

Comprehensive Proteomic Analysis of the Human Amniotic Fluid Proteome: Gestational Age-Dependent Changes

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Amniotic fluid (AF) is a significant contributor to fetal health and constitutes a potential rich source of biomarkers for diagnosis of maternal and fetal disorders. In this study, we performed a comprehensive survey of the proteins expressed in AF, combining gel and liquid-based fractionation approaches coupled with LC-MS/MS analysis. Two-dimensional Liquid Chromatography (2D-LC) analysis identified 118 nonredundant proteins with high confidence. One- and two-dimensional gel electrophoresis and in-gel digestion identified 101 proteins. Combining both sets resulted in 219 proteins, of which 96 are unique to AF; 70, 18, and 35 proteins are present in serum, cervico-vaginal fluid, and all three fluids, respectively. Fluorescence two-dimensional differential in-gel electrophoresis (2D-DIGE) comparison of first-, second-, and third-trimester AF samples revealed that maximal differences in the relative abundance of AF proteins occur between the first and second trimesters. A systematic analysis of proteins present both in AF and maternal serum could lead to the development of new noninvasive diagnostic procedures to monitor fetal status.

Keywords: Amniotic fluid • Proteome • Pregnancy • Biomarkers • Aneuploidy

Introduction

Amniotic fluid (AF) protects the fetus physically and biochemically and assists in its development. AF resides in the amniotic cavity that is lined by the amnion and chorion. The amnion is composed of five layers: the epithelium, basement membrane, compact layer, fibroblast layer, and the spongy layer. The chorion is composed of a reticular layer, basement membrane, and trophoblast layer.¹ Cells from the amnion and chorion layers secrete proteins into the AF that include the extracellular matrix components of the membranes that are crucial for maintenance of their tensile strength.¹ The importance of AF and the viability of the amniotic membranes is underscored by the problem of preterm birth, which occurs in 11.8% of pregnancies in the United States (Births: final data for 2002 Hyattsville (MD) National Center for Health Statistics; 2002). Since current serum-based tests are unable to detect many of the complications of pregnancy, AF is an important potential source of biomarkers for fetal pathology.

In a search for markers of complications during pregnancy, the biochemical composition of AF has been analyzed. In

general, the protein and metabolite composition of AF varies throughout gestation; for example, the concentrations of metabolic enzymes such as γ -glutamyl transferase, alkaline phosphatase, and leucine aminopeptidase increase, as well as the metabolites urea and creatinine.² The increasing levels of enzymes and electrolytes in the later part of gestation correlate with the formation of the fetal kidneys, lungs, and the gastrointestinal tract. Immunoassays have revealed the occurrence of growth factors, innate immunity molecules, and serum components in AF that are believed to be involved in growth, development, and protection of the fetus from infection.³

To date, several groups have reported protein profiles of AF. Park et al. recently described a comparison of proteins from infected and non-infected AF using 2D PAGE and mass spectrometry (MS) analysis.⁴ Tsangaris et al. characterized and functionally annotated the amnion cell proteome. Using 2D SDS-PAGE and MALDI-MS/MS, they discovered 432 gene products.⁵ Nilsson et al. examined AF protein composition using LC-ESI-FT-ICR, identifying 43 proteins.⁶ Using an immunodepletion technique to remove major protein species, Michel et al. compared maternal plasma to AF by isoelectric focusing and LC-MS/MS to find potential markers for premature rupture of membranes (PROM) and described 26 proteins that were specific to AF.⁷

One- and two-dimensional gel electrophoresis offers the advantage of separation of proteins by molecular weight in a

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complex mixture. However, the bias toward high-abundance proteins, as well as a narrow molecular weight range, limits the number of proteins that can be identified. Two-dimensional Liquid Chromatography tandem mass spectrometry (2D-LC-MS/MS), a gel-free method pioneered by Yates and co-workers, has proven to be an effective and robust technique for investigating global changes in protein expression as a function of development and disease.⁸ In this study, we have identified the general protein profile of AF using both in-gel digestion of AF samples and 2D-LC-MS/MS proteomic analysis. To examine the changes in AF protein composition during pregnancy, we compared the protein profiles of each trimester by fluorescence two-dimensional differential in-gel electrophoresis (2D-DIGE).

Materials and Methods

Samples. AF samples from first trimester (11–13 weeks gestational age; GA), second trimester (16–18 weeks GA), and term pregnancies (greater than 18 weeks GA) were obtained following guidelines and applicable local regulatory requirements. This study was approved by the Institutional Review Board of Oregon Health & Science University. All subjects were identified prospectively and gave informed written consent to participate in the study.

Immunodepletion of Abundant Serum Proteins. AF samples were depleted of six major serum proteins (albumin, IgG, IgA, anti-trypsin, transferrin, and haptoglobin) using an Agilent multiple affinity system (Agilent Technologies, Inc., Palo Alto, CA) that is based on antibody-antigen interactions and optimized buffers for sample loading, washing, eluting, and regenerating. AF (4000 mL) was acetone-precipitated for each sample. The pellet was resuspended in Agilent buffer A. Particulates were removed by filtering through a 0.22-mm spin filter for 1 min at 16 000g. A total of 160 μ L of the diluted serum was injected into an Agilent immunoaffinity column (4.6 \times 100 mm) attached to a Waters HPLC system equipped with an autosampler, UV detector, and a fraction collector. The flow rate was set to 0.5 mL/min for the first 10 min with 0% buffer B, and 10–17 min at 1 mL/min with 100% B and 17–28 min at 1 mL/min with 0% buffer B. Low-abundance flow-through fractions 2–5 were collected, concentrated, and buffer-exchanged with 10 mM Tris, pH 8.4, using 5000 MWCO filters. Protein concentration was determined using a Bio-Rad DC protein assay kit.

