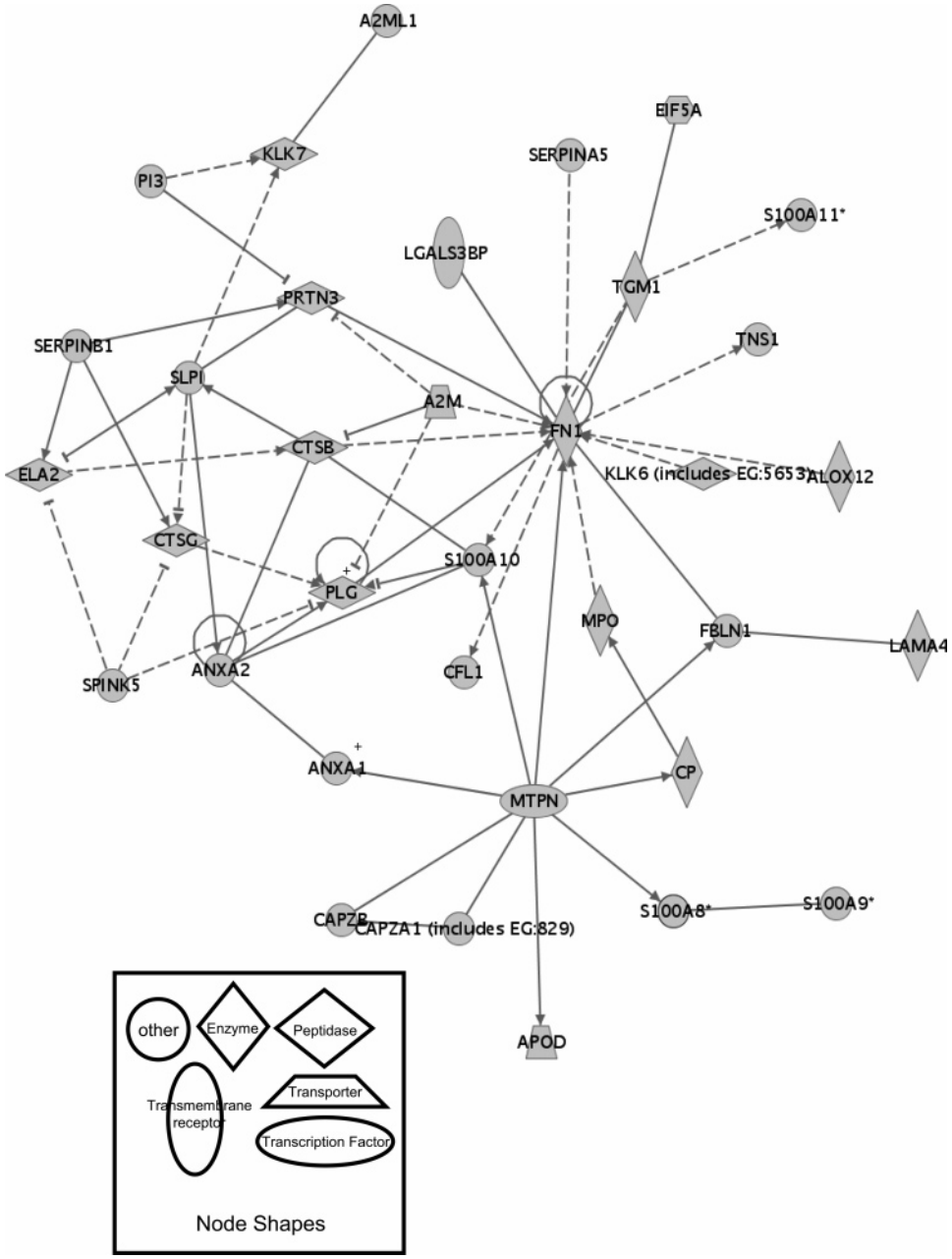

Figure 4. Example of a network constructed based on the gene products identified in CVF. This was the highest scoring network and contains 35 genes/proteins identified in CVF. Full gene names can be found at the gene nomenclature website: www.gene.ucl.ac.uk/nomenclature/. A legend for the node shapes, representing genes, is located at the bottom of the figure. Solid lines represent established direct interactions, dotted lines represent indirect interactions.

the proteins identified in these studies and our study are likely due to changes in the CVF proteome as a result of pregnancy.

