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Isobaric labeling and tandem mass spectrometry: A novel approach for profiling and quantifying proteins differentially expressed in amniotic fluid in preterm labor with and without intra-amniotic infection/ inflammation

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Abstract

Objective. Examination of the amniotic fluid (AF) proteome has been previously attempted to identify useful biomarkers in predicting the outcome of preterm labor (PTL). Isobaric Tag for Relative and Absolute Quantitation (iTRAQ^{TM}) labeling allows direct ratiometric comparison of relative abundance of identified protein species among multiplexed samples. The purpose of this study was to apply, for the first time, the combination of iTRAQ and tandem mass spectrometry to identify proteins differentially regulated in AF samples of women with spontaneous PTL and intact membranes with and without intra-amniotic infection/inflammation (IAI).

Methods. A cross-sectional study was designed and included AF samples from patients with spontaneous PTL and intact membranes in the following groups: (1) patients without IAI who delivered at term ($n = 26$); (2) patients who delivered preterm without IAI ($n = 25$); and (3) patients with IAI ($n = 24$). Proteomic profiling of AF samples was performed using a workflow involving tryptic digestion, iTRAQ labeling and multiplexing, strong cation exchange fractionation, and liquid chromatography tandem mass spectrometry. Twenty-five separate 4-plex samples were prepared and analyzed.

Results. Collectively, 123,011 MS^2 spectra were analyzed, and over 25,000 peptides were analyzed by database search (X!Tandem and Mascot), resulting in the identification of 309 unique high-confidence proteins. Analysis of differentially present iTRAQ reporter peaks revealed many proteins that have been previously reported to be associated with preterm delivery with IAI. Importantly, many novel proteins were found to be up-regulated in the AF of patients with PTL and IAI including leukocyte elastase precursor, Thymosin-like 3, and 14-3-3 protein isoforms. Moreover, we observed differential expression of proteins in AF of patients who delivered preterm in the absence of IAI in comparison with those with PTL who delivered at term including Mimecan precursor, latent-transforming growth factor β-binding protein isoform 1L precursor, and Resistin. These findings have been confirmed for Resistin in an independent cohort of samples using ELISA. Gene ontology enrichment

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analysis was employed to reveal families of proteins participating in distinct biological processes. We identified enrichment for host defense, anti-apoptosis, metabolism/catabolism and cell and protein mobility, localization and targeting.

Conclusions. (1) Proteomics with iTRAQ labeling is a profiling tool capable of revealing differential expression of proteins in AF; (2) We discovered 82 proteins differentially expressed in three clinical subgroups of premature labor, 67 which were heretofore unknown. Of particular importance is the identification of proteins differentially expressed in AF from women who delivered preterm in the absence of IAI. This is the first report of the positive identification of biomarkers in this subgroup of patients.

Keywords: *Proteomic, preterm birth, prematurity, MIAC, microbial invasion of the amniotic cavity, chorioamnionitis, isotopic, multiplex, label, high-dimensional biology, 'bottom-up', 'top-down', computational biology, high-dimensional biology, systems biology, endoplasmic reticulum stress, misfolded protein, unfolded protein*

Introduction

'High-dimensional biology' refers to the use of high throughput techniques that allow simultaneous examination of changes in the genome (DNA), transcriptome (mRNA), proteome (proteins), metabolome (metabolites), lipidome (lipids), glycome (carbohydrates), and other biochemical components in a biological sample. The goal of such approaches is to improve understanding of the physiology and mechanisms of disease, [1–3] to identify biomarkers for diagnostic or prognostic purposes, as well as to develop therapeutic tools [4–45]. A critical aspect of high-dimensional biology is intelligent mining of complex data sets generated with the use of these techniques, collectively referred to as 'omics' sciences. Thus, computational biology is an integral part of high-dimensional biology.

Examination of the amniotic fluid (AF) proteome has been employed as a means to identify biomarkers for spontaneous preterm labor (PTL) and other obstetrical disorders [13,16–18,31,44,45]. Mass spectrometry-based proteomics falls into two broad categories: 'top-down' and 'bottom-up' [46]. In the former, intact proteins are characterized directly via mass spectrometry, whereas in the latter, proteins are first digested, and the resulting peptides are then analyzed to identify and sometimes to quantify proteins (the 'bottom-up' approach is frequently used with liquid chromatography). Although Michaels et al. [47] have pioneered the use of 'bottom-up' proteomics of AF, most approaches to date have used a 'top-down' methodology for analysis of AF. In fact, although the digestion step produces many more fragment products than the original number of proteins, peptides can be more easily and accurately identified in the mass spectrometer in comparison with the intact proteins in the top-down approach. The main disadvantages of the bottom-up approach are that (a) the re-assembly of peptides to deduce their parent proteins is a complex computational task, and (b) quantitation of the proteins is less straightforward. In practice, a 'bottom-up' workflow tends to be lower-throughput than top-down counterparts.

Isobaric Tag for Relative and Absolute Quantitation (iTRAQ^{TM}) is a new technique applicable to 'bottom-up' mass spectrometry [48–50]. Isobaric labeling refers to the derivatization of primary amino groups of peptides in iTRAQ. These isobaric tags do not differ in mass, and upon fragmentation of the isotope encoded reporter ions from the peptides during mass spectrometry, relative quantitative information of each tag can be measured. In iTRAQ, there are four distinct labels. Each is comprised of a 'reporter' element and a 'balance' element. The reporters have approximate masses of 114–117 Da, whereas their corresponding balances have masses of 28–31 Da such that they sum to about 145 Da (hence the term 'isobaric'). In the first stage of tandem mass spectrometry, a peptide species labeled with any of the four reagents will appear at the same mass-to-charge ratio (m/z), whereas at the second stage of mass spectrometry, the labile bond between the reporter and balance elements is broken, producing distinct reporter ion peaks at 114–117 Da. The relative abundance of peptides labeled with the four reagents is directly indicated by the heights of these four peaks. Moreover, as several samples are combined and analyzed together, throughput is substantially increased compared with the analysis of unlabeled samples, which must be accomplished one by one. Thus, although there are 'bottom-up' approaches that do not involve the use of labeling reagents, the two-fold advantages of simpler quantitation and higher throughput due to multiplexing are apparent.

The aim of the present work was to apply, for the first time, iTRAQ-based proteomic analysis of AF of patients with spontaneous PTL with intact membranes to identify differentially expressed proteins in the presence or absence of intra-amniotic infection/inflammation (IAI).

Material and methods

Study design and population

A cross-sectional study was designed by searching our clinical database and bank of biological samples, selecting AF samples from 75 patients with an episode of spontaneous PTL and intact membranes who were classified into: (a) PTL without IAI who delivered at term ($n = 26$); (b) PTL without IAI who delivered preterm (< 37 weeks gestation) within 7 days of amniocentesis ($n = 25$); and (c) PTL with IAI ($n = 24$).

All women provided written informed consent prior to the collection of AF. The collection of AF and its utilization for research purposes were approved by the Institutional Review Boards of Sotero del Rio Hospital (Santiago, Chile) and the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, NIH, DHHS. Many of these samples have been previously used to study the biology of inflammation, hemostasis, metabolomics, proteomics, and growth factor concentrations in normal pregnant women and those with pregnancy complications.

Definitions

Spontaneous PTL was defined by the presence of regular uterine contractions occurring at a frequency of at least two every 10 min associated with cervical change before 37 completed weeks of gestation that required hospitalization. Intra-amniotic infection was defined as a positive AF culture for micro-organisms. Intra-amniotic inflammation was diagnosed by an AF interleukin (IL)-6 concentration > 2.6 ng/ml [51].

Sample collection

AF samples were obtained from transabdominal amniocenteses performed for evaluation of microbial status of the amniotic cavity. Samples of AF were transported to the laboratory in a sterile capped syringe and cultured for aerobic/anaerobic bacteria and genital mycoplasmas. White blood cell count, glucose concentration, and Gram-stain were also performed shortly after collection as previously described [52–54]. The results of these tests were used for clinical management. AF IL-6 concentrations were used only for research purposes. AF not required for clinical assessment was centrifuged at 1300 *g* for 10 min at 4°C and the supernatant was aliquoted and stored at −70°C until analysis.

Overview of the experimental workflow

The basic experimental strategy is to digest AF proteins, identify each peptide in the resulting mixture and measure their concentrations relative to corresponding peptides in a reference sample, and then to compute the relative concentration of the digested proteins from their daughter peptides. From this information, differentially regulated proteins among the three clinical groups can be identified.