Fluorescence Two-Dimensional Differential in-Gel Electrophoresis (2D-DIGE). AF proteins (50 μ g) were labeled with Cy Dye DIGE Fluor minimal dye (Amersham Biosciences) at a concentration of 100–400 pmol of dye/50 μ g of protein. Samples were labeled with Cy3, Cy5, or Cy2 (reference pool), and all three labeled samples were multiplexed and resolved in one gel. Labeled proteins were desalted by acetone precipitation and dissolved in IEF buffer and rehydrated on a 24 or 13-cm IPG strip (pH 4–7) for 12 h at room temperature. The IPG strip was subjected to one-dimensional electrophoresis at 65–70 kVhr and then equilibrated with DTT equilibration buffer and IAA equilibration buffer for 15 min sequentially before second-dimensional SDS-PAGE analysis. The IPG strip was then loaded onto an 8–16% SDS-PAGE gel and electrophoresis conducted at 80–90 V for 18 h to resolve proteins in the second dimension. Gels were scanned in a Typhoon 9400 scanner (Amersham) using appropriate lasers and filters with PMT voltage between 550 and 600. Images in different channels were overlaid using pseudo-colors, and differences were visualized

using ImageQuant software (Amersham Biosciences). Two-dimensional gel image analysis to determine the differentially abundant protein spots was performed using Phoretix 2D evolution, version 2005 (Non-Linear Dynamics, Ltd.). A fixed area was selected from every gel and a cross-stain analysis (CSA) protocol was performed. Background subtraction was done using the ‘mode of non spot’ method. A ratiometric normalization algorithm was applied to account for potential concentration differences in protein labeling. Normalized protein spots in the Cy5 and Cy3 channels were compared to the internal standard (Cy2) to generate a ratio of relative amount. Spots with a ratio of ± 1.5 were considered to be differentially expressed.

Two-Dimensional Liquid Chromatography Tandem Mass Spectrometry (2D-LC-MS/MS) Analysis. One milligram of pooled sample was prepared from immunodepleted first, second, and third (term)-trimester AF samples. The sample was evaporated in a speedvac and dissolved in 100 μ L of digestion buffer containing 8 M urea, 1 M Tris base, 0.1 M methylamine, and 10 mM CaCl₂ (pH 8.5). For reduction and alkylation of cysteine residues, samples were first incubated at 50 °C in 12.5 μ L of 0.9 M DTT for 15 min and, then, in 25 μ L of 1.0 M iodoacetamide in the dark at room temperature for another 15 min. Before the addition of 40 μ L of mass spectrometry-grade trypsin (1 mg/mL) (Promega), an additional 12.5 μ L of 0.9 M DTT, along with 210 μ L of water, was added. Samples were then thoroughly mixed and incubated overnight at 37 °C. Digestion was halted by the addition of 40 μ L of 88–96% formic acid. Digests were desalted prior to 2D-LC analysis using SepPak plus C18 cartridges (Waters). The SepPak eluate (1 mL) was fractionated using an LC equipped with a UV detector and a fraction collector. Sample was injected into a polysulfoethyl column (strong cation-exchange, SCX) (The Nest Group), 2.1-mm i.d. \times 100 mm, 5 mm, 300 Å. Solvent A was 10 mM potassium phosphate (pH 3) and 25% acetonitrile, and solvent B was 10 mM potassium phosphate (pH 3), 350 mM KCl, and 25% acetonitrile. A 95-min gradient was employed for fractionation of peptides with 10 min with buffer A, a ramp to 50% B over 45 min, followed by a 20-min ramp to 100% B and a ramp back to 100% A over 0.1 min, and maintained for 20 min. Column flow rate was 200 mL/min, and 80 fractions were collected and stored at –20 °C. The SCX fractions were evaporated, dissolved in 100 mL of 0.1% TFA, and desalted using a 96-well Vydac silica C18 spin column (The Nest Group). After elution with 80% acetonitrile and 0.1% formic acid, fractions were consolidated into 43 fractions, evaporated, and dissolved in 20 μ L of 5% formic acid.

Mass Spectrometry. 2D-LC fractions and gel digests were further separated using a Nanoease C18 75- μ m i.d. \times 15-cm fused silica capillary column (Waters, Inc., Milford, MA) and a 95-min water/ACN gradient. The mass spectrometer was calibrated using Glu1 Fibrinopeptide B. An MS/MSMS survey method was used to acquire spectra. Masses from m/z 400 to 1500 were scanned for MS survey and masses from m/z 50 to 1900 for MS/MS. A total of 9397 MS/MS spectra were acquired from the 2D-LC fractions. Raw MS/MS spectra were preprocessed with ProteinLynx Global Server v.2.1 software (Waters, Inc., Milford, MA).

Gel Electrophoresis and 2D-LC Protein Identification. Raw mass spectra from either gel-electrophoresis-based or 2D-LC experiments were preprocessed by de-isotoping and centroiding the raw data. Tandem mass spectra from the 2D-LC experiment were searched against a combined database con-

taining known contaminants and forward and reverse entries of the Swiss-Prot human database (version 46.6). Peptide matching was performed using three independent search engines: Sequest, X! Tandem, and OpenSea. All search engines were configured using the protocol as outlined in ref 9. Peptide identifications from all search engines were combined into protein identifications using Scaffold software (v1.3.5, Proteome Software, Portland, OR). All the proteins with at least two independent peptide identifications (probability ≥ 0.8) were considered to be highly likely to be present in the sample. Proteins and peptides from gel electrophoresis experiments were identified by matching the preprocessed tandem mass spectra to either IPI or Swiss-Prot database selected for human species. Peptide identification was performed by combining the results of two independent search engines: ProteinLynx Global Server v2.1 (Waters) and OpenSea (v1.3.1).^{10,11} ProteinLynx was configured to use 0.1 and 0.15 Da as parent and fragment ion mass tolerances, respectively. OpenSea was configured with the same protocol used for 2D-LC. Peptide identifications from both search engines were combined into protein identifications using Microsoft Excel. Protein identifications that had two or more independent peptides that were matched by both ProteinLynx (significance score > 10.6) and OpenSea (significance score > 100)^{10,11} were considered as high-confidence identifications. High-confidence protein identifications from both 2D-LC and gel-electrophoresis experiments were combined into a nonredundant list of proteins present in AF.

