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Maternal serum proteome changes between the first and third trimester of pregnancy in rural Southern Nepal

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Abstract

Characterization of normal changes in the serum proteome during pregnancy may enhance understanding of maternal physiology and lead to the development of new gestational biomarkers of health. In 23 Nepalese pregnant women who delivered at term two-dimensional difference in-gel electrophoresis (DIGE) was used to assess changes in relative protein abundance between paired serum samples collected in the first and third trimesters. One-hundred and forty-five of over 700 protein spots in DIGE gels (pI 4.2–6.8) exhibited nominally significant ($p < 0.05$) differences in abundance across trimesters. Additional filtering using a Bonferroni correction reduced the number of significant ($p < 0.00019$) spots to 61. Mass spectrometric analysis detected 38 proteins associated with gestational age, cytoskeletal remodeling, blood pressure regulation, lipid and nutrient transport, and inflammation. One new protein, pregnancy-specific β -glycoprotein 4 was detected. A follow-up isotope tagging for relative and absolute quantitation (iTRAQ) experiment of six mothers from the DIGE study revealed 111 proteins, of which 11 exhibited significant ($p < 0.05$) differences between trimesters. Four of these proteins: gelsolin, complement C1r subcomponent, α -1-acid glycoprotein, and α -1B-glycoprotein also changed in the DIGE analysis. Although not previously associated with normal pregnancy, gelsolin decreased in abundance by the third trimester ($p < 0.01$) in DIGE, iTRAQ and Western analyses. Changes in abundance of proteins in serum that are associated with syncytiotrophoblasts (gelsolin, pregnancy specific β -1 glycoprotein 1 and β -2-glycoprotein I) probably reflect dynamics of a placental proteome shed into

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Appendix. Supplemental Information A supplemental figure and a file containing methodological details and supporting data can be found online.

maternal circulation during pregnancy. Measurement of changes in the maternal serum proteome, when linked with birth outcomes, may yield biomarkers for tracking reproductive health in resource poor settings in future studies.

Keywords

pregnancy proteins; maternal serum proteomics; DIGE; iTRAQ; gelsolin; pregnancy-specific β -glycoprotein 4

1. Introduction

Efforts to characterize the human plasma proteome and discover clinically useful biomarkers have generated wide interest in the biomedical research community.[1] This pursuit is based on the hypothesis that distributions of individual or clustered protein concentrations in plasma or serum reflect or predict the status of tissues from which they originate. Cancer and cardiovascular disease biomarkers are extensively sought but remain elusive.[2, 3] Although most often used for disease detection, prognosis and treatment, the same mass spectrometry-based technologies can be used to study changes during normal developmental processes.[4] Pregnancy is a life stage of special interest because maternal and fetal metabolism undergo alterations that can be influenced by genetic, nutritional and environmental factors.

In parallel with improving antenatal care, a need exists to discover and develop gestational indicators to monitor pregnancy, detect complications and predict adverse outcomes. One approach is to harness the discovery potential of serum proteomics to identify biomarkers; however, only sparse data exist to describe normal changes of the maternal serum proteome during pregnancy. To our knowledge, no study has utilized difference in gel electrophoresis (DIGE) and isotope labeling for relative and absolute quantitation (iTRAQ) to prospectively characterize changes in the serum proteome associated with normal, advancing gestation. In this investigation, we applied DIGE and tandem 8-plex iTRAQ to survey changes in the maternal serum proteome between the first and third trimesters of term pregnancies. The study was carried out in a small cohort of women living at low-altitude in a chronically undernourished rural plains community, typical of Southern Nepal, where antenatal care coverage is low, more than 90% of births occur at home, and where low birth weight and infant mortality rates are excessive [5]. In such settings, it may be possible to identify proteomic biomarkers for tracking health, nutritional status and disease during pregnancy that could, in the future, guide care.

2. Methods

2.1. Study subjects

Subjects (n=24) delivering a term birth (37 weeks' gestation) were sampled from a sub-cohort of 740 pregnant women from whom blood had been collected and processed to serum while participating in a larger (n=4996) five-arm, placebo-controlled trial in Southern Nepal that assessed the impact of antenatal micronutrient supplementation on birth size. The design

and procedures are described elsewhere.[6] Briefly, in the first and third trimesters, at ~9 and ~32 weeks, respectively, maternal health was assessed during the trial by obtaining a weekly history of morbidity symptoms, measuring anthropometry and determining micronutrient status following phlebotomy. Indicators of preeclampsia (blood pressure, proteinuria) and gestational diabetes (urine glucose) were evaluated at each visit, both of which served as exclusion criteria.