We feel confident that none of the proteins identified in CVF in this study were the result of seminal contamination. Subjects confirmed abstinence from unprotected sexual intercourse for at least two weeks prior to sample collection. As well, we analyzed each CVF sample in this study for KLK3 (prostate-specific antigen; PSA), an excellent marker for seminal contamination with levels ranging from 10^7-10^8 μg/L in seminal plasma. We identified trace amounts of KLK3 in these CVF samples (<5 μg/L). Much larger levels would have been expected if the samples were contaminated with seminal fluid.

Our data were also searched against the following nonhuman databases using X!tandem: *S. cerevisiae*, *S. pombe*, *Bacillus substilis*, *Clostridium perfringens*, *Deinococcus radiourans*, *E. coli* K12, *Francisella tularensis tularensis*, *Geobacter sulfurreducens Haemophilus influenzae*, *Listeria innocua*, *Mycobacterium leprae*, *Mycobacterium tuberculosis* CDC1551, *Mycoplasma pulmonis*, *Staphylococcus aureus* MW2, *Streptococcus pneumoniae* TIGR4, *Streptococcus pyogenes* M1 GAS, and *Yersinia pestis* CO92. No significant matches were identified.

We identified members of the kallikrein family of serine proteases in human CVF by ELISA. Kallikreins are a group of 15 secreted serine proteases, with tryptic or chymotryptic activity.[21] KLK1, 5, 6, 7, 8, 10, 11, 12, 13, and 14 have all been found in human CVF by ELISA, with KLK6 (median 600 μg/L), 11 (median 1690 μg/L), 12 (median 3188 μg/L), and 13 (median 1750 μg/L) found at highest levels.[20] In this study, we positively

identified KLK 6, 7, 10, 11, 12, and 13 in CVF (Table 2, Supplementary Figure 1). Five of the 6 KLKs were identified by more than 2 peptides.

Two different sample fractionation methods were used. SDS-PAGE fractionation of an individual CVF sample and a pooled sample was performed. In the individual sample, we identified 282 unique proteins, 180 (64%) of which were identified by 2 or more unique peptides (Table 1). We also identified 181 unique proteins in the pooled CVF sample, 130 (72%) of which were identified by 2 or more unique peptides (Table 1). SCX liquid chromatography was performed for the pooled CVF sample only, and 442 unique proteins were identified. One-hundred ninety-two (44%) of these proteins were identified by more than 2 unique peptides (Table 1). The reproducibility between duplicates prepared by SCX was 54% (Figure 1A).

Prefractionation of samples by SCX yielded a greater number of proteins and better reproducibility when compared to 1D-SDS-PAGE. This is not surprising given that SDS-PAGE is known to have a relatively low dynamic range, resulting in the identification of primarily high abundance proteins.[22] There are also limitations with respect to the amount of protein that can be loaded onto an SDS-PAGE gel.[23] Chromatographic fractionation methods such as SCX have been shown to result in identification of larger numbers and lower abundance proteins.[24−26]

The study by Dasari et al.[16] employed SCX and SDS-PAGE fractionation of CVF, yielding similar results to our study with respect to the number of proteins identified using each method. A larger number of proteins were identified as a result of SCX fractionation when compared with SDS-PAGE fractionation. Another recent study[26] used various fractionation approaches to characterize the urinary proteome. Comparing 2D-LC−MS/MS using SCX, with 1D-LC−MS/MS and 1D-SDS-PAGE-LC−MS/MS, this group also identified the largest number of proteins using the 2D-LC−MS/MS (SCX) approach.[26] This study also compared reproducibility between experiments using SDS-PAGE separation vs SCX separation and found 25% greater reproducibility with SCX fractionation, compared with SDS-PAGE separation,[26] in accordance with our own findings (Figure 1A, B).

Classification of the identified proteins by Genome Ontology (GO) indicated that 30% of the proteins in CVF are extracellular or membranous (Figure 2), as would be expected for an extracellular biological fluid. Analysis of the proteins identified in CVF reveals distinct protein subgroups: host defense, proteolysis, enzyme inhibition, cellular adhesion, and the cytoskeleton.