Data Analysis. Functional annotation was obtained by assignment of Gene Ontology (GO) terms for the identified proteins using the DAVID program.¹² Ninety-five percent of the proteins could be assigned to GO categories (biological significance). To classify and annotate the identified proteins for fetal biology, the proteins were further analyzed using Bioinformatic Harvester, SOURCE,¹³ STRING,¹⁴ Swiss-Prot, OMIM, and the EBI-Hinxton "Uniprot, Swiss-Prot, TrEMBL" database, as well as Pubmed searches using key words such as "fetus".

Results and Discussion

The AF is known to provide nutrients, electrolyte balance, protection from physical stress, and defense against infection.³ While there has been progress in understanding the protein composition of AF by immunoassay and single proteomic strategies, previous studies have only identified as many as 43 proteins. To gain greater insight into the functions of AF and identify potential biomarkers of pathology, we endeavored to generate a more comprehensive protein profile. Additionally, AF proteome analyses to date have examined term samples and identified some candidates with potential diagnostic utility. To expand the current understanding of AF composition, we studied AF from all three trimesters and used two distinct proteomic techniques to gain a broader protein profile.

In-Gel Digestion and LC-MS/MS. We first conducted in-gel digestion and tandem LC-QTOF-MS/MS of AF proteins resolved by 1D and 2D SDS-PAGE. To detect lower-abundance proteins in AF, we fractionated the most abundant proteins from the sample for both in-gel analysis and 2D-LC. Following 1D and 2D gel electrophoresis, spots were removed from the gels, trypsin-digested, and analyzed by Q-TOF mass spectrometry. Spectra were processed into a peak list by ProteinLynx, and analyzed by the database search program OpenSea, which searched the processed data against a Swiss-Prot database. Identifications from the database were based on 2 or more

peptide matches per protein. In total, 171 proteins were identified from the spectra.

2D-LC Analysis of the AF Proteome. In addition to in-gel digest identification, we employed 2D-LC analysis of a pool of three samples (one from each trimester) to obtain a more comprehensive list of the protein composition of AF. With the major protein species removed, the pooled sample was trypsinized, reduced, alkylated, and desalted prior to analysis using multidimensional chromatography. The fractions were then applied to a Q-TOF mass spectrometer and analyzed. 2D-LC spectra were processed by ProteinLynx, and searched against a Swiss-Prot database using OpenSea, X!Tandem, and Sequest programs. The Scaffold program (Proteome Software, Portland, OR) was used to combine the peptide and protein identifications from all three search engines, and 37% of experimental spectra were matched to 118 noncontaminant proteins with at least two independent peptide identifications per protein.

To understand the quantitative contributions of individual proteins to AF, we measured the spectral counts of tryptic peptides in the pooled sample. Liu et al. showed that the total number of peptide identifications in a complex peptide mixture analyzed by 2D-LC linearly correlated over a 2-order-of-magnitude dynamic range with the abundance of the proteins in a quantitative approach called spectral counting.¹⁵ The proteins identified by 2D-LC are listed in Table 1 and are ranked by abundance according to their spectral counts.

We combined the protein identifications from 2D-LC and gel electrophoresis-based experiments into a comprehensive list of proteins found in AF (Supporting Information Table 1). Of the 171 proteins found by in-gel analysis and the 118 proteins discovered by 2D-LC, 70 proteins were common to both lists. Thus, these complementary approaches identified 219 AF proteins. The in-gel identification method has the advantage of focusing proteins in a band or spot on a gel, but is limited by molecular weight and hydrophobicity. Also, there is high loss of peptides during the processing of low-abundance proteins from gel pieces. Conversely, the 2D-LC approach analyzes a complete protein set from a sample, but detection appears to be limited by the efficiency of elution of peptides from the cation-exchange column. Furthermore, competitive ionization of multiple proteins in a particular fraction results in the obscuring of signals of proteins that ionize poorly. The list also includes proteins previously identified by others that are present in amnion cells⁵ and AF.^{4,6,7}

To determine which proteins are unique to AF compared to serum and cervico-vaginal fluid (CVF), we cross-referenced the set of AF proteins that we identified (Supporting Information Table 1) with a curated HUPO plasma proteome¹⁶ and the CVF proteome.⁹ Of the AF proteins observed in this study, 96 are unique to AF, 70 were also seen in serum, 18 in CVF, and 35 in all three fluids (Table 1, Supporting Information Table 1, and Figure 2). As the HUPO plasma proteome is based on a compilation of data from diverse subjects, we also compared the AF proteins identified here to second-trimester maternal serum proteome that we have characterized (data not shown). Of the 392 proteins identified in maternal serum, we found that 69 proteins are common between AF and second-trimester maternal serum.

Functional Annotation of the AF Proteome. We have categorized the identified proteins by functional class. The list of proteins were processed by DAVID and sorted by GO terms. The results are listed in the Supporting Information table.