For the present study, 8 women were selected from a placebo group and 16 women were sampled from an arm receiving a daily supplement of folic acid (400 µg). Within the former group, one serum pair was mismatched, leaving 7 women with valid paired sera in this group. One subject, in the latter group, who delivered a still birth was included in the analysis. Both groups received 1000 µg of vitamin A daily following their first trimester baseline assessment, in response to earlier findings in this setting indicating that risk of maternal mortality could be lowered with this supplement.[7] To maximize information obtainable in this initial study, subjects within each group were selected based on being high or low in baseline folate status and responsive or not in their serum folate concentration to the daily folic acid supplement. We collectively term the resulting six allocation, status and response strata (each of n=3 or 4) as nutrient subgroups, whose membership was taken into consideration during the statistical analysis (see below). Supplemental Table 1 provides anthropometric data characterizing study women for all groups combined and evidence of their comparability to the large trial population cohort from which they were selected.

2.1.1 Serum depletion—Serum samples were kept frozen on liquid nitrogen following processing in the field and during transport in vapor phase shippers, which were protective for up to 2 weeks, from Nepal to Baltimore, Maryland (USA). No evidence of thawing was observed upon transfer for storage at –80°C in Baltimore. A protease inhibitor (Complete™, Roche Diagnostics) was added immediately upon thawing. Serum (50 µL) was depleted of albumin, immunoglobulin IgA, IgG, anti-trypsin, transferrin and haptoglobin using a Multiple Affinity Removal Column (4.6 × 100 mm, Agilent Technologies) according to the manufacturer's instructions. The low abundance protein (LAP) fraction was concentrated by TCA/acetone precipitation.

2.1.2 DIGE labeling of low abundance serum proteins—Precipitated LAP samples were dissolved in labeling buffer (7 M urea /2 M thiourea /4% CHAPS /30 mMTris-HCl (pH 8.5)) to a uniform final protein concentration (1 mg/mL). Sample quality was evaluated by 1D SDS-PAGE to verify the multiple affinity removal column performed to the manufacturer's nominal specification of 98% target serum protein depletion. Non-saturation fluorescent dye labeling (cy-2, cy-3, cy-5) was performed according to the manufacturer's instructions (GE Healthcare). Equal amounts of LAP from all subjects and both trimesters were combined into a single pooled sample and labeled with cy-2 to prepare the internal standard. First and third trimester LAP fractions from each subject were respectively labeled with cy-3 and cy-5. Equal amounts (15 µg each) of cy-3 and cy-5 labeled protein from the same subject were combined with the cy-2-labelled internal standard for separation by IEF. Details regarding electrophoretic separations are provided in the Supplemental File.

Fluorescent gel images were acquired using a Typhoon fluorescence imager (500 V, GE Healthcare Systems).

2.1.3 Statistical analysis of DIGE images—Fluorescent gel images (pI 4.2–6.8) were analyzed using the Decyder software suite (GE Healthcare). Paired t-tests were initially performed without regard to nutrient subgroup using the Biological Variance Analysis (BVA) module in Decyder, to characterize differences in the log standardized spot abundance between trimesters and target spots for mass spectrometric analysis.

Differences between first and third trimester spot abundance were then recalculated taking nutrient subgroup differences into consideration. Linear mixed effects models were used to assess the effects of gestational age on paired differences in serum protein spot abundance across nutrient subgroups using a random effect for each. The p-values reported for the pregnancy effect are derived from testing the intercept (technically, a fixed effect) being equal to zero. That is, departures from the null hypothesis indicate systematic increases or decreases in spot abundance differences that cannot be explained by differences in nutrient subgroup status. Only spots with at least three observed paired values (i.e., in the first and third trimester) in at least four subgroups were considered for the analysis. False discovery rates were assessed via q-values derived from the p-value distribution across all spots.[8] Analyses were carried out in the open source statistical environment R (<http://cran.r-project.org/>).

2.1.4 Excision of 2D gel plugs and in-gel digestion of proteins—Independently of subgroup status, proteins in spots exhibiting differences in relative abundance ($p < 0.05$) and cy-2 spot volumes exceeding 4.5×10^4 fluorescence units were targeted for identification. Fifty-eight spots were manually excised from silver or Coomassie stained gels. After destaining, proteins were digested overnight at 37°C with trypsin (80 ng, Promega, V511A).[9]

2.1.5 LC-MS/MS identification of peptides in gel spots—Data dependent collision induced dissociation (CID) spectra were acquired using an LTQ mass spectrometer (Thermo-Fisher, USA). Details regarding MS instrument parameters, nano-LC separation and Mascot searching of the Swiss-Prot database are provided in the Supplemental File.

2.2. Preparation of serum proteins for tandem 8-plex iTRAQ analysis

To corroborate DIGE results, depleted first and third trimester serum pairs from six mothers were reanalyzed in a tandem 8-plex iTRAQ experiment (Applied Biosystems-MDS Sciex). The word “tandem” indicates that one pooled sample is included in both of the two separate LC-MS/MS runs that comprise two 8-plex iTRAQ analyses. This common sample enabled normalization of data from the separate iTRAQ analyses. Precipitated LAP was dissolved in TEAB (triethyl ammonium bicarbonate, 0.5 M) supplemented with 0.1% SDS to a final concentration of 0.5–1 mg LAP/mL. Protein concentration was measured using the Bradford assay and BSA as a calibration standard.