Figure 1 illustrates schematically the workflow used to implement this strategy. First, a pooled reference sample was created by mixing a small amount of protein from each of the AF samples. This mixture was then separated into replicate aliquots. Samples were depleted of albumin and Immunoglobulin G (IgG) so that proteins at lower concentrations would be unmasked and therefore more easily detected. Individual samples were digested with trypsin. The resulting tryptic peptides were then labeled with one of three iTRAQ isobaric labeling reagents [each reagent produces a tandam mass spectrometry (MS/MS) reporter ion at 115, 116, or 117 Da], whereas an aliquot of the reference pool was always labeled with the iTRAQ reagent producing a 114 Da reporter ion. Groups of four samples, comprising the pooled reference sample (114) and three individual samples (115, 116, and 117), were combined together into a ''4-plex.'' Each of these multiplexed mixtures was then subjected to strong cation exchange (SCX) fractionation in order to simplify the peptide mixture for subsequent analysis. Each of the SCX fractions was subjected to liquid chromatography MS/MS (LC-MS/MS) analysis. The eluent from the LC column was spotted onto matrix-assisted laser desorption ionization (MALDI) target plates, and subsequently subjected to tandem mass spectrometry analysis (MS/MS). In the first stage of mass spectrometry, a precursor ion has the same mass from all four samples in the mix due to the fact that the iTRAQ reagents are constructed with a reporter unit and a balance unit, the sum of whose masses is the same for all four of the reagents. However, upon collisional dissociation for MS/MS analysis, the reporter unit produces a low-mass peak whose intensity indicates the relative amount of peptide in the mixture that was labeled with the corresponding reagent. Thus, the MS/MS spectrum contains two kinds of information: (a) a ladder of peaks characteristic of the peptide sequence that is used to identify the peptide, and (b) the four reporter ion peaks at 114–117 Da that are used to quantify the relative amount of peptide from the four samples in the mix. The following sections describe in detail the experimental workflow (summarized in Figure 1).

Amniotic fluid protein extraction and digestion [55]

AF samples were depleted of albumin using Vivapure immunoaffinity anti-human serum albumin capture resins (Satorius Stedium, Bohemia NY, USA) and IgG with ultralink immobilized protein G (Pierce, Rockford, IL, USA). Protein concentrations of individual depleted AF samples were determined with bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Forty micrograms of total protein from each sample was precipitated by incubation with 4.5 ml of chilled acetone at 4°C for 3 h, followed by centrifugation (3000 rcf) for 30 min. Triethylammonium bicarbonate (0.5 M) and 2% sodium dodecylsulfate were added to dissolve the protein pellet before mixing it with a reducing reagent [50 mM Tris-(2-carboxyethyl)

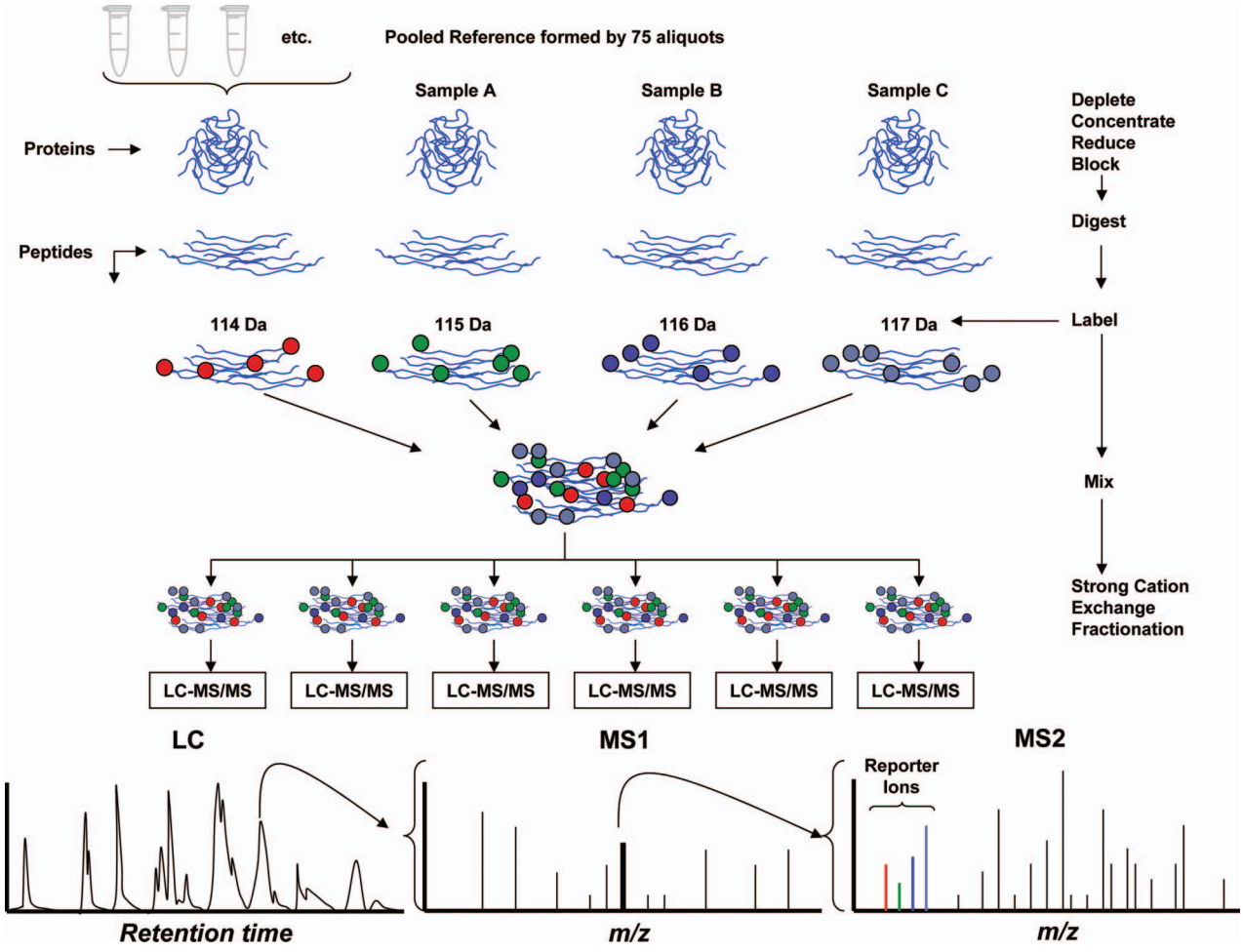

Figure 1. Diagram of the iTRAQ workflow. The sequence of the workflow is indicated by the arrows in the right side. *Pooled reference*: Small aliquots from each of the 75 samples of amniotic fluid were pooled to create a reference sample. *Deplete-concentrate-reduce-block*: The amniotic fluid samples were depleted of albumin and IgG, subsequently concentrated and reduced. *Digestion*: The samples were subjected to trypsin digestion. *Labeling*: The reference sample and three individual samples (denoted A, B, and C in the figure) were labeled with iTRAQ reagents (the numbers 114–117 refer to the reporter mass of the individual iTRAQ reagent). *Mixing and fractionation*: The four labeled samples were mixed and subjected to strong cation exchange (SCX) fractionation to simplify the mixture prior to conducting liquid chromatography and mass spectrometry (LC-MS/MS). Each SCX fraction was subjected to LC-MS/MS. *Liquid chromatography*: Proteins were separated by liquid chromatography. The retention time is displayed in the horizontal axis. *MS1*: The first stage of mass spectrometry for a specific retention time is indicated schematically. Each peak in the mass spectrum (horizontal axis is *m/z*) corresponds to an individual peptide. *MS2:* A single peak is selected for collisional dissociation and the fragments subjected to a second stage mass spectrometry (tandem MS/MS). The reporter ions from the iTRAQ label appear in the low mass range, distinct from peptide fragment peaks. The pattern of peaks in the final mass spectrum is matched to a database in order to identify the parent peptide. The intensities of the reporter ion peaks indicate the relative abundance of the four samples.

phosphine]. The reduction was completed by incubating the solution at 60°C for 1 h. The reduced cysteine residues were then blocked with 200 mM of methyl methanethiosulfonate, at room temperature for 10 min. Trypsin digestion of proteins in each sample was performed with 10 μg of trypsin (1 μg/μl) (Worthington Biochemical Corp., Lakewood, NJ, USA) in the presence of $CaCl_2$ overnight at 37°C.

Reference pool and quality control

To generate a pooled sample as a reference standard for each iTRAQ 4-plex, 17 μg of each depleted sample was combined into a pool which was then separated into 25 aliquots of 40 μg each. Each of these pooled reference standards was subsequently processed as described above.

Internal protein concentration standards were provided for quality control by spiking known amounts of non-human protein into each individual sample, including the pooled reference standard samples. Two micrograms of Phage T4 (New England BioLabs, Ipswich, MA, USA) and 0.2 μg of *Escherichia coli* RecA (New England BioLabs, Ipswich, MA, USA) was added into each sample of 40 μg of AF protein [56,57].