Table 1. AF Proteins Identified by 2D-LC^a

Swiss-Prot/ TrEMBL accession no.	description	MW (kDa)	pI	normalized spectrum counts	functional category	found in other studies	found in Serum/ CVF
P02774	Vitamin D-binding protein precursor	52.95	5.4	10.11	Cell communication	c, a, b	S, C
P08833	Insulin-like growth factor binding protein 1 precursor	27.88	5.1	7.66	Cell communication	c, a	
P02760	AMBP protein precursor [Contains: Alpha-1-microglobulin	38.98	6	6.72	Cell communication	c, a, b	S
P02751	Fibronectin precursor	262.58	5.5	6.06	Cell communication	d	S
P00450	Ceruloplasmin precursor	122.19	5.4	5.32	Cell communication	c	S, C
P02763	Alpha-1-acid glycoprotein 1 precursor	23.49	4.9	4.78	Cell communication	a	S, C
P02452	Collagen alpha 1 (I) chain precursor	138.87	5.7	4.64	Cell communication	d	S
P02790	Hemopexin precursor	51.66	6.6	3.27	Defense response	c	S, C
P04217	Alpha-1B-glycoprotein precursor	54.25	5.6	2.93	Defense response	c, a	S, C
P01042	Kininogen precursor	71.93	6.3	2.76	Enzyme activity	c	S, C
P02749	Beta-2-glycoprotein I precursor	38.28	8.3	2.42	Cell communication		S, C
P61769	Beta-2-microglobulin precursor	13.7	6.1	1.91	Signal transduction	c, a	
P02753	Plasma retinol-binding protein precursor	22.99	5.8	1.85	Cell communication		S
P09486	SPARC precursor	34.61	4.7	1.82	Development	d	S
P19652	Alpha-2-acid glycoprotein 2 precursor	23.59	5	1.74	Cell communication		S
P00747	Plasminogen precursor	90.55	7	1.71	Enzyme activity		S, C
P02771	Alpha-fetoprotein precursor	68.66	5.5	1.68	Cell communication	b	S
P01236	Prolactin precursor	25.86	6.5	1.51	Signal transduction		
P02461	Collagen alpha 1 (III) chain precursor	138.54	6.2	1.4	Cell communication		
P02647	Apolipoprotein A-I precursor	30.76	5.6	1.37	Cell communication	d	S, C
P80370	Delta-like protein precursor	41.12	5.4	1.31	Development		
P25311	Zinc-alpha-2-glycoprotein precursor	33.85	5.6	1.08	Cell proliferation		
P20908	Collagen alpha 1 (V) chain precursor	183.6	4.9	1.03	Cell communication		
P02765	Alpha-2-HS-glycoprotein precursor	39.31	5.4	1	Metabolism	a, d	S, C
P13727	Eosinophil granule major basic protein precursor	25.21	6.2	0.97	Defense response		
P04196	Histidine-rich glycoprotein precursor	59.56	7.1	0.85	Enzyme activity	c	S
Q16610	Extracellular matrix protein 1 precursor	60.66	6.3	0.85	Signal transduction		S, C
P01033	Metalloproteinase inhibitor 1 precursor	23.15	8.5	0.8	Metabolism		S
P43652	Afamin precursor	69.05	5.6	0.74	Cell communication		S
P08603	Complement factor H precursor	139.11	6.2	0.71	Defense response	c	S, C
P12111	Collagen alpha 3 (VI) chain precursor	343.53	6.4	0.68	Cell communication	c	
P41222	Prostaglandin-H2 D-isomerase precursor	21.01	7.7	0.68	Cell communication		S
P23142	Fibulin-1 precursor	77.24	5.1	0.63	Cell communication	c	S
P17936	Insulin-like growth factor binding protein 3 precursor	31.64	9	0.63	Development		
P01034	Cystatin C precursor	15.78	9	0.63	Enzyme activity		
P18065	Insulin-like growth factor binding protein 2 precursor	35.12	7.5	0.57	Cell communication		S
Q16270	Insulin-like growth factor binding protein 7 precursor	29.11	8.3	0.57	Cell communication	d	
Q12841	Follistatin-related protein 1 precursor	34.97	5.4	0.57	Cell proliferation	c	
P01008	Antithrombin-III precursor	52.59	6.3	0.57	Enzyme Activity	c	S, C
P98160	Basement membrane-specific heparan sulfate proteoglycan core protein precursor	468.79	6.1	0.48	Cell communication	c	
P10451	Osteopontin precursor	35.4	4.4	0.46	Cell communication	c	
P09466	Glycodelin precursor	20.61	5.4	0.46	Development		
P12109	Collagen alpha 1 (VI) chain precursor	108.53	5.3	0.37	Cell communication	d	
P51884	Lumican precursor	38.41	6.2	0.37	Cell communication	c	S
Q14766	Latent transforming growth factor beta binding protein, isoform 1L precursor	173.2	5.3	0.37	Enzyme activity		
P22692	Insulin-like growth factor binding protein 4 precursor	27.92	6.8	0.37	Metabolism		
P62328	Thymosin beta-4	4.9	5	0.37	Metabolism		C
Q12805	EGF-containing fibulin-like extracellular matrix protein 1 precursor	54.62	5	0.34	Defense response		S
P10909	Clusterin precursor	52.48	5.9	0.34	Signal transduction	c	S
P04004	Vitronectin precursor	54.29	5.6	0.31	Cell communication	c	S, C
Q15582	Transforming growth factor-beta induced protein IG-H3 precursor	74.66	7.6	0.31	Cell proliferation	d	S
P61626	Lysozyme C precursor	16.52	9.4	0.31	Enzyme activity		C
P00751	Complement factor B precursor	85.52	6.7	0.31	Metabolism	c, a	S
P13987	CD59 glycoprotein precursor	14.16	6	0.28	Defense response	c	C
P35555	Fibrillin-1 precursor	312.28	4.8	0.28	Development		
P08294	Extracellular superoxide dismutase [Cu-Zn] precursor	25.86	6.1	0.28	Metabolism		
Q9HC84	Mucin-5B precursor	590.47	6.2	0.26	Cell communication		C
P07998	Ribonuclease pancreatic precursor	17.63	9.1	0.26	Enzyme activity		
P02788	Lactotransferrin precursor	78.32	8.5	0.23	Defense response	a	S, C
Q9UBC9	Small proline-rich protein 3	18.14	8.9	0.23	FNA ^b		C
Q9UBC9	Small proline-rich protein 3	18.14	8.9	0.23	FNA ^b		C
P01344	Insulin-like growth factor II precursor	20.12	9.5	0.23	Signal transduction		
Q14118	Dystroglycan precursor	97.56	8.7	0.23	Signal transduction		
P00748	Coagulation factor XII precursor	67.8	8	0.2	Enzyme activity		S
P07602	Proactivator polypeptide precursor [Contains: Saposin A	58.09	5.1	0.2	Metabolism		