For all samples ($n=12$), LAP (30 µg) was distributed into separate tubes. Aliquots (6 µg) from each individual sample were also combined into a single pooled sample that was then

divided into two equal samples for labeling. Samples were diluted with TEAB to yield a uniform final protein concentration (0.33 mg/mL).

2.2.1 Protein digestion, iTRAQ labeling and strong cation exchange (SCX)

fractionation—Samples were reduced with tris-(2-carboxyethyl) phosphine, alkylated with methyl methanethiosulfonate (MMTS) and digested overnight at 37°C with trypsin at a substrate-to-enzyme ratio of 20:1. Solutions were concentrated by vacuum centrifugation to ~40 µL before labeling. Details of the sample labeling and SCX fractionation methods are provided in the Supplemental File.

2.2.2 LC-MS/MS analysis of SCX fractions—Dry SCX fractions were dissolved in 0.1% HCOOH. LC-MS/MS analysis was performed using an Eksigent nano-LC system interfaced with a Q-TOF mass spectrometer (QstarPulsar™, Applied Biosystems-MDS Sciex). Chromatographic and MS/MS data acquisition parameters are provided in the Supplemental File.

2.2.3 8-plexiTRAQ analysis—MS/MS spectra were searched against the Swiss-Prot database (SP 54.6, n=290484 protein sequences, n=107100015 amino acid residues) using the Protein Pilot™ (v2.0.1) and Paragon™ search algorithms (Applied Biosystems-MDS Sciex) with the following parameters: species = human, enzyme = trypsin (one missed cleavage), cysteine static modification with MMTS and iTRAQ (peptide labeled at N-terminal and lysine). Raw peptide identification results from Paragon™ searches were processed by the ProGroup algorithm. ProGroup uses peptide identification results to specify proteins that are detected for a given confidence threshold. The peptide confidence threshold cutoff was >99%. Protein Pilot™ was used to calculate normalized protein abundance metrics.

2.3. Western blot analysis

To provide cross-validation, two separate pools of non-depleted serum from the first and third trimesters were prepared for Western analysis by combining equal volumes from each subject (n=23). Pooled serum was diluted in Laemmli buffer, boiled and loaded (1.5 µL serum equivalents/well) on a 10% SDS-PAGE gel. Technical replicate samples (n=3) for each trimester were loaded in adjacent wells. Proteins were resolved at 100 V for 1 hr and subjected to semi-dry transfer to a nitrocellulose membrane (0.45 µm). After blocking with 5% non-fat dry milk (NFDM) in tris buffered saline (TBS), membranes were incubated for 1 hr with primary antibodies diluted 1:1000 in 5% NFDM-TBS. Antibodies were purchased from Abcam Inc. (Cambridge, MA, USA) and numbers following the proteins refer to the vendor's catalog number: Alpha-2-macroglobulin (A2M, ab36995), ceruloplasmin (ab51083), gelsolin (ab11081), apolipoprotein A-I (Apo A-I, ab52945), α-1-acid glycoprotein (ab440). Membranes were washed with TBS and incubated for 1 hr with the appropriate antibody-HRP conjugate diluted 1:3000 in 5% NFDM-TBS. Chemiluminescent images were acquired using a Versadoc Imaging system (BioRad, Hercules, CA, USA).

3. Results

3.1. DIGE analysis

Of approximately 700 initially detected gel spots (pI 4.2–6.8), 172 exhibited nominally significant ($p < 0.05$) paired differences in relative abundance between trimesters. More stringent filtering of spot data and re-calculation of p-values using mixed effects models taking nutrient subgroup status into consideration reduced the number of eligible detected spots to 260 and the number of significant ($p < 0.05$) spots to 145. Additional filtering using a Bonferroni correction for multiple comparisons reduced the number of significant ($p < 0.00019$) spots to 61. A (pI 4–7) silver stained DIGE gel image for one woman is presented in Figure 1. A reconstructed image of this gel is provided in Supplemental Figure 1 where color is used to indicate differentially abundant spots that are not otherwise apparent in Figure 1.

3.1.1 LC-MS/MS identification of proteins excised from 2D gels—Fifty-eight spots exhibiting significant paired differences in abundance were selected from locations shown in Figure 1 for LC-MS/MS analysis. The number of spots excised was limited to 58 by our ability to visually match preparative gel spots to the DIGE master image. Thirty-eight proteins were detected on the basis of identifying ($p < 0.05$) two or more unique peptides exhibiting Mascot search scores exceeding the identity score. Mascot search results and protein data base accession numbers of proteins are provided in the Supplemental File.