Labeling with multiplex reagents

Seventy microliters of iTRAQ reagent (isobaric reagents 114, 115, 116, and 117 from Applied Biosystems, Framington, MA, USA) in ethanol was added to each sample and incubated at room temperature for 1 h. Following this derivatization step, the four samples were mixed, dried, and re-suspended in 3 ml of SCX loading buffer [25% v/v acetonitrile (ACN), 10 mM KH_2PO_4, pH 3.0, with phosphoric acid]. In each mix, the reference pool was labeled with the 114 reagent. For the other three reagents, individual samples were randomly assigned such that each mix contained one sample from each of the three clinical groups (except that one mix contained two samples from the term delivery group and none from the group with IAI, due to the unequal numbers of samples in the three groups).

Strong cation exchange chromatography

Each peptide mixture was separated on an SCX chromatography column (Polysulfoethyl column, The Nest Group, Southborough, MA). The column was first equilibrated with SCX loading buffer. The pH of the iTRAQ labeled sample was verified to be less than 3.3. Additional loading buffer was added if necessary to adjust the pH. The sample was then loaded at a rate of $\approx$1 drop/s onto the column and washed with 1 ml of SCX loading buffer to remove salts, trys-(2-carboxyethyl) phosphene and unincorporated iTRAQ reagent. Peptides were eluted with a linear gradient of 0–1000 mM KCl (in 25% v/v ACN, 10 mM KH_2PO_4, pH 3.0). Six pooled fractions of the elutant were collected for analysis with LC-MS/MS.

Liquid chromatography

The SCX fractions were re-suspended in 40 μl of high performance liquid chromatography (HPLC) loading buffer (94.9%:5%:0.1% water:acetonitrile: trifluoroacetic acid containing 2 mM ammonium acetate). Twenty-five microliters of each fraction were injected on an UltiMate 3000 Chromatography System equipped with a Probot fraction collector (Dionex LC-Packings, Hercules, CA). HPLC fractions were spotted directly onto the MALDI target. An integrated syringe pump on the fraction collector enabled the mixing of the HPLC eluent and MALDI matrix prior to deposition onto the MALDI plate. To ensure good mass calibration of the MALDI MS spectra, an internal molecular mass standard [ACTH (18–39) peptide; $MH^+ = 2465.199$ Da] was added to the matrix solution at a concentration of 400 femtomole/μl. Peptides were separated over a 35-min elution gradient and spotted onto the MALDI target at intervals of 30 s. Protein elution from the column was monitored with ultraviolet light at a wavelength of 214 nm using a 45-nl flow cell. Prior to each set of six HPLC injections representing an iTRAQ mix, a mixture of peptide standards (Michrom Bioresources, Auburn, CA, USA) was injected to monitor the consistency of HPLC retention times. Each HLPC run was spotted in a 6×35 spot format (210 fractions) such that three injections were accommodated on a single MALDI plate. Thus, two such plates were required to collect the chromatographic separation of each complete iTRAQ mix represented in six SCX fractions.

Mass spectrometry

Mass spectrometry analysis was performed with a Sciex/AB 4800 MALDI MS/MS instrument (MDS/Sciex, Concord, Ontario, Canada). First, LC-MS data were collected from all the six HPLC injections. To ensure the accuracy of mass measurements, two internal standards were used for mass calibration, a 2465.199 Da peptide standard that was co-infused with the matrix during deposition onto the MALDI target, and a 568.136 Da matrix trimer ion. The settings for acquisition and processing methods in the Sciex/AB 4800 software were optimized for the reliable detection of monoisotopic peaks to slightly below the intensity threshold to be used later for MS/MS precursor selection. This threshold was important for the correct distinction of useful components from chemical noise.

In order to maximize MS/MS performance and efficiency, a custom MS/MS precursor selection program was used to generate a non-redundant precursor list from the two dimensional chromatography space ensuring that each precursor was analyzed from the SCX-HPLC fraction from which the best quantification was possible. In order to maintain the consistency of precursor selection across all iTRAQ mixes, exclusion and inclusion lists were generated and dynamically updated for the next mix in the queue. Precursor ions on these lists were identified through their accurate mass, HPLC retention time and SCX fraction number and categorized as: (a) precursors suitable for quantification (fully alkylated, fully iTRAQ-labeled peptides) – constituting the inclusion list; (b) precursors not suitable for quantification (incomplete alkylation/iTRAQ labeling, chemical by-products, peptides from trypsin, or unidentified precursors) – constituting the exclusion list; and (c) opportunistic precursors that match precursor selection criteria but were not included in either list. Opportunistic precursors that could be identified in Mascot searches against the human Uniref protein database were used to build and update the inclusion and

exclusion lists depending on how they matched to criteria (a) and (b).

Mass spectrometry data were acquired in two phases. The first phase started with the acquisition of data from the first iTRAQ mixture with empty inclusion and exclusion lists. Precursors identified from this mixture were sorted into the inclusion and exclusion lists using the criteria previously described. The remaining mixtures were then run sequentially with the dynamically updated exclusion and inclusion lists. Items on the inclusion list were preferentially selected for MS/MS, whereas items on the exclusion list were not selected. The exclusion and inclusion lists continuously grew until the last iTRAQ mixture in the queue was analyzed.

A second phase of mass spectrometry analysis was performed in order to ensure a uniform likelihood for the detection of precursors across all 25 4-plexes. In each 4-plex, precursors were targeted for acquisition that had been added to the inclusion list later during the first phase. Also, precursors that yielded poor signal-to-noise for quantification in the first phase were re-analyzed with a longer signal integration time in the second phase (increasing the number of laser shots to about 5000, as compared to about 2000 during the first phase). By completing the two phases, a highly overlapping set of proteins were measured in each iTRAQ 4-plex.

Data management

From each LC-MS/MS run in the Sciex/AB 4800 database, MS/MS peak lists were extracted (limited to the *m*/*z* region above 100 Da) using the instrument software. Ninety-four peaklist files were generated. Typically, each 4-plex was represented by four files (two sets of SCX fractions: 1–3 and 4–6, and for each of these two spectrum acquisition passes). Peaklist files and associated experimental data were indexed into a MySQL database to facilitate data management. Custom-written Perl programs were used to perform database queries to: (a) extract and format peaklist files for input to protein identification engines; (b) extract and normalize reporter ion intensities for each peptide; and (c) correlate quantitation and identification information.

Protein identification

A single large peaklist file was extracted from the database as input for protein identification. All MS/MS spectra were analyzed using Mascot software version 2.2.03 (Matrix Science, London, UK) and X!Tandem version 2007.01.01.2 (www.thegpm.org). X!Tandem was set up to search the ipi.HUMAN database (version 3.28, 136040 entries) with reverse search included. All searches were conducted assuming trypsin cleavage. The same was the case for Mascot searches. Mascot and X!Tandem searched the database with a fragment ion mass tolerance of 0.40 Da and a parent ion tolerance of 100 PPM. Fixed modifications specified for both Mascot and X!Tandem included MMTS on cysteine residues and iTRAQ modification of lysine and the N-terminus. Oxidation of methionine and iTRAQ modification of tyrosine were specified for both search engines as variable modifications.

Scaffold (version 01.07.00, Proteome Software, Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm [58]. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (which takes into account the Mascot and X!Tandem probabilities) [59]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. *De novo* sequencing using PEAKS Software (Bioinformatics Solutions, Inc., Waterloo, Ontario, Canada) was used to confirm peptide identity for precursors not identified by database search, yet determined to be of interest in differential expression analyses [60].

Quality control

Tryptic peptides derived from the spiked-in quality-control proteins Phage T4 and *E. coli* recA were identified by database search with X!Tandem. Mean reporter ion ratios for these peptides relative to the pooled reference 114 reporter ion were computed for each sample individually. Because the concentrations of the spiked standards were the same for pooled reference and experimental samples, these ratios should theoretically be near 1.0. AF samples whose mean spike ratios deviated by more than 50% from unity were excluded from further consideration (seven from patients with PTL who delivered at term, four from patients who delivered preterm without IAI, and three from patients who delivered preterm with IAI).

Determination of differential expression

Statistical analysis. To compensate for experimentally induced variation in total peptide concentrations, a global median normalization (or standardization) was performed such that the median of the sample/reference ratios was unity [61,62]. Relative peptide

expression values were determined by computing the ratio of the peak intensity for each reporter ion corresponding to an individual sample to that of the corresponding reference pool reporter ion. MS/MS spectra in which no reference reporter ion at 114 Da was observed were excluded from the determination of relative abundance.

The amount of an identified protein in a sample relative to the reference pool was determined by computing the median of all of the relative peptide expression values observed in a sample corresponding to that protein. For pair-wise comparisons among the three clinical groups, the median of the individual sample protein expression values was formed for each group and the ratio of medians represented the relative protein expression ratio. *p*-values were computed with the Mann–Whitney rank sum test. A p-value < 0.05 was considered significant.