Host Defense. As mentioned, a primary function of CVF is host protection from microorganisms.[6] Proteins such as haptoglobin,[27] neutrophil defensin,[13,28] lysozyme,[8] and lactoferrin[9,10] have previously been shown to be present in CVF, contributing to host defense. Analysis by Ingenuity software has shown that 75 of the identified proteins are associated with immune response (Figure 3). In addition to the proteins mentioned, several other proteins known to be associated with host defense have been identified in our study, such as: azurocidin[29] (CAP-37), dermcidin,[30] and galectin-3 binding protein[31] (Supplementary Table 1).

Proteolysis. Many proteolytic enzymes, particularly serine (e.g., kallikreins) and cysteine (e.g., cathepsins) proteases, were identified in CVF (Supplementary Table 1). Other enzymes identified include members of the carboxypeptidase family, matrix metalloproteinase-9, myeloperoxidase, neutrophil collagenase, and neutrophil gelatinase. Members of the cathepsin family have previously been shown to be present in vaginal fluid; increased cathepsin B levels are associated with cervical carcinoma.[32] Matrix metalloproteinase-9 is known to be present in CVF, and levels have been studied in pregnant women with bacterial vaginosis.[33]

We here show that many members of the kallikrein family are present in CVF, as demonstrated by two independent methods (ELISA and mass spectrometry).

Enzyme Inhibition. Given that many enzymes are present in CVF, it is expected that enzyme inhibitors would also be present to control enzyme activity. The biological network constructed from CVF proteins in Figure 4 highlights some enzyme−inhibitor interactions, such as: inhibition of cathepsin B by alpha-2-macroglobulin, SPINK-5 inhibition of plasmin, and elafin inhibition of KLK7. Two main families of inhibitors were identified in CVF: inhibitors of serine proteases and inhibitors of cysteine proteases. Serine protease inhibitors including anti-trypsin, anti-thrombin, elafin, and serpins B3, B4, B9, B12 were identified, as well as the cysteine protease inhibitors cystatin A and B (Supplementary Table 1). Anti-trypsin and cystatin A have recently also been identified in CVF by Di Quinzio et al.[18]

Cell-Adhesion and the Cytoskeleton. We identified, in CVF, several proteins associated with the cytoskeleton, or involved in cell−cell adhesion. Analysis using Ingenuity software revealed that 96 genes were found to be associated with cellular movement, 85 with cell-to-cell signaling, and 75 with cellular assembly and organization (Figure 3). Cytoskeletal proteins include cornifin B, envoplakin, fibronectin, myosin, and periplakin. Members of the desmoglein and desmocollin cell−cell adhesion molecule family were also identified (Supplementary Table 1). Fibronectin has previously been recognized as a component of CVF and is currently used as a marker for preterm labor in pregnant women.[14] The vaginal epithelium undergoes changes throughout the menstrual cycle,[34] including keratinization and shedding of epithelial cells upon decreasing estrogen levels.[35] Given these physiological changes, it is not surprising that cytoskeletal and cell−cell adhesion proteins are present in this fluid. The loss of cell−cell contacts accompanies the shedding of epithelial cells, resulting in enrichment of CVF for these proteins.

In conclusion, we present here the most comprehensive list of human CVF proteins to date. This information should be useful in the understanding of CVF function and in the identification of CVF components that may serve as biomarkers for gynecological malignancies, perivaginal infections, or conditions associated with pregnancy.

Abbreviations: CVF, cervico-vaginal fluid; ACN, acetonitrile; TFA, trifluoroacetic acid; DTT, dithiolthreitol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; 1D, one-dimensional; 2D, two-dimensional; ESI, electrospray ionization; SCX, strong cation-exchange; MS/MS, tandem mass spectrometry; GO, Genome Ontology; LC, liquid chromatography; HPLC, high-performance liquid chromatography.

Supporting Information Available: Supplementary Table 1A provides a list of all proteins identified in our study, along with accession numbers, the number of peptides identified, peptide sequences, and which experiment they were obtained from. Supplementary Table 1B outlines the overlap between proteins identified by our study and by 3 other studies by Dasari et al.,[16] Pereira et al.,[17] and Di Quinzio et al.[18]

Supplementary Table 1C is a list of all proteins identified in this study with their specific subcellular localizations, according to GO. This material is available free of charge via the Internet at http://pubs.acs.org.

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