Table I contains spot identification numbers, the 34 proteins detected, paired average DIGE abundance ratios and the corresponding p- and q-scores taking nutrient subgroup status into consideration. Spots 100, 140–142, 145, 148 and 208 were excluded from the mixed effect analysis on the basis of an insufficient number of data points but proteins detected in them are reported in the Supplemental File. A DIGE paired ratio of two and negative two indicate a 2-fold increase and decrease in relative spot abundance, respectively. The individual log standardized abundance of spot 86 in Table I clearly exhibits relative decreases in the third trimester in Figure 2.

Pregnancy specific β -1-glycoprotein 4 (PSG4_human) was detected in spot 148 on the basis of identifying four different peptides exceeding the Mascot identity score. These data are the first mass spectrometric evidence for the existence of this protein. Hemopexin, A2M and heparin cofactor 2 were co-detected on the basis of identifying five, three and two peptides, respectively. Although spot 148 exhibited a 1.8-fold decrease ($p = 0.0003$) in abundance between trimesters, an insufficient number of data points nonetheless resulted in its exclusion from the statistical analysis that considered nutrient subgroup status.

Spot 000 in Figure 1, corresponding to the abundant green spot (~40 kDa, pI ~4.1) in Supplemental Figure 1, consistently appeared during preliminary analyses. Although the pI range 4.0–4.2 was not included in quantitative analyses, the green color indicates that the protein in this spot is more abundant in the first trimester. On the basis of peptide identifications, the protein in spot 000 was inferred to be α -1-acid glycoprotein 1 (A1AG1_human), an acute-phase reaction protein also known as orosomucoid-1.

3.2. Tandem 8-plex iTRAQ

One-hundred and eight proteins were identified of which 11, reflecting immune, enzymatic, transport and other functions, differed in abundance between trimesters ($p < 0.05$, paired t-tests) and are listed in Table II. Peptide identification and relative abundance iTRAQ data are provided in the Supplemental File.

3.3. Western blot analysis of non-depleted, pooled sera

Western blot analysis of non-depleted, pooled sera ($n=23$) from the first and third trimesters (Figure 3) detected trends in abundance for A2M, ceruloplasmin, gelsolin, Apo A-I and α -1-acid glycoprotein that were generally consistent with those measured by DIGE and iTRAQ.

4. Discussion

Gestational changes in the serum proteome were surveyed in a small sample of Nepalese pregnant women among a larger cohort that participated in a micronutrient supplementation trial. The study area is typical in demographic, nutritional and health characteristics to the southern plains of Nepal, where over 60% of the population resides, and the contiguous rural State of Bihar to the south. For this reason, the study area is one that resonates with a larger, generally under nourished and little researched rural region of Gangetic South Asia, and a site where nutrition and health intervention trials have been carried out over the past two decades to address inequities in nutrition and health. Thus, the proteomic findings are likely to reflect patterns that may exist in comparable populations in this rural region.

Our results suggest that pregnancy has a dramatic effect on the relative abundance of many proteins in the plasma proteome. Out of about 700 gel spots initially detected, 260 emerged following data filtering and 145 exhibited a nominal ($p < 0.05$) or more significant change between trimesters. Variation during gestation that we could attribute to nutrient subgroup membership, given subjects were participating in a larger supplementation trial, was accounted for using linear mixed effects models to avoid bias away from the null hypotheses related to gestation-related changes (i.e., minimizing type I errors) that could arise from nutrient-related effects. The analysis reveals strong pregnancy-related signals in the data: under the global null of no change in any spot, only 13 (5% of 260) compared to 145 spots would have been expected to yield a p-value less than 0.05. To address the multiple comparisons issue, we calculated false discovery rates and q-values, a preferred approach in high-throughput experiments (RNA expression, proteomics, etc) for gauging the number of false positives to be expected from a list of differences in protein abundance reported as significant. In contrast to studies in search of therapeutic targets or diagnostic markers, that set stringent significance levels and minimize family-wise error rates (for example via the Bonferroni method), and where the emphasis is to avoid all false positives, our present study has sought to identify an initial list of proteins that could be assessed in the future, a goal that allows some false positive findings to be considered acceptable. Remarkably, despite the limited sample size, 61 gel spots showed changes large enough to yield significant p-values even when we applied the Bonferroni correction to control the family-wise error rate ($p < 0.05/260 = 0.00019$).