Analysis for over-representation of biological processes
An analysis was undertaken to determine which biologic processes were overrepresented in patients with PTL as a function of the proteins identified to be differentially regulated. Gene ontology (GO) terms that were enriched among the set of differentially regulated proteins in IAI relative to all proteins identified in all samples in the present study. We used BiNGO (version 2.0) [63] to calculate GO term enrichment, and CytoScape (version 2.5.1) [64] to visualize the resulting network of GO biological processes. We then used the IPIhuman database version 3.35 to convert protein references to corresponding gene names. Redundant gene references were removed in order to avoid bias due to multiple protein database references to the same gene. Distributions were tested for statistical significance using the hypergeometric test (equivalent to an exact Fisher test [65]), and the Benjamini and Hochberg [66] correction method for false discovery rate (FDR) was applied. GO terms that were enriched at the 95% confidence level after correction for multiple comparisons were accepted as significant.

Results

The demographic and clinical characteristics of the patients included in this study are described in Table I. The gestational age at amniocentesis did not differ significantly among the groups. Among patients with PTL who delivered preterm, gestational age at delivery was not significantly different between patients with and without IAI.

Protein identification

A total of 123,011 MS/MS spectra were acquired across the two mass spectrometry phases from 25 iTRAQ 4-plex mixtures, representing AF samples from 75 patients. Of these spectrum observations, 26,801 spectra produced confident peptide identifications. Three hundred nine distinct proteins were identified with greater than 95% confidence and at least two distinct identified peptides, with an estimated FDR of 1.06%. Additionally, 16 proteins (S100A12, RETN, FK506, HIST1H1D, YWHAB, BASP1, SERPINB1, ARHGDIA, PGD, MRLC2, CTSL1, FKBP1A, NME1, YWHAQ, CALRETICULIN, and CPM) which were provisionally identified by database searching based on one peptide each were validated by *de novo* sequencing and subsequently included in the differential expression analysis.

Table I. Demographic and clinical characteristics of patients with spontaneous preterm labor with intact membranes.

	PTL without IAI, term delivery ($n = 26$)	p	PTL without IAI, preterm delivery ($n = 25$)	p^a	PTL with IAI, preterm delivery ($n = 24$)	p^b
Maternal age (years)	21 (16–38)	NS	28 (16–45)	NS	23 (17–41)	NS
Parity	1 (0–5)	NS	1 (0–4)	NS	1 (0–4)	NS
Previous preterm delivery	15.4 (4/26)	NS	36 (9/25)	NS	13 (3/23)	NS
GA at amniocentesis (weeks)	29.4 (24–33.7)	NS	30 (23.4–33.4)	NS	30 (23.1–33.3)	NS
AF WBC count (cells/mm^3)	2 (0–35)	NS	3 (0–18)	< 0.001	325 (0–9000)	< 0.001
AF IL-6 (ng/ml)	0.6 (0.05–1.3)	0.009	1.1 (0.1–2.5)	< 0.001	57.9 (5.6–246.8)	< 0.001
Tocolysis	34.6 (9/26)	NS	48 (12/25)	NS	29.2 (7/24)	NS
Amniocentesis-to-delivery interval (days)	65 (33–109)	< 0.001	6 (0–7)	0.02	1 (0–7)	< 0.001
GA at delivery (weeks)	38.6 (37.3–40.1)	< 0.001	31 (23.4–33.9)	NS	30.1 (23.1–34)	< 0.001
Birth weight (g)	3045 (2580–4230)	< 0.001	1630 (550–1990)	NS	1380 (660–2490)	< 0.001

Values expressed as percentage (number) or median (range).
p, comparison between PTL who delivered at term and PTL who deliver preterm without IAI.
p^a, comparison between PTL who delivered preterm without IAI and PTL with IAI.
p^b, comparison between PTL who delivered at term and PTL with IAI.
PTL, preterm labor; IAI, intra-amniotic infection/inflammation; GA, gestational age; AF, amniotic fluid; WBC, white blood cell; IL, interleukin; NS, not significant.

Differential regulation: preterm delivery with intra-amniotic infection/inflammation

Table II catalogs proteins that have been reported in the literature to be differentially regulated in the AF of women with preterm delivery in the presence of IAI. Table III lists proteins which were determined in the present study to be differentially regulated between patients who delivered preterm with infection and/or inflammation in comparison with those in the remaining two groups. Criteria for inclusion in Table III were: (a) confirmed protein identity; (b) p-value for differential expression of the protein of less than 0.05; and (c) the fold-change in relative abundance for the protein between the two groups of at least 1.5. Seventy-seven proteins were found to be up-regulated in the AF of patients with IAI, but only six were down-regulated. All except five of the proteins listed in Table II that had previously been determined to be associated with infection/inflammation related PTL and delivery are found in Table III. Moreover, one of those (azurocidin) that does not appear in Table III nonetheless was detected, identified and was differentially regulated, but was excluded from the Table because it was detected in a number of samples insufficient to generate statistical significance.

Differential expression: preterm delivery without intra-amniotic infection/inflammation

Table IV lists proteins which were determined to be differentially regulated between patients who delivered preterm without IAI in comparison to those of patients with PTL without IAI that delivered at term. Four proteins were found to be up-regulated in patients in PTL/delivery without IAI: Resistin (RETN), Thymosin-like 3 (TMSL3), Antileukoproteinase (SLPI), and Growth-inhibiting protein 12 (also known as Lactoferrin, LTF). Two proteins were down-regulated: latent-transforming growth factor β-binding protein isoform 1L (LTBP1) and Mimecan precursor (OGN).

Gene ontology analysis

The subset of genes listed in Table III ($n = 83$) was compared for enrichment against the basis set of genes ($n = 447$) corresponding to the proteins identified in the entire experiment. Figure 2 shows the resulting network. Colored nodes indicate GO terms that are significantly enriched.

Table II. Proteins previously reported in the literature to be differentially up-regulated in the amniotic fluid of patients with IAI.

Protein name in present study	Protein name as cited	Reference
CAPG macrophage-capping protein	Macrophage capping protein	[18]
LCN2 neutrophil gelatinase-associated lipocalin precursor	Neutrophil gelatinase-associated lipocalin	[18]
MPO isoform H17 of Myeloperoxidase precursor	Myeloperoxidase precursor	[18]
LCP1 plastin-2	L-plastin	[18]
⋆	Azurocidin	[18]
CAMP antibacterial protein FALL-39 precursor	Antibacterial protein FALL-39	[18]
†	Gp-340 variant protein	[18]
†	Similar to mouse von Ebner salivary gland protein, isoform 2	[18,45]
SERPINB1 leukocyte elastase inhibitor	Leukocyte elastase inhibitor	[18]
S100A8 protein S100-A8	Calgranulin A (S100-A8)	[18,80]
S100A9 protein S100-A9	Calgranulin B (S100-A9), calprotectin	[18,77,80]
S100A12 protein S100-A12	Calgranulin C (S100-A12)	[13]
DEFA1; LOC653600; LOC728358 neutrophil defensin 1 precursor	Neutrophil defensins 1-3	[13,18,77]
†	Bactericidal/permeability-increasing protein (BP1)	[77]
‡	Insulin-like growth factor binding protein 1 (IGFBP-1) fragments	[18,45]
MMP8 neutrophil collagenase precursor	MMP-8	[81]
MMP9 matrix metalloproteinase-9 precursor	MMP-9	[75]
‡	MMP-2	[82]
TIMP1 metalloproteinase inhibitor 1 precursor	Metalloproteinase inhibitor 1 (TIMP-1)	[75]
MIF Macrophage migration inhibitory factor	Macrophage migration inhibitory factor	[78]
†	C-reactive protein (CRP)	[74,79]
LTF growth-inhibiting protein 12	Lactoferrin	[76]

⋆differentially expressed, but not statistically significant.
†not observed.
‡observed, not differentially expressed.

Table III. Proteins differentially expressed in amniotic fluid samples of patients with preterm delivery with IAI, compared with patients without IAI who delivered at term.