Table 1. (Continued)

Swiss-Prot/ TrEMBL accession no.	description	MW (kDa)	pI	normalized spectrum counts	functional category	found in other studies	found in Serum/ CVF
P01019	Angiotensinogen precursor	53.14	5.9	0.2	Metabolism	c	S
P01833	Polymeric-immunoglobulin receptor precursor	83.3	5.6	0.17	Cell communication	c	C
P02766	Transthyretin precursor	15.87	5.5	0.17	Cell communication	c, a	S
P02768	Serum albumin precursor	69.35	5.9	0.17	Cell communication	b, d	S, C
P05156	Complement factor I precursor	65.7	7.7	0.17	Defense response		S
Q15113	Procollagen C-proteinase enhancer protein precursor	47.95	7.4	0.17	Development		S
P11464	Pregnancy-specific beta-1-glycoprotein 1 precursor	47.21	8.3	0.17	FNA ^b	c	
P29279	Connective tissue growth factor precursor	38.05	8.4	0.17	Metabolism		
Q9UGM5	Fetuin-B precursor	42.08	5	0.17	Metabolism		
P02671	Fibrinogen alpha/alpha-E chain precursor	94.96	5.7	0.14	Cell proliferation		S, C
P00746	Complement factor D precursor	26.99	8.1	0.14	Defense response		S
P02748	Complement component C9 precursor	63.16	5.4	0.14	Defense response	c	S
P07476	Involucrin	68.45	4.6	0.14	Development		C
P05090	Apolipoprotein D precursor	21.26	5.1	0.14	Metabolism	c	S
P01243	Chorionic somatomammotropin hormone precursor	25	5.3	0.14	Signal transduction	a	
P08123	Collagen alpha 2(I) chain precursor	129.44	9	0.14	Cell communication	d	
P05362	Intercellular adhesion molecule-1 precursor	57.81	8.3	0.11	Cell communication		
P24593	Insulin-like growth factor binding protein 5 precursor	30.55	8.6	0.11	Cell communication		
P02679	Fibrinogen gamma chain precursor	51.5	5.4	0.11	Cell proliferation	c	S, C
Q07654	Trefoil factor 3 precursor	8.62	5.7	0.11	Defense response		
P35321	Cornifin A	9.86	8.9	0.11	Development		C
Q95633	Follistatin-related protein 3 precursor	27.64	6.4	0.11	FNA ^b		
P31949	Calgizzarin	11.72	6.6	0.11	Metabolism		
P61916	Epididymal secretory protein E1 precursor	16.55	7.6	0.11	Metabolism		
O43278	Kunitz-type protease inhibitor 1 precursor	58.38	5.9	0.09	Enzyme activity		
P04278	Sex hormone-binding globulin precursor	43.76	6.2	0.09	Cell communication		S
P16070	CD44 antigen precursor	81.54	5.1	0.09	Cell communication		
Q13421	Mesothelin precursor	69.03	6	0.09	Cell communication	c	
P01028	Complement C4 precursor	192.75	6.7	0.09	Defense response	c	S, C
P08174	Complement decay-accelerating factor precursor	41.38	7.8	0.09	Defense response		
P31025	Von Ebner's gland protein precursor	19.23	5.4	0.09	Defense response	c, a	
Q10588	ADP-ribosyl cyclase 2 precursor	35.72	8	0.09	Defense response		S
P03973	Antileukoproteinase 1 precursor	14.31	9.1	0.09	Enzyme activity		C
P04080	Cystatin B	11.12	7	0.09	Enzyme activity	a	C
P02652	Apolipoprotein A-II precursor	11.16	6.3	0.09	Metabolism		S
P10599	Thioredoxin	11.59	4.8	0.09	Metabolism		C
Q961Y4	Carboxypeptidase B2 precursor	48.39	7.6	0.09	Metabolism		S
P11684	Uteroglobin precursor	9.98	5	0.09	Signal transduction		
P55290	Cadherin-13 precursor	78.27	4.8	0.09	Cell organization		
P22105	Tenascin-X precursor	464.42	5.2	0.06	Cell communication		S
P80188	Neutrophil gelatinase-associated lipocalin precursor	22.57	9	0.06	Cell communication		S, C
P01024	Complement C3 precursor	187.15	6	0.06	Defense response	c	S, C
P01860	Ig gamma-3 chain C region	32.31	7.9	0.06	Defense response		S
P48745	NOV protein homolog precursor	39.14	8.1	0.06	Development		
P03952	Plasma kallikrein precursor	71.35	8.6	0.06	Enzyme activity		S
P17900	Ganglioside GM2 activator precursor	20.8	5.2	0.06	Enzyme activity		
P04156	Major prion protein precursor	27.64	9.1	0.06	Metabolism		
P07988	Pulmonary surfactant-associated protein B precursor	42.1	5.3	0.06	Metabolism	c	
P01215	Glycoprotein hormones alpha chain precursor	13.06	8.5	0.06	Signal transduction		
Q14767	Latent transforming growth factor-beta-binding protein 2 precursor	195.04	5.1	0.06	Signal transduction		
P00734	Prothrombin precursor	70.02	5.6	0.06	Enzyme activity	c	S
P02787	Serotransferrin precursor	77.03	6.8	0.06	Defense response	a, c, d	S, C
P09228	Cystatin SA precursor	16.43	4.8	0.06	Enzyme activity		
Q03591	Complement factor H-related protein 1 precursor	37.64	7.7	0.06	Cell communication		S