An important feature of these data are that they reflect plasma proteome dynamics from a group of pregnant women reflecting the general health of a larger, rural population of pregnant women in Southern Nepal. Most of the 38 proteins detected in DIGE gel spots have been functionally associated with advancing gestational age, cytoskeletal remodeling, blood pressure regulation, lipid and nutrient transport, inflammation and blood coagulation. A previously unreported decrease in gelsolin in the third trimester was observed. Corroboration of this result by Western blot analyses using serum that was not depleted supports the interpretation that this feature of the DIGE data is not a serum immuno-depletion artifact. Interpretation of the DIGE data is complex because more than one protein was detected in 63% of MS-analyzed spots. However, the multiple confirmatory approaches (iTRAQ and Western) used to detect differentially abundant proteins can be coupled with some knowledge of physiology to draw inferences about changes occurring with advancing pregnancy.

4.1. Pregnancy zone protein

Pregnancy zone protein (PZP, 161 kDa, pI 5.9) is a metalloprotease inhibitor that participates in maintaining immuno-tolerance of the fetus. Plasma concentrations increase during gestation, reaching 1 mg/mL in late pregnancy. [15–18] PZP was detected in four spots (9, 10, 58, 199 in Table I) exhibiting up to 3.3-fold increases in abundance. However, PZP was co-detected with up to three other proteins (A2M, complement factor H, ceruloplasmin), leaving uncertainty about which protein(s) drive DIGE spot abundance changes.

Serum A2M concentration is reported to not change between the first and third trimesters, consistent with iTRAQ and Western blot data (Supplemental File).[15, 19] Although complement factor H plasma concentrations can widely vary (110–615 µg/mL), they are reported to be unchanged during normal pregnancy, consistent with iTRAQ data.[20] Ceruloplasmin serum concentrations increase from 2–3 mg/mL in the first trimester to 4 mg/mL in late pregnancy.[21] However, Western blot and iTRAQ analyses did not detect differences ($p < 0.05$) in ceruloplasmin. The late first trimester timing of the initial blood draw (~ 10 week' gestation) may have precluded detecting changes occurring earlier in pregnancy. PZP antibodies were not available to perform Western blotting, but iTRAQ analyses detected a mean 38% increase ($p < 0.059$) with advancing gestation. These data collectively indicate increased PZP is the most likely contributor to the increased abundance of spots (9, 10, 58, 199) in Table I.

4.2. Known pregnancy-associated proteins detected

Changes in abundance were measured for spots in which only one protein was detected. Blood concentrations of most of these proteins are known to change during pregnancy. For example, angiotensinogen (spot 101a, Table I) increased with gestational age, consistent with iTRAQ data (Table II) and published reports.[22] The abundant (200 mg/L serum) protein β -2-glycoprotein I was detected in three DIGE gel spots (131, 202 and 234, Table I). This protein, also known as apolipoprotein H, is expressed by placental syncytiotrophoblasts, increases in serum in the third trimester and has four N-glycosylation sites.[23, 24] Abundance increased with gestational age in spots 131 and 234, but decreased

in spot 202 (at higher pI), and no change was detected by iTRAQ analysis (Table II). This change in the distribution of β -2-glycoprotein I, with increasing abundance at lower pIs (spots 131 and 234), suggest increased N-glycosylation with gestational age. The iTRAQ analysis is less amenable to detecting changes in protein post-translational modification state and missed these subtle changes. Beta-2-glycoprotein was observed as three 2D-gel spots in one of the earliest serum proteome studies.[25] It is a major antigen in anti-phospholipid syndrome, an autoimmune disease associated with fetal loss.[26]

The decrease in α -1-acid glycoprotein 1 (spot 000, Figure 1, at <pI 4.2) in the third trimester was supported by Western and iTRAQ analyses and is consistent with reported gestational trends.[27] Western blot and iTRAQ analyses supported the increased abundance of Apo A-I (28 kDa, pI 5.28) observed in DIGE spot 152b. Apo A-I is a component of the high density lipoprotein complex, transports cholesterol and protects low density lipoprotein from oxidative stress. Serum levels are reported to increase up to 1.3-fold from the first to second trimester, and nearly 3-fold by the third trimester.[28] These data demonstrate the use of DIGE to detect expected gestational trends in relative protein abundance; however, the purpose of using DIGE is to find new trends and proteins.

4.3. Novel serum pregnancy-associated protein

Two proteins were detected for which there was either only transcriptional evidence of existence (pregnancy specific β -1 glycoprotein 4) or no information was available about normal differential abundance between the first and third trimesters (gelsolin).