		Samples					
		Protein ID				Differential Expression	
Accession	Gene name, description	U_p	P_p	N_i	N_o	R	p-value
IPI00007047	S100A8 protein S100-A8	10	1.000	19	17	inf*	4.4 E − 05
IPI00218131	S100A12 protein S100-A12	1	0.610	4	4	Inf*	2.7 E − 02
IPI00027769	ELA2 leukocyte elastase precursor	2	0.998	9	9	27.4	2.0 E − 02
IPI00006988	RETN resistin precursor	1	0.610	15	14	23.5	8.0 E − 04
IPI00180240, IPI00220828, IPI00719405, IPI00815642, IPI00815829, IPI00815835, IPI00816008, IPI00816288, IPI00816464	TMSL3 thymosin-like 3	6	1.000	14	13	12.1	2.0 E − 03
IPI00007244, IPI00236554, IPI00236556	MPO isoform H17 of Myeloperoxidase precursor	28	1.000	18	17	8.2	2.1 E − 03
IPI00005721, IPI00021827	DEFA1; LOC653600; LOC728358 neutrophil defensin 1 precursor	4	1.000	17	16	7.5	5.5 E − 04
IPI00019600	UBE2V2 ubiquitin-conjugating enzyme E2 variant 2	2	0.997	4	4	6.5	2.9 E − 02
IPI00027462	S100A9 protein S100-A9	14	1.000	20	18	5.2	3.8 E − 04
IPI00028064	CTSG cathepsin G precursor	4	1.000	10	10	5.0	4.6 E − 03
IPI00018534, IPI00020101, IPI00152906, IPI00303133, IPI00329665, IPI00419833, IPI00554798, IPI00719084, IPI00794461, IPI00815755	HIST1H2BL histone H2B type 1-L	4	1.000	18	17	4.6	2.5 E − 03
IPI00292532	CAMP antibacterial protein FALL-39 precursor	5	1.000	4	4	4.5	2.9 E − 02
IPI00027509	MMP9 matrix metalloproteinase-9 precursor	9	1.000	17	17	4.5	8.2 E − 04
IPI00019502	MYH9 Myosin-9	8	1.000	8	7	4.5	1.2 E − 03
IPI00171611, IPI00216402, IPI00219038, IPI00465070, IPI00719351	HIST2H3C; HIST2H3A histone H3.2	5	1.000	16	15	4.3	8.4 E − 03
IPI00018873, IPI00472879	PBEF1 isoform 1 of nicotinamide phosphoribosyltransferase	3	1.000	9	8	3.9	1.2 E − 03
IPI00419585	PPIA; LOC653214; LOC654188 peptidyl-prolyl cis-trans isomerase A	8	1.000	19	17	3.8	4.3 E − 05
IPI00465248	ENO1 Isoform α-enolase of α-enolase	7	1.000	13	11	3.7	5.4 E − 03
IPI00759644	FK506 binding protein12	1	0.610	17	15	3.7	7.3 E − 05
IPI00217466	HIST1H1D histone H1.3	1	0.610	6	5	3.5	1.7 E − 02
IPI00021439, IPI00021440	ACTB actin, cytoplasmic 1	16	1.000	21	19	3.5	8.9 E − 06
IPI00220644, IPI00479186	PKM2 isoform M1 of Pyruvate kinase isozymes M1/M2	5	1.000	12	11	3.4	3.9 E − 04
IPI00010471	LCP1 plastin-2	14	1.000	19	17	3.3	3.2 E − 04
IPI00453473	HIST1H4E; HIST1H4F; HIST1H4A; HIST1H4K; HIST1H4C; HIST1H4L; HIST2H4A; HIST1H4D; HIST2H4B; HIST1H4H; HIST1H4B; HIST1H4I; HIST1H4J; HIST4H4 histone H4	7	1.000	17	16	3.2	3.8 E − 03
IPI00012011, IPI00784459	CFL1 cofilin-1	2	0.998	16	14	2.9	2.4 E − 04
IPI00298860	LTF growth-inhibiting protein 12	36	1.000	21	18	2.9	3.2 E − 04
IPI00021085	PGLYRP1 peptidoglycan recognition protein precursor	2	0.998	15	14	2.9	7.8 E − 05
IPI00218834, IPI00640044, IPI00796109	FCGR3A low affinity immunoglobulin-γ-Fc region receptor III-A precursor	3	1.000	18	16	2.9	6.1 E − 04
IPI00013508, IPI00759776	ACTN1 α-actinin-1	3	1.000	4	4	2.8	2.9 E − 02
IPI00178083, IPI00218319, IPI00218320, IPI00382894	TPM3 29 kDa protein	8	1.000	12	11	2.7	1.3 E − 03
IPI00788802	TKT transketolase variant (Fragment)	9	1.000	16	15	2.6	3.4 E − 04

(*continued*)

Table III. (*Continued*).

		Samples					
		Protein ID				Differential Expression	
Accession	Gene name, description	U_p	P_p	N_i	N_o	R	p-value
IPI00216318, IPI00640721, IPI00645801, IPI00759832, IPI00795517	YWHAB isoform long of 14-3-3 protein β/α	2	0.610	13	12	2.6	4.0 E – 05
IPI00299547	LCN2 neutrophil gelatinase-associated lipocalin precursor	6	1.000	13	13	2.6	7.0 E – 03
IPI00291005	MDH1 malate dehydrogenase, cytoplasmic	3	1.000	7	7	2.5	4.1 E – 03
IPI00027341	CAPG macrophage-capping protein	5	1.000	19	17	2.5	2.7 E – 04
IPI00217987, IPI00645887	ITGAM integrin α-M precursor	5	1.000	7	6	2.5	1.2 E – 03
IPI00418471, IPI00827679	VIM vimentin	11	1.000	17	15	2.4	4.5 E – 05
IPI00002147	CHI3L1 chitinase-3-like protein 1 precursor	4	1.000	17	16	2.4	5.2 E – 04
IPI00293276, IPI00790382	MIF macrophage migration inhibitory factor	2	0.998	19	17	2.4	2.1 E – 03
IPI00465028, IPI00797687	TPI1 triosephosphate isomerase 1 variant	7	1.000	14	13	2.3	4.2 E – 04
IPI00032292, IPI00642739	TIMP1 Metalloproteinase inhibitor 1 precursor	5	1.000	19	17	2.3	1.6 E – 05
IPI00027444	SERPINB1 leukocyte elastase inhibitor	1	0.610	11	10	2.3	2.3 E – 05
IPI00219757, IPI00796076	GSTP1 glutathione S-transferase P	4	1.000	18	16	2.3	2.3 E – 05
IPI00299024	BASP1 brain acid soluble protein 1	1	0.610	14	13	2.3	1.9 E –03
IPI00021263, IPI00790768	YWHAZ 14-3-3 protein zeta/delta	6	1.000	19	18	2.2	1.6 E – 03
IPI00010270	RAC2 Ras-related C3 botulinum toxin substrate 2 precursor	3	1.000	8	7	2.2	2.1 E – 02
IPI00019755	GSTO1 glutathione transferase omega-1	2	0.998	19	17	2.1	1.7 E – 06
IPI00783881	SFTPA1 similar to Pulmonary surfactant-associated protein A1 precursor	5	1.000	9	8	2.1	3.6 E – 02
IPI00075248, IPI00386621, IPI00794543	CALM2; CALM1; CALM3 calmodulin	2	0.998	17	16	2.1	9.1 E – 04
IPI00807640	HSPA1B; HSPA1A heat shock 70 kDa protein 1B	5	1.000	19	17	2.1	8.5 E – 05
IPI00465439	ALDOA fructose-bisphosphate aldolase A	9	1.000	11	10	2.1	7.9 E – 04
IPI00219525, IPI00747533	PGD 6-phosphogluconate dehydrogenase, decarboxylating	1	0.610	15	14	2.1	5.1 E – 03
IPI00003815, IPI00793767, IPI00794402, IPI00796541	ARHGDIA Rho GDP-dissociation inhibitor 1	1	0.610	14	13	2.1	2.0 E – 04
IPI00033494	MRLC2 myosin regulatory light chain	1	0.610	10	9	2.0	5.7 E – 03
IPI00216298, IPI00552768	TXN thioredoxin	2	0.991	12	11	1.9	4.5 E – 03
IPI00025252, IPI00657680	PDIA3 protein disulfide-isomerase A3 precursor	5	1.000	16	15	1.9	6.4 E – 04
IPI00465436	CAT catalase	7	1.000	16	16	1.9	1.9 E – 03
IPI00219365	MSN moesin	11	1.000	16	15	1.9	7.6 E – 04
IPI00169383	PGK1 phosphoglycerate kinase 1	4	1.000	12	12	1.9	5.0 E – 04
IPI00216691	PFN1 profilin-1	3	1.000	14	13	1.9	1.3 E – 03
IPI00012887, IPI00640540	CTSL1 cathepsin L precursor	1	0.610	19	17	1.9	3.9 E – 03
IPI00003362	HSPA5 HSPA5 protein	8	1.000	16	15	1.8	9.0 E – 04
IPI00453476	hCG_2015269 similar to phosphoglycerate mutase 1 (phosphoglycerate mutase isozyme B) (PGAM-B) (BPG-dependent PGAM 1) isoform 1	6	1.000	15	14	1.8	6.3 E – 04
IPI00413778	FKBP1A FKBP1A protein	1	0.610	8	7	1.8	9.3 E – 03
IPI00013808	ACTN4 α-actinin-4	8	1.000	18	16	1.8	4.4 E – 05
IPI00012048, IPI00026260, IPI00029091, IPI00375531, IPI00604590, IPI00790856, IPI00791971, IPI00795292, IPI00798187	NME1 nucleoside diphosphate kinase A	1	0.610	6	6	1.8	1.3 E – 02
IPI00220642	YWHAG 14-3-3 protein-γ	1	1.000	15	14	1.7	7.0 E – 06
IPI00004573	PIGR polymeric-immunoglobulin receptor precursor	5	1.000	17	15	1.7	3.3 E – 02
IPI00302592, IPI00333541, IPI00644576	FLNA filamin A, α	5	1.000	14	13	1.7	1.0 E – 03

(*continued*)

Table III. (*Continued*).