^a Identified proteins are listed with their Swiss-Prot or TrEMBL accession numbers. Molecular weight (kDa) and pI calculated using the CalPI/MW tool available on the Swiss-Prot Web site. Functional annotation was performed using procedures outlined in the Materials and Methods section. Spectral counts of each protein were normalized with total number of matched spectra in the experiment (3512). The proteins in the table are ordered by the decreasing normalized spectral counts. AF proteins that we identified that were also found in other studies of AF are marked accordingly: a, ref 4; b, ref 6; c, ref 7; and d, ref 5. AF proteins that are also present in serum (S) and CVF (C) are also marked. ^b FNA, function not assigned.

Further sorting for relation to fetal development was also carried out based on KEGG pathway terms and the Bioinformatic harvester Web site using SOURCE, STRING, and TrEMBL. The subgroups assigned were Development, Transport and localization, Signal transduction, Complement and coagulation, Defense response, and Metabolism (Figure 1).

1. Development. We have identified a set of proteins that may have a role in the development of several different tissues

(Table 1 and Supporting Information Table 1). Tetranectin, placental anticoagulant protein IV, Fetuin B, α 2 HS glycoprotein, and cartilage oligomeric matrix protein are involved in skeletal development. Biotinidase, cadherin 13, prion, and prostaglandin D2 synthase have possible functions in central nervous system development. Alanyl aminopeptidase may be involved in angiogenesis. The regulation of extracellular matrix is a key feature in the development of many tissues. Cartilage

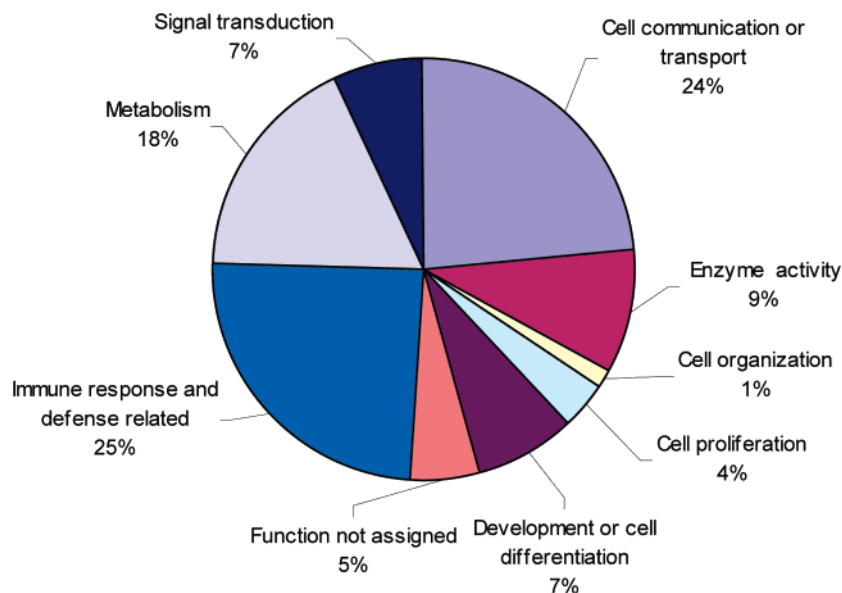


Figure 1. Functional annotation of the AF proteome. Identified AF proteins were categorized using GO terms, KEGG terms, SOURCE, and STRING. Percentages of the total from each group are labeled. The protein functional groups are development or cell differentiation, cell communication or transport, enzyme activity, signal transduction, immune and defense-related, cell proliferation, cell organization, and metabolism.

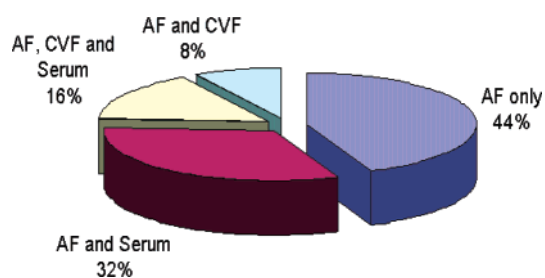


Figure 2. Proteome overlap between AF, serum, and CVF: AF proteins found in this study were cross-referenced with serum and CVF proteomes.

oligomeric matrix protein, extracellular matrix protein 1, and certain collagen isoforms may participate in the formation of connective tissue. Fibronectin is known to organize the extracellular matrix. TIMP-1 inhibits metalloproteases that degrade extracellular matrix proteins. Chitinase 3 like 1 has been reported to remodel tissue. Other proteins in this group, such as nidogen 1, nidogen 2, and E-cadherin, are involved in cell adhesion. The extracellular matrix is a fundamental component of the basement membrane of amniotic membranes. One of the most studied extracellular matrix proteins in relation to amniotic membrane disorders is collagen. Collagen membrane levels,¹⁷ isoforms,¹⁸ and changes in its metabolism¹⁹ can contribute to preterm premature rupture of membranes (PROM). Another report has suggested that matrix metalloproteases not bound to TIMP can degrade the fetal membrane extracellular matrix, resulting in PROM.²⁰