Pregnancy specific β -1 glycoprotein 4 (PSG4) was detected in spot 148 (Table I and Supplemental File) on the basis of identifying four peptides exceeding the Mascot identity score to provide the first MS-based evidence for this protein beyond its transcript. Pregnancy specific β -glycoprotein (PSG) transcripts are produced by ten genes of unknown function for which little protein level data exist.[29–5 31] Transcripts are synthesized by placental syncytiotrophoblasts, appear early in pregnancy and are detected in maternal serum.[32, 33] Shed syncytiotrophoblasts enter maternal circulation and are a potential source of PSGs detected in proteomic studies.[34] While iTRAQ analysis did not detect PSG4, it did detect a 27% ($p < 0.005$) increase in PSG1 with increasing gestational age. This confirms the initial report of PSG1 detection in maternal serum.[13] PSG1 transcripts are elevated in placentas obtained from women with preeclampsia and PSG1 protein has been detected in amniotic fluid.[35, 36]

Gelsolin (83 kDa, pI 5.7) was the only protein detected in spot 86 (Table I and Figure 2) and was co-detected with coagulation factor XIII β chain in spots 190 and 242, all of which decreased 20–80% in abundance with gestational age. Gelsolin decreased by 72% in iTRAQ (Table II) and 56% in Western analyses (Figure 3, $p < 0.007$ for both), in support of the DIGE trend. Gelsolin exists in two forms: cytosolic and secreted.[37] The 5–9 peptides identified in spots 86, 190 and 242 are common to both; however, iTRAQ identified eight peptides of which one (PNSMVVEHPEFLK, Supplemental File) is unique to the secreted form. Although other aspects of its biochemistry are well known, normal gestational changes of serum gelsolin have not been reported.[38–40]

Gelsolin is involved in regulating Ca^{2+} channels in syncytiotrophoblasts covering the placental villous tree.[40, 41] It cleaves and caps actin filaments during cytoskeletal remodeling and muscle tissue is a major source of gelsolin (~200 $\mu\text{g/mL}$ plasma).[42, 43] Actin is released into circulation by trophoblasts.[44, 45] Gelsolin-actin complexes are rapidly cleared from circulation but serum gelsolin decreases during acute and chronic inflammation concomitant with increases in actin filaments. [46–50] Saturation of the actin-scavenger system produces angiopathic effects.[51, 52] Depressed plasma gelsolin in traumatic injury is prognostic of survival.[49, 53] Gelsolin is a biomarker of inflammation. [38, 40, 54]

Because pregnancy is a state of increased inflammation, during which placental and fetal cells are shed into circulation, serum gelsolin concentration might be expected to decrease with advancing gestational age. Decreased gelsolin abundance measured by DIGE, iTRAQ and Western blot might have resulted from the progressive loading of actin scavenging systems by the release of proteins from the placenta and fetus into maternal circulation with increasing gestational age.[55, 56]

Changes in the serum concentration of gelsolin during pregnancy may also be related to its participation in a recently suggested mechanism for regulating the bioactivity of lysophingolipids such as sphingosine 1-phosphate (S1P).[57] S1P stimulates uterine contraction and plays a physiologic role in gestation and parturition.[58, 59] Gelsolin contains a phosphatidylinositol 4,5-bisphosphate (PIP2) domain that binds lipid signaling molecules, including S1P.[57] S1P binding to gelsolin inhibits both actin severing (by gelsolin) and S1P-mediated cell activation, thus generating a potentially homeostatic relationship. If gelsolin becomes depleted in late pregnancy, the resulting increased S1P availability might contribute to a signal cascade resulting in uterine contraction.[60]

It is difficult to generally compare changes observed here by DIGE in normal pregnancies in Nepal to those in studies of Down syndrome, preeclampsia or HELLP syndrome.[10, 13, 14, 61] These other studies use case-control experimental designs and focus on the first two trimesters. For example, DIGE studies by Nagalla et al. found gelsolin to be 1.5-fold greater ($p < 0.04$) in mothers carrying fetuses with Down syndrome.[13] Using iTRAQ, Kolla et al. (2010) observed gelsolin to be less abundant ($p < 0.001$) in mothers carrying Down syndrome fetuses.[62] DIGE studies of preeclampsia report variable differences in gelsolin between cases and controls.[63] Complex physiologic responses resulting from hypertension and inflammation in preeclampsia may confound the detection of more subtle changes indicative of normal gestational development. However, many proteins (egapolipoproteins, A2M, PZP, hemopexin) detected in these studies were also detected in our work.

4.4. Conclusions

Conventional serum biomarker research is predicated on the detection of proteins released from tissues of varying status or origin to detect disease. The use of serum proteomics to discover gestational biomarkers is similarly predicated on the detection of proteins transported in maternal circulation that originate in the fetus, placenta and maternal tissues at different stages of normal development. The changes in abundance of serum proteins associated with syncytiotrophoblasts (gelsolin, pregnancy specific- β glycoproteins, β -2-

glycoprotein I and PZP) suggest our DIGE studies, to some degree, may reflect the normal dynamics of the maternal placental proteome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

A2M	α -2-macroglobulin
DIGE	difference in gel electrophoresis
HELLP	hemolysis, elevated liver enzymes and low platelet count
IEF	isoelectric focusing
iTRAQ	isotope tagging for relative and absolute quantification
LAP	low abundance protein
PZP	pregnancy zone protein

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Fig. 1. Peptides located at numbered positions in this silver stained 2D gel image were identified by LC-MS/MS to detect proteins in depleted maternal serum that exhibit differences in relative abundance measured by DIGE between the first and third trimesters of pregnancy. These arbitrary numbers are used to indicate proteins detected in the corresponding gel spots in Table I. A Precision Plus Protein Standard Ladder (BioRad 161-0363) was used.