		Samples					
		Protein ID				Differential Expression	
Accession	Gene name, description	U_p	P_p	N_i	N_o	R	p-value
IPI00291175, IPI00307162	VCL isoform 1 of Vinculin	3	1.000	13	12	1.7	1.6 E − 02
IPI00027846	MMP8 neutrophil collagenase precursor	4	1.000	5	5	1.7	7.9 E − 03
IPI00018146, IPI00796727	YWHAQ 14-3-3 protein theta	2	0.610	12	12	1.6	1.2 E − 02
IPI00479145	KRT19 keratin, type I cytoskeletal 19	5	1.000	19	17	1.6	6.0 E − 03
IPI00019038	LYZ lysozyme C precursor	4	1.000	18	16	1.6	2.4 E − 03
IPI00025512	HSPB1 heat-shock protein β-1	2	0.998	10	11	1.6	6.2 E − 03
IPI00000874, IPI00640741, IPI00641244	PRDX1 peroxiredoxin-1	3	0.998	16	16	1.6	3.9 E − 03
IPI00029997	PGLS 6-phosphogluconolactonase	3	1.000	14	12	1.6	2.7 E − 02
IPI00022488	HPX hemopexin precursor	19	1.000	19	18	0.7	1.4 E − 02
IPI00297646	COL1A1 collagen α-1(I) chain precursor	10	1.000	19	17	0.6	2.4 E − 02
IPI00025465, IPI00830128	OGN mimecan precursor	4	1.000	10	9	0.6	1.7 E − 02
IPI00026270	CPM carboxypeptidase M precursor	1	0.610	15	15	0.5	4.4 E − 05
IPI00010341	PRG2 bone-marrow proteoglycan precursor	3	1.000	16	14	0.5	8.3 E − 03
IPI00219832	ABP1 isoform 2 of Amiloride-sensitive amine oxidase [copper-containing] precursor	13	1.000	20	17	0.5	7.7 E − 05

U_p is the number of peptides contributing to protein identification probability (P_p). N_i and N_o are the numbers of samples in which peptides from each protein were observed in patients delivering preterm with IAI and patients with preterm labor who delivered at term, respectively. A relative peptide expression value is determined by normalizing to the reference pool reporter ion intensity. The expression of a protein in a sample relative to the reference pool is determined by computing the median of all relative peptide expression values corresponding to that protein. The median of these individual values is formed for the two groups (N_i and N_o), and their ratio is the protein expression ratio R (values >1.0 correspond to over-expression in the IAI group compared with the term delivery group). p-values were computed with the Mann–Whitney two-sided rank sum test. Protein identities for S100A12, RETN, FK506, HIST1H1D, YWHAB, BASP1, SERPINB1, ARHGDIA, PGD, MRLC2, CTSL1, FKBP1A, NME1, YWHAQ, and CPM were manually validated. Criteria for inclusion in this list were (a) confirmed protein identity, (b) p-value for differential expression of the protein of less than 0.05, and (c) the fold-change between groups of at least 1.5. *The median value among the term delivery group was zero, whereas the median value in the IAI group was finite.

Table IV. Proteins differentially expressed in amniotic fluid samples of patients with preterm delivery without IAI, compared with patients who delivered at term.

		Samples					
		Protein ID				Differential Expression	
Accession	Gene name, description	U_p	P_p	N_i	N_o	R	p-value
IPI00006988	RETN Resistin precursor	1	0.610	15	14	5.3	0.039
IPI00180240, IPI00220828, IPI00719405, IPI00815642, IPI00815829, IPI00815835, IPI00816008, IPI00816288, IPI00816464	TMSL3 thymosin-like 3	6	1.000	14	13	2.6	0.005
IPI00008580	SLPI Antileukoproteinase precursor	2	0.998	8	7	2.1	0.040
IPI00298860	LTF Growth-inhibiting protein 12	36	1.000	20	18	1.9	0.027
IPI00220249, IPI00410152, IPI00784258	LTBP1 Latent-transforming growth factor β-binding protein, isoform 1L precursor	2	0.998	4	4	0.6	0.029
IPI00025465, IPI00830128	OGN Mimecan precursor	4	1.000	10	9	0.6	0.025

Headings, calculations, and criteria for inclusion are as described for Table III.

Discussion

Principal findings of this study

(1) Unbiased analysis using liquid chromatography-tandem mass spectrometry with isobaric labeling (iTRAQ) of the AF proteome of women with PTL identified proteins differentially regulated in women with IAI and in those without IAI who delivered preterm; (2) iTRAQ identified many proteins not previously known to be differentially expressed in

cases of IAI and confirmed the differential expression of many proteins previously reported in the context of hypothesis-driven research; (3) importantly, this proteomic approach ('bottom-up') allowed the identification for the first time of candidate proteins which are differentially regulated in women with PTL leading to preterm delivery in the absence of IAI; and (4) a systems biology approach indicated that two biological processes – actin cytoskeleton organization biogenesis and protein targeting – were found to be prevalent in the AF of patients with PTL and IAI, a novel observation with biological implications. Collectively, these observations suggest that a 'bottom-up' proteomic experimental strategy is a powerful approach for the discovery of potential biomarkers in PTL as well as to gain an understanding in the mechanisms of disease.

'Top-down' or 'bottom-up' proteomic approaches for the analysis of amniotic fluid proteome in preterm labor? The term 'top-down' proteomics refers to the analysis of intact proteins in biological matrices [46]. The general approach consists in detecting differentially regulated proteins between two clinical conditions (health *vs*. disease) followed by identification of such proteins or peptides. This method has been successfully used in identifying biological markers in PTL with IAI [18]. The main challenge in 'top-down' proteomics in the absence of front-end separation of complex protein mixtures is protein identification which is often unsuccessful [46]. To address this challenge, 'bottom-up' proteomics was developed [67]. It reverses the treatment of the problem by identifying proteins first, and then asking the question: 'which of the identified proteins is differentially regulated between clinically-relevant subgroups'. This is accomplished by digestion of proteins in the biological matrix, peptide identification using tandem mass spectrometry, and determination of differentially regulated proteins among two or more clinical conditions. This approach overcomes the main bottleneck of 'top-down' approaches, namely protein identification. Moreover, the recent addition of isobaric labeling technologies [48–50] (e.g. iTRAQ) to 'bottom-up' approaches facilitates both the throughput and quantitation capabilities of LC-MS/MS based studies, and has recently been used successfully to identify biomarkers [62,68–73]. This report represents the first utilization of iTRAQ-based LC-MS/MS approach to characterize the differential expression of proteins in the AF of patients with PTL.

Proteins differentially regulated in intra-amniotic infection/inflammation. We have found a large number of proteins that are differentially regulated (down as well as up) in the AF of women with IAI. This includes most of the proteins previously identified via hypothesis-driven research [13,18,45,74–82], listed in Table II. We interpret this recapitulation of the results of many hypothesis-driven experiments as a validation of the previous findings with an unbiased, discovery-based proteomic approach. Importantly, we also identified differential regulation of many proteins which have not been previously implicated in the process of IAI. These findings can serve as the basis to determine the clinical value of each protein, or a proteomic ''fingerprint'' formed by multiple proteins, as possible biomarkers. Moreover, it opens new lines of investigation to explore the role of each newly identified protein in this study in preterm parturition.

Gene ontology enrichment analysis. The breadth and scope of the present findings allow for the first time a systems-biology approach to the proteomic characterization of AF of patients with PTL with IAI. Four main branches are apparent in this network, which are labeled 'host defense', 'mobility, localization and targeting', 'anti-apoptosis', and 'metabolism/catabolism'. Within each of these branches there are clusters of GO terms that are significantly enriched and functionally inter-related (Figure 3).

Metabolism and catabolism. Parturition is an extremely energy-intensive process, so it is not surprising that biological processes relating to energy production are overrepresented. There are 26 gene products (Supplementary Table S1) which were identified to participate in the metabolism/catabolism cluster in Figure 2. Among these, there are several that appear in at least 15 of the 16 significantly enriched nodes of this branch. They include α-enolase, triosphosphate isomerase, 6-phosphogluconate dehydrogenase (decarboxylating), pyruvate kinase isozymes M1/M2, fructose-bisphosphate aldolase A, 6-phosphogluconolactonase, malate dehydrogenase (cytoplasmic), and phosphoglycerate kinase 1. It is interesting that this cluster converges on glycolysis, whose importance in energy production is self-evident (See Figure 2). The precise reason for the over-expression of these proteins in AF requires further study. It may be expected that the over-expression would occur in the myometrium, cervix, membranes, and the human fetus. Thus, the change in expression in AF may reflect the activity in membranes and the human fetus, among other sites.