2. Transport and Localization. The identified transport and localization proteins that are relevant to fetal growth and development include nutrient/cofactor transport (vitamin and mineral), lipid transport, and hormone carriers (Table 1 and Supporting Information Table 1). Afamin, Vitamin D binding protein, retinal binding protein, ceruloplasmin, and transferrin transport Vitamin E, Vitamin D, Vitamin A copper, and iron, respectively. In some cases of PROM, it has been observed that there are low maternal and fetal serum levels of copper.²¹

Consistent with the observation that AF contributes to fetal lung development, lipids that compose fetal lung surfactant are derived from apolipoproteins.²² Several apolipoproteins (Apo A1, Apo A2, Apo A4, and Apo H) are among the lipid transport molecules we found in AF. Other identified lipid transport proteins include phospholipid transfer protein, lipocalin, and α -2-glycoprotein. The identified hormone carriers T4-binding globulin and transcortin associate with thyroid hormone and steroid hormones, respectively. T4 deficiency during gestation is known to affect neural development.²³ Transcortin is believed to participate in glucocorticoid-induced maturation of fetal tissues.²⁴ As transcortin associates with glucocorticoids as well as progestins, it is possible that it may have a multifunctional involvement in fetal development.

3. Signal Transduction. We have identified both growth factors (IGF-II, growth hormone, latent TGF- β , and prolactin) and growth factor-binding proteins (IGFBP-1, IGFBP-2, and mac-25/IGFBP-7) in AF (Table 1 and Supporting Information Table 1). The identified growth factor-binding proteins form complexes with their respective growth factors and prolong their half-lives. A recent study has reported that levels of IGF-II, IGFBP-1, and IGFBP-3 affect birth weight.²⁵

4. Complement and Coagulation. We have detected a wide variety of complement and coagulation proteins (Table 1 and Supporting Information Table 1). Since all of the complement pathway proteins are known to be part of the acute-phase response, they may have a protective function for the developing fetus. As many of the proteins of the complement cascade are present in AF, it is possible that they may prevent intra-amniotic infection and the subsequent complications that can result. Several of the coagulation proteins we have identified are also involved in hemostasis and blood pressure regulation. We speculate that a balance of these molecules may be required for prevention of preeclampsia. Since proteases and protease inhibitors such as inter-alpha inhibitor H2 may be involved in development, it is possible that they are multifunctional.