Fig. 2.

The log standardized abundance of DIGE spot 86 (gelsolin) for 23 women, stratified by nutrient subgroup status, exhibits marked decreases in relative abundance in the third trimester. The designations “F” and “P” indicate membership in daily folate or placebo allocation groups as described in the methods. The designations “1” and “3” respectively indicate folate insufficiency (serum <7.8 n moles/L) or sufficiency (serum folate >22.5 n moles/L) at the time of blood sample collection, while the order of appearance indicates the trimester (first or third).

Protein	Mean Band Intensity Ratio (T3/T1)	Average band intensity difference (T3-T1) relative to avg. T1 band intensity	Mean (T3-T1) band intensity $\pm \sigma_{(n-1)}$ (n=3)	95% CI difference of means	p*
α-2-macroglobulin (A2M)					
	1.01	0.005	11 $\pm$ 378	-595 to 617	0.962
Ceruloplasmin					
	0.82	-0.179	-175 $\pm$ 145	-408 to 59	0.107
Gelsolin					
	0.54	-0.46	-183 $\pm$ 42	-250 to -115	0.002
Apolipoprotein A-I					
	2.06	1.057	242 $\pm$ 95	90 to 394	0.012
α-1-acid glycoprotein					
	0.75	-2.255	-419 $\pm$ 163	-250 to -115	0.002
* t-test on technical replicates of 1.5 μ L pooled (N=23) whole serum, 5 degrees of freedom.					
** Bands were not detected in the 1 st trimester (T1) sample. An average band intensity, equal to 0.5 x 3 $\sigma_{(n-1)}$, was used to perform a t-test.					

Fig. 3.

Three technical replicates of non-depleted serum (1.5 μ L serum per lane), respectively pooled (n=23) from the first and third trimester, were analyzed by Western blotting to detect differences in protein abundance. The average relative intensities of gelsolin, Apo A-I and α -1-acid glycoprotein are significantly different ($0.05 < p$, t-test) between trimesters. **A.)** A2M (~161 kDa). **B.)** ceruloplasmin (~120 kDa). **C.)** gelsolin (~83 kDa). **D.)** Apo A-I (~28 kDa). **E.)** α -1-acid glycoprotein (~22 kDa).

Table I
Maternal Serum Proteins Detected in Differentially Abundant DIGE Spots^a

Maternal serum proteins detected in DIGE gel spots exhibiting significant differential abundance between the first and third trimester determined using a mixed effect model. Protein database accession numbers, identified peptides and Mascot scores are provided in the Supplemental File.

Gel Spot Number	Protein Name (Number of peptides identified)	Relative Abundance (3 rd vs 1 st trimester)	p value (mixed effect model corrected)	q value
9	complement factor H (11), PZP (2), A2M (2)	3.0	6.61E-07	2.59E-06
10	complement factor H (12), PZP (5)	3.3	1.33E-07	9.11E-07
58	A2M (12), complement factor H (4), ceruloplasmin (3), PZP (2)	2.0	3.81E-06	1.05E-05
70	A2M (8), complement component C7 (2)	1.3	0.001314	0.001302
80	complement factor B (8), A2M (2)	1.3	0.770255	0.266381
86	gelsolin (9)	-1.8	1.40E-08	1.93E-07
88	serum amyloid P component (4), A2M (3), complement C1r (2)	-1.2	0.06277	0.034188
90	Inter- α -trypsin-inhibitor heavy chain H4 (12), afamin (7)	-2.5	4.73E-09	9.73E-08
91	inter- α -trypsin-inhibitor heavy chain H4 (14), afamin (8)	-1.7	1.12E-06	3.70E-06
96	A2M (8), complement factor B (5), serotransferrin (2)	1.5	0.006025	0.004675
101a	angiotensinogen (2)	2.4	2.46E-09	6.73E-08
101	serotransferrin (19), A2M (5)	5.0	0.798142	0.26954
116	pro-thrombin (2), anti-thrombin III (5), complement C3 (4)	1.6	0.249033	0.108942
131	β -2-glycoprotein 1 (3)	1.6	6.17E-05	9.96E-05
133b	transthyretin (2), β -2-glycoprotein 1 (2)	3.7	2.29E-07	1.11E-06
133	complement C3 (2), anti-thrombin III (2)	1.9	0.001055	0.001113
147	hemopexin (3)	-1.4	8.12E-06	2.09E-05
152b	Apo A-IV (18), Apo A-I (3), haptoglobin (4)	2.5	1.67E-08	1.95E-07
152	hemopexin (3), A2M (3)	-2.4	2.08E-09	6.73E-08
153	pigment epithelium derived factor (4), sex hormone binding globulin (2), complement factor H related protein 1 (2)	2.1	1.75E-07	1.03E-06
170	A2M (3), hemopexin (2)	-1.3	0.000969	0.001035
176	Apo A-I (7), tetranectin (2)	-2.0	0.063327	0.034264
178	Apo A-I (6), plasma retinol-binding protein (2)	-1.4	0.019808	0.013033
189	α -1B-glycoprotein (8)	-2.0	4.55E-05	7.79E-05
190	gelsolin (5), coagulation factor XIII β chain (3)	-1.4	0.000316	0.0004
191	hemopexin (3), heparin cofactor 2 (3)	1.5	0.000439	0.000523
193	hemopexin (6)	-1.9	2.12E-09	6.73E-08
198	complement C1r subcomponent (3)	-1.3	0.000182	0.000245
199	A2M (16), complement factor H (2), PZP (2)	1.5	7.37E-05	0.000112
200	A2M (16)	1.2	0.043152	0.024992
202	β -2-glycoprotein 1 (3)	-1.6	2.58E-06	7.57E-06
203	hemopexin (3)	1.8	9.96E-06	2.37E-05