Mobility, localization, and targeting. There are two sub-clusters within this branch of Figure 2. One sub-cluster is related to protein localization and targeting and the second sub-cluster is related to cell mobility and localization. The term 'targeting' in GO refers to the process of locating specific proteins to particular

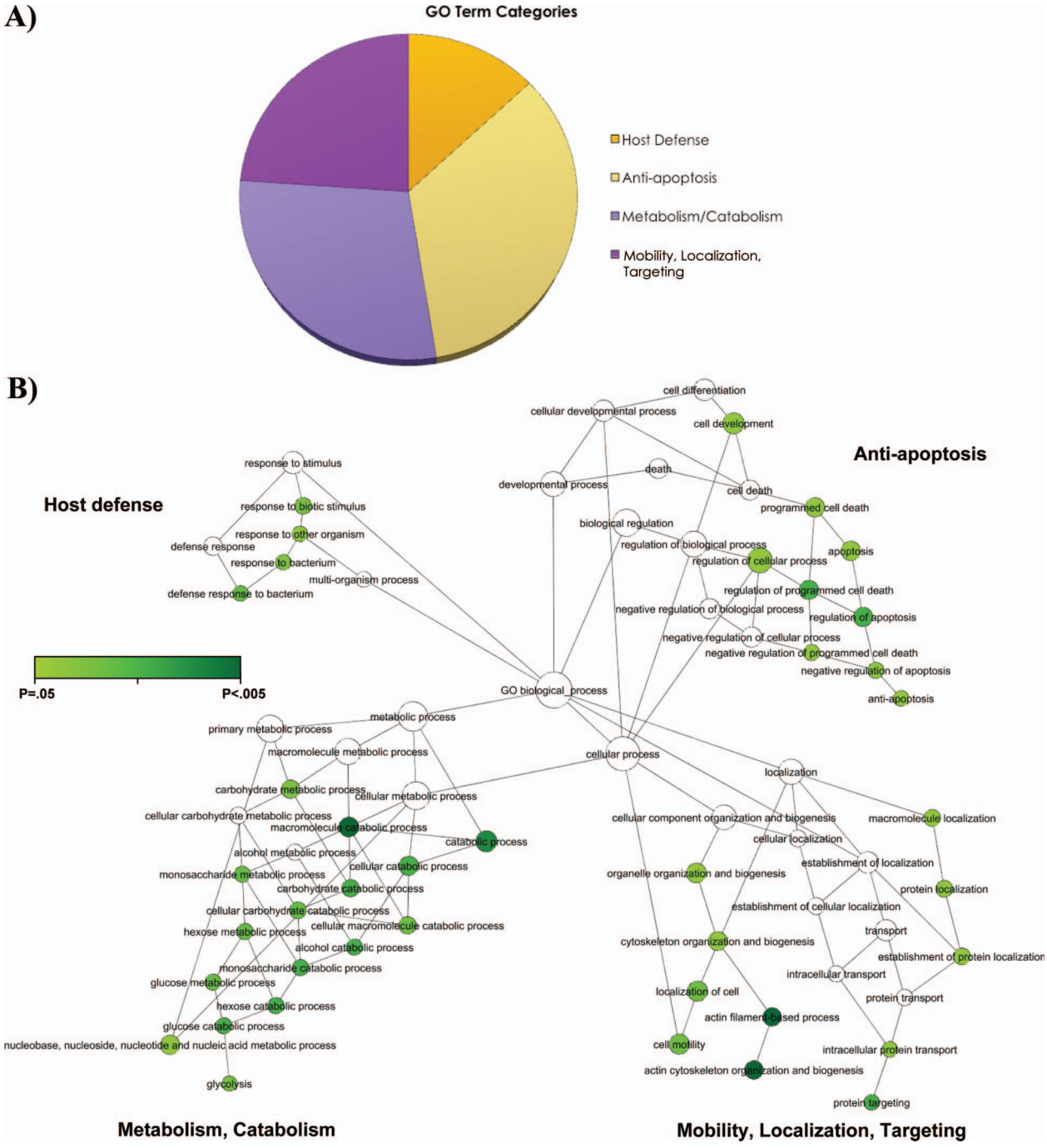

Figure 2. A description of the gene ontology biological processes over-represented in the amniotic fluid of women with spontaneous preterm labor in the presence of intra-amniotic infection/inflammation. (A) Pie chart that displays the main four gene ontology terms that were significantly enriched in the amniotic fluid of women with preterm labor with IAI: (1) metabolism/catabolism; (2) mobility, localization and targeting; (3) anti-apoptosis; and (4) host defense. (B) The four main branches in the network diagram represent four gene ontology terms described in the pie chart. Color nodes represent the biological processes that are significantly over-represented (the darker the color, the greater the significance is). The open nodes represent biological processes that did not reach statistical significance. As described in detail in the text, BiNGO (version 2.0) was used to calculate GO term enrichment, and CytoScape (version 2.5.1) was used to visualize the resulting network of GO biological processes.

membrane-bounded subcellular organelles, such as the peroxisome, and it usually requires an organelle specific protein sequence motif. It is not surprising that host response to infection or inflammation, as well as parturition, requires the re-orientation/re-organization of proteins within the cell.

The protein localization and targeting sub-cluster (Table S2) includes α-filamin-A, myosin-9

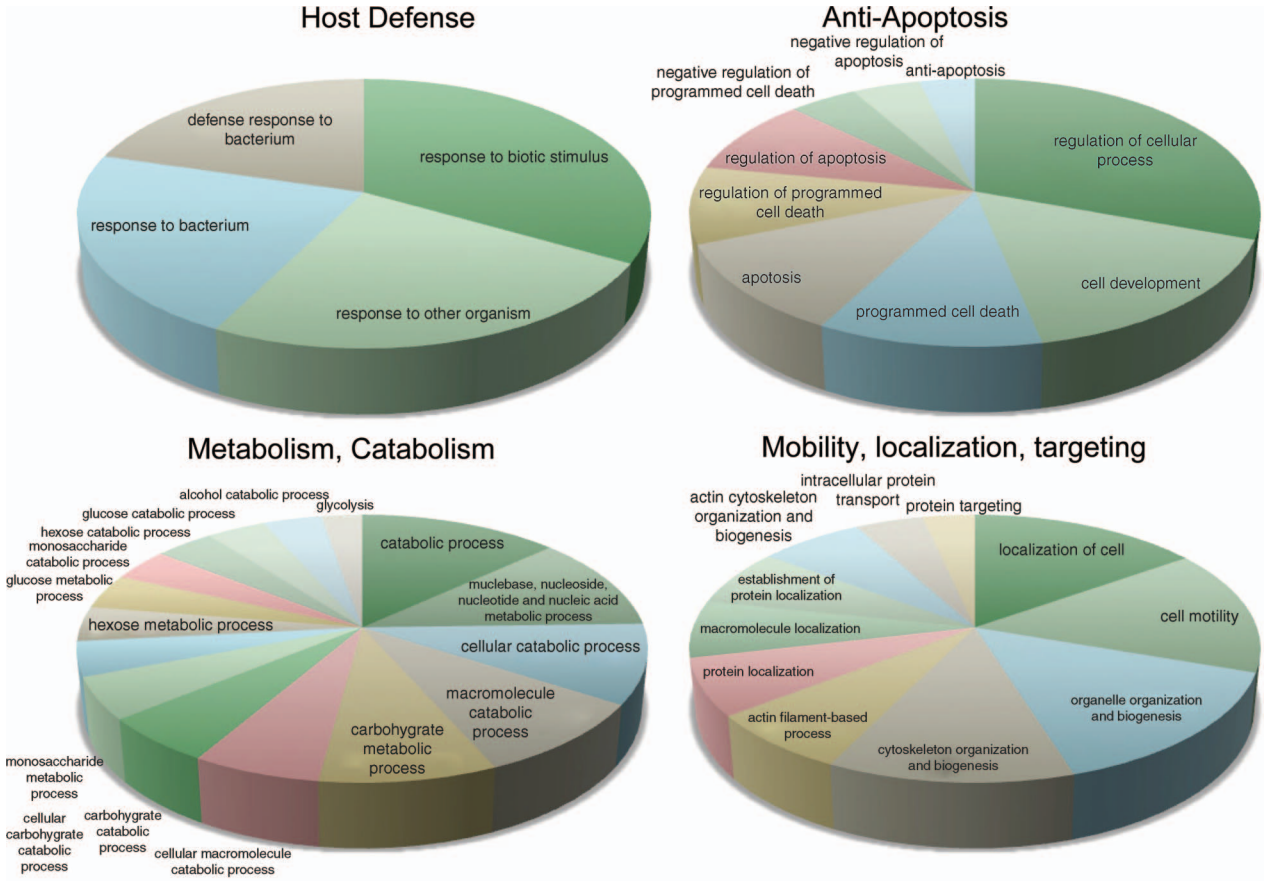

Figure 3. Clusters of biological processes identified in the main four gene ontology terms. The four pie charts represent the main gene ontology terms over-represented in the amniotic fluid of women with spontaneous preterm labor in the presence of intra-amniotic infection/inflammation. Each pie chart displays clusters of biological processes that are significantly enriched and functionally inter-related.

