# Comparative Proteomics of Umbilical Cord and Maternal Plasma

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## Introduction

Recent studies have identified that human umbilical cord blood has potential as a source of biomarkers and biologically active molecules (Correale et al. 2009, Choolani et al. 2009). In this report, the fetal cord plasma proteome was compared to the proteome of the mother's plasma, which was drawn immediately after delivery. Detecting the minor differential expression between these proteomes is complicated by the presence of high-abundance proteins. Employing ProteoMiner™ protein enrichment technology, we were able to significantly reduce high-abundance proteins, revealing the nuances of protein expression in cord and maternal plasma. A list of differentially expressed proteins was generated. These proteins may play a role in the remarkable bioactivities of cord blood.

## Methods

### Subjects and Sample Collection

All subject-related procedures complied with the instructions of the local ethics committee. Samples were collected from ten women during natural childbirth. The women were 20 to 30 years of age and all delivered successfully.

Blood was collected immediately after delivery from the umbilical vein and the maternal cubital vein into blue-topped Vacutainer tubes (Becton, Dickinson and Company) containing sodium citrate using standard technique. After collection, tubes were stored for 2 hr at room temperature prior to centrifugation. Centrifugation was carried out at room temperature in a horizontal rotor for 20 min at 1,500–1,800 x g. Plasma was aspirated without disturbing the cell layer and frozen at –20ºC prior to being transported. Transportation to the proteomics laboratory was carried out on ice without thawing. Upon arrival at the laboratory, plasma was aliquoted, and stored at –80ºC until analysis.

### High-Abundance Protein Depletion

Plasma samples were centrifuged at 10,000 x g for 10 min to sediment the precipitates. Then 900 µl of each sample was treated to deplete high-abundance proteins using a ProteoMiner protein enrichment kit (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions. The process yielded 300 µl of depleted fraction per sample.

### Sample Preparation for 2-D Electrophoresis

To prepare the sample for 2-D electrophoresis, 125 µl of depleted fraction was diluted eight-fold with deionized water and protein concentration was determined using the *RC DC*™ protein assay (Bio-Rad) according to the manufacturer's instructions. Seven dilutions of BSA from 0.1 to 1.48 mg/ml were used to prepare the standard curve.

The volume of the depleted fraction containing 1 mg of protein was treated using the ReadyPrep™ 2-D cleanup kit (Bio-Rad). Proteins were redissolved in a IEF sample buffer to a final concentration of 10 µg/µl.

### 2-D Electrophoresis

For all electrophoretic procedures, the PowerPac™ 1000 power supply (Bio-Rad) was used.

Solubilized protein samples (500 µg of total protein per sample) were applied on the acidic end of 18 cm, pH 5–8 ReadyStrip™ IPG strips (Bio-Rad) using the cup-loading method. The strips had been previously rehydrated in buffer containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 1.25% (w/v) Bio-Lyte® 3/10 ampholytes (Bio-Rad), and 65 mM DTT. The focusing conditions were as follows: 1 hr at 250 V, 5 hr linear voltage gradient from 250 to 3,500 V, and 50 kV-hr at 3,500 V. IEF was performed using the PROTEAN® IEF cell (Bio-Rad).

After completion of IEF, the strips were equilibrated for 12 min in buffer containing 6 M urea, 2% (w/v) SDS, 120 mM Tris-HCl (pH 8.8), 20% (w/v) glycerol, and 2.5% (w/v) DTT, then transferred to the same buffer with 365 mM acrylamide replacing DTT for an additional 10 min. Equilibrated IPG strips were then subjected to SDS-PAGE to resolve proteins in the second dimension.

Second dimension electrophoresis was performed using the PROTEAN® Plus Dodeca™ cell (Bio-Rad) at 65 V with 20 x 20 cm, 12% polyacrylamide slab gels (30% C, 2.6% T) freshly prepared according to standard protocol.

Gels were then placed in fixing solution (25% (v/v) isopropanol, 10% (v/v) acetic acid), and incubated overnight with gentle shaking.

The following day, gels were stained with Flamingo™ fluorescent gel stain (Bio-Rad) according to the manufacturer's protocol. Protein spots were visualized with a fluorescent imager using the following acquisition mode: a green (532 nm) excitation laser in combination with a 555 nm longpass emission filter, 450 V detector, 100 µm resolution.

#### Image Analysis

2-D gel images were analyzed with PDQuest™ software (Bio-Rad). Differentially expressed proteins were identified on 2-D maps of cord and maternal samples. Protein spot volume determination was performed by calculating the total signal intensity inside the specified region (in image units, ODs or counts) by multiplying the intensity by the area in $mm^2$.