Gel Spot Number	Protein Name (Number of peptides identified)	Relative Abundance (3 rd vs 1 st trimester)	p value (mixed effect model corrected)	q value
204	hemopexin (5)	−1.4	1.71E-05	3.56E-05
205	A2M (16), complement C3 (4), apo A-I (2)	−1.2	0.013752	0.009425
210b	transthyretin (6)	1.3	0.013494	0.009326
210	hemopexin (4), complement C3 (3)	−1.3	1.64E-06	5.17E-06
212	complement C3 (3), carboxypeptidase N catalytic chain (2)	−1.3	5.10E-06	1.35E-05
213	hemopexin (2), A2M (2)	−1.2	0.000558	0.000628
220	complement factor H (2)	1.9	0.004102	0.003374
222	complement factor H related protein 1 (4)	−1.3	0.023942	0.015383
224	ficolin-3 (4)	1.4	0.041195	0.0242
232	A2M (8)	1.3	0.004822	0.003927
234	β-2-glycoprotein 1 (6)	1.8	1.76E-07	1.03E-06
238	complement factor B (12), A2M (2)	1.3	0.000109	0.000155
239	complement factor B (12)	1.3	5.48E-05	9.01E-05
242	gelsolin (5), coagulation factor XIII β chain (2)	−1.2	0.000551	0.000628
244	inter-α-trypsin inhibitor family heavy chain H4 (5)	−2.7	4.92E-05	8.26E-05

^aProtein database accession numbers, identified peptides and Mascot scores are provided in the Supplemental File.

Table II
iTRAQ top 23 proteins modulated between trimesters I and III.^a

Differentially abundant proteins detected by tandem 8-plex iTRAQ. Protein database accession numbers, identified peptides and individual relative protein abundance data are in the Supplemental File.

Accession Number	Protein	Mean (T3–T1)	σ (n–1)	p
P11464	pregnancy-specific β -1-glycoprotein 1	0.75	0.39	0.005
P06396	gelsolin	–0.32	0.17	0.007
P05155	plasma protease C1 inhibitor	–0.18	0.10	0.008
P80108	phosphatidylinositol-glycan-specific phospholipase D	0.15	0.09	0.010
P04196	histidine-rich glycoprotein	–0.43	0.27	0.012
P00736	complement C1r subcomponent	–0.16	0.11	0.018
P04003	C4b-binding protein α chain	–0.26	0.20	0.025
P02649	apolipoprotein E	0.24	0.19	0.028
P02763	α -1-acid glycoprotein 1	–0.32	0.28	0.037
P51884	lumican	–0.15	0.13	0.040
P04217	α -1B glycoprotein	0.21	0.20	0.046
P20742	pregnancy zone protein	0.38	0.38	0.059
P01019	angiotensinogen	0.34	0.27	0.063
Q14520	hyaluronan-binding protein 2	0.13	0.14	0.066
P00748	coagulation factor XII	0.14	0.14	0.067
P36980	complement factor H-related protein 2	0.17	0.19	0.086
P02647	apolipoprotein A-I	0.30	0.35	0.089
P19652	α -1-acid glycoprotein 2	–0.28	0.33	0.090
P05452	tetranectin	–0.29	0.35	0.092
P02774	vitamin D-binding protein	0.28	0.34	0.095
P04278	sex hormone binding globulin	0.39	0.47	0.096
P01042	kininogen-1	0.15	0.18	0.097
P13671	complement component C6	–0.08	0.10	0.099

^aPeptides identification scores and relative abundance data are provided in the Supplemental File.