5. Defense Response. In addition to the complement system, there are several immunoglobulin chains that are components

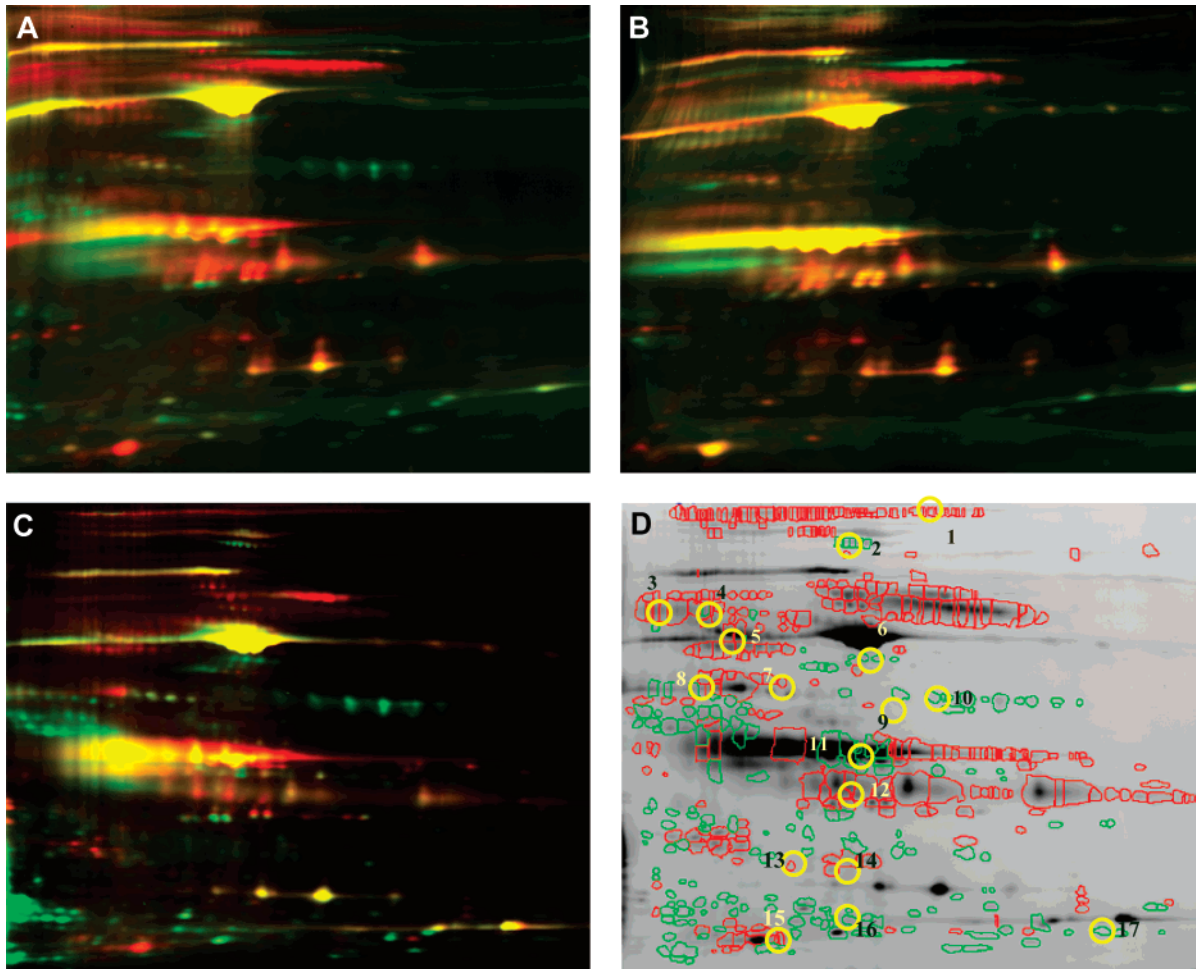


Figure 3. Protein expression in AF from each trimester was compared by 2D-DIGE analysis of immunodepleted AF samples. Equal amounts of protein (50 μ g) were labeled with 400 pmol Cy5 and Cy3. Samples for each experiment were first subjected to isoelectric focusing between pH 4 and 7. In the second dimension, the labeled proteins were separated by SDS-PAGE in an 8–20% Tris-glycine gradient. (A) First trimester (Cy3-green), term (Cy5-red); (B) second trimester (Cy3), term sample (Cy5); (C) first trimester (Cy3), second trimester sample (Cy5); (D) differential expression map comparing first and second trimester proteins (analysis of C). Outlined spots indicate a difference of at least 1.5 \times change of abundance between the samples (first and second trimester). Identified differentially abundant proteins (circled) are listed in Supporting Information Table 2.

of AF (Table 1 and Supporting Information Table 1). Presumably, these immunoglobulins can also provide protection against infection. There have been reports indicating the presence of antiviral IgGs in AF that may have a protective function.²⁶ Previous work by our group revealed several protein markers associated with intra-amniotic infection, including calgranulin A, calgranulin B, azurocidin, and L-plastin.²⁷ Some of the defense response proteins form complexes with other molecules. Examples include placental protein 14 that binds to fetuin and orosomucoid 1 that can associate with AMBP, fetuin, and complement 8 proteins. The association of these proteins suggests coordination of function to maintain fetal homeostasis. Thus, these protein complexes have the potential to be biomarkers in their own right, where altered associations may have physiological consequences.

6. Metabolism. A subset of the metabolic proteins of AF protects against oxidative stress and has implications for fetal development (Table 1 and Supporting Information Table 1). Among these proteins are glutathione peroxidase, superoxide dismutase, gamma glutamyltransferase 1 enzyme, and 1 gamma glutamyltransferase-like 4 proteins. These enzymes might protect the developing fetus against reactive oxygen species that

can cause developmental defects. Also, there is evidence that reactive oxygen species can harm the basement membrane and contribute to preterm PROM.²⁸ Among the set of proteins we have identified in AF that are involved in protein catabolism is ubiquitin. While the function of extracellular ubiquitin is unknown, the serum protein amyloid beta precursor, in conditions such as Alzheimer's and Down syndrome, is known to be covalently modified by a mutant form of ubiquitin.²⁹

Two-Dimensional Differential in-Gel Electrophoresis (2D-DIGE) Analysis. The protein composition of AF is known to change during the course of pregnancy.² We used differential dye labeling of proteins resolved by 2D gel electrophoresis (2D-DIGE) to determine the general differences in AF protein composition over time. Figure 3 shows three gels that compare protein expression in first trimester to term (Figure 3A), second trimester to term (Figure 3B), and first to second trimester (Figure 3C). The largest protein abundance changes appeared to be between the first and second trimesters (Figure 3C). We also analyzed the differential expression of the spots from each of the gels to obtain a quantitative comparison of the data. Image analysis of differential expression between Cy5- and Cy3-labeled proteins of the three gels (Figure 3) indicates that 572

of the 755 protein spots between the first and second trimesters, 447 of the 556 spots between the first trimester and term, and 430 of the 575 spots between second trimester and term are differentially present. This approach also identified a set of proteins consistently expressed in all three trimesters. Vitamin D binding protein, transthyretin, and several IGFBPs were present at high levels throughout gestation as was also observed by LC-MS/MS. These proteins may have utility as controls for the normalization of differentially abundant biomarkers in diagnostic multianalyte assays.

The changes seen in expression of the proteins we identified by 2D-DIGE is indicative of the role the molecules have in fetal development. Among the proteins that show increased level are Apo A1, Apo A2, IGFBPs, gamma glutamyl transferase 4, and Pigment epithelial derived factor (Supporting Information Table 2). The increase of apolipoproteins between the first and second trimesters may contribute to the fetal lung development that occurs at this stage.²² The increase in the level of gamma glutamyl transferase 4 is consistent with previously published data showing increases in metabolic enzymes during pregnancy.²

The relative amounts of kininogen, ceruloplasmin, angiotensinogen, alpha 2 HS glycoprotein, orosomucoid, and ubiquitin are decreased between the first and second trimesters. The significance of this change is not clear, as the function of these molecules is unknown. It is likely that the immediate expression of these proteins is most important during early fetal development that occurs during the first trimester. While several of these proteins are also abundant in serum, their differential presence in AF indicates that their expression is regulated throughout development. All of the identified differentially expressed proteins are listed in Supporting Information Table 2.

In this study, we have used in-gel digestion followed by tandem MS/MS and 2D-LC analysis to obtain a general protein profile of AF. While we have detected AF proteins that are low abundance, we do not rule out the possibility that ELISA or other immunoassays may detect more individual proteins. The most abundant of the AF proteins we identified by LC-MS/MS are Vitamin D binding proteins and IGFBPs. As IGFBP levels in AF correlate with birth weight, their presence in AF suggests a nutritive function of these proteins.²⁵ We speculate that Vitamin D binding protein may have a similar role in fetal development. We have detected dynamic alterations of the protein profile that occur between the first and second trimesters using 2D-DIGE. For the proteins that we have observed in both AF and CVF (Table 1), it is possible that amnion, placenta, chorion, or decidual cells may secrete these proteins into both the AF and CVF.⁹ The proteins and functional annotations we have listed here may be used for the development of a systemic profile that can be used to determine the status of the fetus.

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Supporting Information Available: Comprehensive lists of proteins found in AF and the identified differentially

abundant proteins. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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