polymeric-immunoglobulin receptor, and a group of 14-3-3 family proteins. The finding that this group also includes PDIA3 (protein disulfide isomerase family A, member 3) suggests that endoplasmic reticulum (ER) stress [83] may be an additional biological process implicated in PTL associated with IAI leading to preterm parturition. ER stress results from the accumulation of unfolded/misfolded proteins in the ER as a consequence of an imbalance between the protein-folding load and the capacity of the ER to fold them [84]. PDIA3 protein interacts with lectin chaperones, calreticulin and calnexin, to modulate folding of newly synthesized glycoproteins [85]. PDIA3 participates in electron transport within the mitochondria and can lead to the H_2O_2 generation during the process of proteins folding. In cases of inflammation, increased activity of this pathway leads to enhanced H_2O_2 generation and oxidative stress [86]. A relationship between ER stress and inflammation is now well recognized [84]. Yet, this study represents the first evidence of a link between ER stress and preterm parturition.

The second sub-cluster (Table S2) relates to cell mobility and localization, and includes actin as well as actin-interacting species such as cytoplasmic actin 1, α-actinin-4 and -1, myosin-9, macrophage migration inhibitory factor, macrophage capping protein, integrin α-M and plastin-2. Also prominent in this sub-group is a set of gene products including Calgranulin B, Vinculin, Profilin-1, Cofilin-1, Leukocyte elastase and Moesin.

Anti-apoptosis. A cluster of anti-apoptotic proteins (Table S3) was overrepresented in the AF of women with PTL and IAI. This observation represents the first report that this pathway is active in PTL associated with IAI. Among the proteins participating in this pathway are: myeloperoxidase, heat shock protein HSPA1B, two members of the 14-3-3 family, Cofilin-1, Glutathione S-transferase (GST), Rho GDP association inhibitor, and macrophage migration inhibitory factor.

Why should gene products involved in anti-apoptosis be over-expressed in the AF in women who have IAI? A possible explanation is that IAI is characterized by an influx of neutrophils into the amniotic cavity in the context of infection [54,74,87–96], and that such neutrophils play a key role in host defense. It is now well-established that neutrophils

engaged in host protection undergo delayed apoptosis [97–100]. This allows prolonged survival of neutrophils favoring an increased number and life span of these key cells. It is interesting that several of the proteins that are identified through GO as being involved in promoting anti-apoptosis also play a role in the regulation of oxidative stress. For example, myeloperoxidase has anti-apoptotic properties [101], and also participates in the generation of reactive oxygen species by neutrophils [102]. The latter is reflected in GO by its annotation as involved in defense response and oxidation reduction. Thus, computational biology methods allow elucidation of pleiotropy (one protein exerting multiple functions).

GST belongs to a family of enzymes comprising cytosolic, mitochondrial, and microsomal proteins [103]. These enzymes catalyze the conjugation of reduced glutathione via a sulfhydryl group, on a wide variety of substrates, including peroxidised lipids [104] and xenobiotics [105]. GSTs may also bind toxins [106] and function as transport proteins. Just as in the case of MPO, this group of enzymes has multiple properties, including anti-apoptosis and control of redox.

Heat shock protein (HSP)A1B (also known as HSP70) was differentially expressed in the AF of women with IAI. This observation is in keeping with our previous report [107]. The precise role of HSP70 in AF is unknown. It is possible that HSP70 participates in stimulating prostaglandin (PG) production during PTL associated with IAI [38,108–117]. Indeed, HSP70 induces cyclooxygenase enzyme COX-2 protein expression and PGE2 production in human umbilical vein endothelial cells [118]. Further studies are required to examine the role of HSP70 in COX-2 expression and PG production by gestational tissues.

The 14-3-3 proteins, first identified in 1967, are a family of acidic regulatory peptides found in plants and animals [119]. These proteins are highly conserved and seven isoforms have been identified in mammals (β, γ, ε, σ, ζ, τ and η) [120,121]. 14-3-3 proteins can interact with many cellular proteins including transcription factors, enzymes, cytoskeletal proteins, signaling molecules, apoptosis factors, and tumor suppressors, as a result of their specific phospho-serine/phospho-threonine binding activity [120,121]. Because 14-3-3 interactions are primarily phosphorylation-dependent, the 14-3-3 proteins have been implicated as important components of crucial biological processes that are regulated by phosphorylation, including signal transduction, cell-cycle regulation metabolism control and apoptosis [119,122,123]. This is the first report documenting an association between the up-regulation of members of the 14-3-3 protein families and PTL.

Host defense. This cluster (Table S4) includes several proteins known to be up-regulated in the AF of women delivering preterm with IAI, including neutrophil defensins [77], lactoferrin [76], FALL-39 antimicrobial protein [18], and Calgranulin C [13,18]. Also included in this cluster are peptidoglycan recognition protein, bone-marrow proteoglycan, and two heat shock proteins (HSPB1 and HSPA1B). The role of antimicrobial peptides [76,77,124–127] and pattern recognition receptors [128–136] as well as heat shock proteins in host defense against IAI [107], has been subject of previous communications by our group, and the interested reader is referred to those publications for details.

Proteins differentially regulated in patients with preterm labor leading to preterm delivery without intra-amniotic infection/inflammation. Of major importance is that we were able to identify and provide quantitative assessment of a cluster of proteins that are differentially regulated in women destined to deliver preterm in the absence of IAI. Such proteins are listed in Table II. Notably, it includes SLPI, Leukocyte elastase inhibitor, a γ-chain of hemoglobin, and Resistin. The identification of Resistin as a potentially differentially regulated protein in this study has allowed us to conduct a targeted approach in which AF Resistin concentrations were measured (using ELISA) in the three clinical groups included in this study [137]. That study found that AF Resistin concentration was higher in women with IAI, as well as in those with PTL without IAI leading to preterm delivery within 48 h and 7 days of amniocentesis, than in those who delivered at term [137]. Therefore, the differential regulation of Resistin in AF found in this study has already been confirmed in an independent set of patients. We have previously reported the behavior of SLPI in PTL [138] as well as hemoglobin [139,140]. Further studies are required to determine if the concentration of these proteins, alone or in combination, have prognostic value.

'Systems biology' analysis of preterm delivery in the presence of intra-amniotic infection/inflammation. Proteomic studies such as the present one offer a 'wide-angle' picture of protein expression within a system; in this case, AF in women presenting with PTL. Among women diagnosed with IAI who delivered preterm there is a large number of proteins that appear to be differentially regulated in comparison with patients without IAI, within a constellation of an even larger number of proteins that were sampled experimentally. The scope of such studies permits a statistical analysis to suggest the significance of biological processes that are important in the presence of IAI. We use the term 'systems biology' here as it relates to such a holistic as opposed to

a reductionistic approach to understanding biological process underlying preterm delivery with IAI.

Strengths and weaknesses of this study. The major strength of this study is that it is the first 'bottom-up' proteomic approach used in the characterization of AF in the setting of PTL, and that differentially regulated proteins have been found in women who delivered preterm with and without IAI. Moreover, since some of the proteins found to be differentially regulated with this approach have been previously reported to participate in preterm parturition, this may be considered validation of our findings and approach. A potential weakness of 'bottom-up' proteomics is that because protein identification is predicated on the observations of tryptic peptides, the method may be unable to distinguish isoforms, fragments, or other post-translational modification from intact proteins which may be found in health or disease. Gravett et al. [18] and our group [45] have previously reported an example of such case when fragments of insulin-like growth factor binding protein (IGFBP)-1 which is proteolytically degraded in cases of infection, were found in AF [141]. Such findings would be more difficult to detect with 'bottom-up' proteomics. Consequently, 'bottom-up' and 'top-down' proteomics are complementary methods that may be used in concert to discern the full complement of proteins differentially regulated in a biologic matrix.

Conclusions

This work provides evidence that a 'bottom-up' proteomics approach can be used to discern differentially regulated proteins in the AF of women with PTL. We have identified a large number of proteins which have been implicated in PTL through decades of hypothesis-driven research, and have also discovered proteins which thus far have not been implicated in preterm parturition. These observations confirm the power of the combined use of proteomics and computational biology as a discovery platform to gain insight into the complex biological processes of preterm parturition. The complexity of such processes has eluded definition using conventional reductionist, hypothesis-driven research. It is important to note that discovery-based approaches are fundamentally hypothesis-generating methodologies, and thus, provide new hypotheses which can be tested using conventional approaches.

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