#### Protein Identification

Protein spots were excised from the stained gels, washed twice with 50% (v/v) acetonitrile (ACN) in 0.1 M ammonium bicarbonate for 20 min at 56°C, and air dried. The dried gel pieces were swollen in a minimal volume of digestion buffer containing 50 mM ammonium bicarbonate and 15 mg/ml trypsin (sequencing grade), and incubated for 2 hr at 56°C or 18 hr at 36°C. Next, 5 µl of 0.5% (v/v) trifluoroacetic acid in 10% (v/v) ACN was added and mixed thoroughly. The extracted peptides were analyzed using an ultraflex MALDI-TOF mass spectrometer (Bruker Corporation). Peptide mass fingerprint spectra were obtained using an ultraflex TOF/TOF instrument (Bruker) in the reflectron TOF operation mode. The mass spectrometer was equipped with a pulsed UV laser, a two-stage gridless reflector, a 2 GHz digitizer, a LIFT-TOF/TOF unit to analyze fragment ions of selected peptide ions, and multichannel-plate detectors for linear and reflector mode measurements. All measurements were carried out in positive ionization mode using a reflector voltage of 20 kV. Database searches were performed using Mascot (www.matrixscience.com). The following search parameters were applied: NCBInr was used as the protein sequence database (http://ncbi.nlm.nih.gov) restricted to *Homo sapiens* taxon; a mass tolerance of 50 ppm and one incomplete cleavage were allowed; acetylation of the N-terminus, alkylation of cysteine by carbamidomethylation, and oxidation of methionine were considered as possible modifications. The Mascot-delivered probability based score was regarded as a quality parameter for the correct identification.

## Results

Originally, ten pairs of umbilical and maternal samples were collected. After 2-D electrophoresis, two pairs were eliminated from the study due to observed protein degradation. Therefore, this report is based on the comparison of eight pairs of maternal and umbilical plasma samples. Analyzed in duplicate, these samples yielded 32 large 2-D gels.

The effectiveness of ProteoMiner protein enrichment technology in the depletion of major proteins from cord plasma was confirmed. Figure 1 compares representative gels derived from a cord blood sample. Treatment with the ProteoMiner kit substantially improved our ability to identify and analyze differentially expressed proteins especially at medium and lower concentrations.

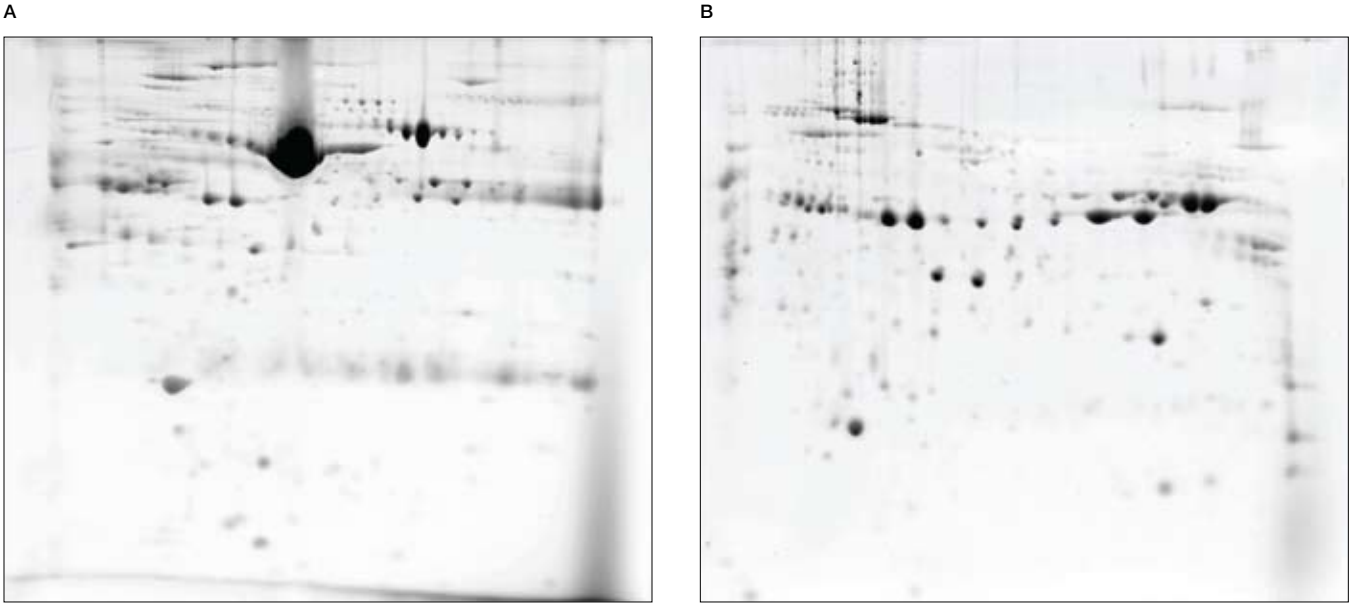


**Fig. 1. 2-D gel image of a representative cord blood plasma sample. A**, unfractionated plasma sample; **B**, plasma sample after treatment with ProteoMiner kit.

Each sample was analyzed in duplicate. Pairwise statistical analysis of spot volumes using PDQuest software identified several differentially expressed spots. These are shown on representative gels in Figure 2.

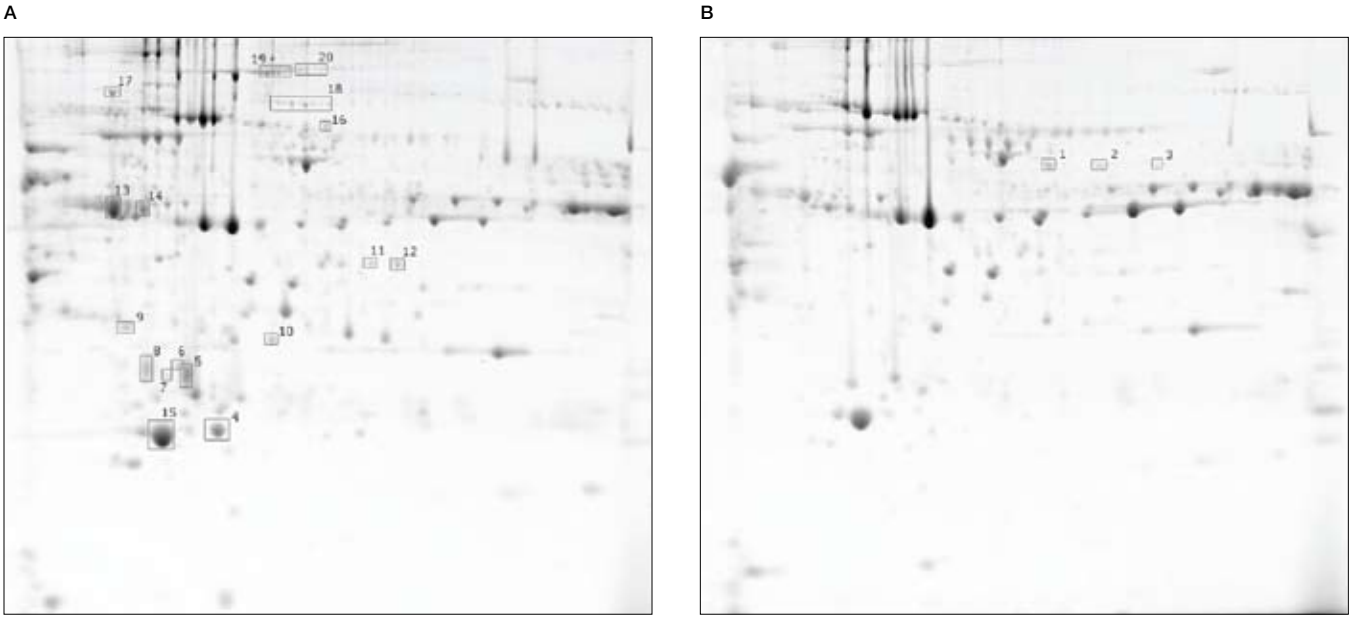


**Fig. 2. Identification of differentially expressed proteins between cord blood plasma and maternal plasma. A,** 2-D gel analysis of cord blood plasma proteins; **B,** 2-D gel analysis of corresponding maternal plasma proteins. Numbered boxes indicate differentially expressed proteins.

Differentially expressed proteins were identified using peptide mass fingerprinting by MALDI-TOF mass spectrometry. Protein identification results are summarized in Table 1.

**Table 1. Proteins differentially expressed between cord and maternal blood plasma.**

| Arbitrary ID Number | Protein | UniProt Accession Number | Relative Level in Cord Blood |
|---|---|---|---|
| 1–3 | Pregnancy-specific β-1-glycoprotein 4 | Q00888 | Reduced |
| 4 | Apolipoprotein A-I (1-242) | P02647 | Elevated |
| 5 | Complement C4B1 fragment | Q6U2M5 | Elevated |
| 6 | Actin, cytoplasmic 1 (β-actin) | P60709 | Elevated |
| 7 | Antithrombin-III | P01008 | Elevated |
| 8 | Not identified | — | Elevated |
| 9 | Clusterin | P10909 | Elevated |
| 10 | Apolipoprotein E (Apo-E) | P02649 | Elevated |
| 11–12 | Complement factor H-related protein 1 | Q03591 | Elevated |
| 13–14, 17 | Alpha-1-antitrypsin | P01009 | Elevated |
| 14* | Vitamin D-binding protein | P02774 | Elevated |
| 15 | Apolipoprotein A-I mature | P02647 | Elevated |
| 16* | Coagulation factor XIII B chain | P05160 | Elevated |
| 16* | Gelsolin | P06396 | Elevated |
| 18 | Fibrinogen alpha chain | P02671 | Elevated |
| 19–20 | Complement factor H | P08603 | Elevated |
| 20* | Alpha-2-macroglobulin | P01023 | Elevated |

* Mixture

## Conclusions

This study identified proteins that are differentially expressed between cord and maternal blood plasma. ProteoMiner protein enrichment technology was used to deplete high-abundance proteins, enabling a more detailed analysis of differential protein expression that would otherwise be impossible. The list of proteins generated provides a foundation for further study of the therapeutic potential of human umbilical cord blood.

## References

Choolani M et al. (2009). Proteomic technologies for prenatal diagnostics: Advances and challenges ahead. Expert Rev Proteomics 6, 87–101.

Correale M et al. (2009). Troponin in newborns and pediatric patients. Cardiovasc Hematol Agents Med Chem 7, 270–278.



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