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Original Article

Altered Proteome Profiles in Maternal Plasma in Pregnancies With Fetal Growth Restriction

Haptoglobin α2 Isoform as a Potential Biomarker

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Abstract

Fetal growth restriction (FGR) affects 3–5% of pregnancies and is associated with increased perinatal morbidity and mortality. Currently, there is no reliable biochemical test to differentiate a pathological FGR from a nonpathological one. The objective of this study was to screen whole maternal plasma to identify differentially expressed relatively abundant proteins associated with FGR. We analyzed maternal plasma from FGR ($n = 28$) and healthy ($n = 22$) pregnancies using two-dimensional gel electrophoresis (2D-GE) followed by software image analysis. Three spots with molecular weight (M_r) 18 kDa corresponding to haptoglobin (hp) α2, as identified by LC-MS/MS and immunoblotting, showed differential expression patterns in FGR. The distribution of hp α2 variants in maternal plasma samples showed the hp α2 variant 1 was low in 72% of FGR, medium in 16%, whereas high in 12%. In comparison, hp α2 variant 1 was high in (41%) of controls, medium in 41%, and low in 18% of cases. Based on the software image analysis, the mean spot volume for hp α2 variant 1 was 0.12 (SD = 0.18) for FGR compared to 0.26 (SD = 0.19) for control ($p = 0.006$). Given that hp turnover is indicative of its maturation process and is traceable in plasma by its dominant/suppressed variants, we propose that hp α2 is an important potential target for evaluation of its clinical and pathophysiological role and as a diagnostic biomarker in FGR.

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Introduction

Fetal growth restriction (FGR) is a pregnancy condition where estimated fetal weight is below the 10th percentile expected for gestational age (GA) ***(1,2)***. The condition affects 3–5% of pregnancies, and is associated with high perinatal morbidity and mortality ***(3)***. FGR infants may also have an increased risk of adverse neurodevelopmental outcomes and of cardiovascular and metabolic diseases later in life ***(4,5)***. Currently, fetal size is routinely ascertained using imaging technology; however, the differentiation of normal, constitutionally small fetuses from those with pathologic FGR remains a clinical challenge. Identification of proteins that can be used as biomarkers is an antecedent step in the development of non-invasive diagnostic or prognostic tests for FGR. Discovery of a candidate biomarker protein or a group of proteins that are associated with the pathophysiology of FGR, should lead to the development of rapid detection and quantification methods, for example immunoassays ***(6,7)***, for possible clinical screening. The measurement of biomarkers combined with advanced ultrasonographic (US) biometry and fetal Doppler technology in a temporal manner will provide the ability to more accurately and precisely detect the presence of FGR.

Clinically significant FGR is caused by reduced fetal growth resulting from maternal conditions such as undernutrition, smoking, drugs, and prepregnancy or pregnancy diseases, that include chronic hypertension or preeclampsia ***(8)***. Although the etiology and pathophysiology of FGR may be varied ***(2)***, it is widely accepted that poor placental development and or placental disease is the major common factor that is associated with abnormal fetal growth ***(8,9)***. Once congenital infection and chromosomal or congenital anomalies have been ruled out, FGR is mostly a result of uteroplacental insufficiency ***(10)***. Proteomic analysis of maternal plasma may also provide us with better understanding of the pathophysiology underlying FGR.

Profiles of plasma proteins can illustrate changes owing to altered metabolism and/or disease process. They may have a causal relationship and/or indicate disease severity. Differential expression of proteins in blood plasma has been widely used to study various diseases ***(11–13)***. The significance of several plasma peptides in gestational diseases with respect to biomarker discovery, and the importance of proteomics in identifying these candidate proteins have been well denoted ***(14)***. Analysis of global plasma proteins is a challenge because of the complexity of plasma proteome's large dynamic range, chemical similarity of multiple protein species and in addition presence of major plasma proteins such as albumin and γ globulin. To overcome these difficulties, prefractionation and depletion is often employed to adequately quantify detectable spots. Because these strategies often lead to high variability, this study is focused on rapid screening of whole plasma using two-dimensional gel electrophoresis (2D-GE) as a proteomic approach to detect changes in plasma proteome profiles associated with FGR.

Materials and Methods

Materials

All chemicals for 2D-GE and LC-MS/MS analysis were of electrophoretic or analytical grade. The total plasma protein concentrations were measured using BCA Protein Assay Kit (Pierce Biotech Inc., Rockford, IL), following the microplate technique as outlined by the manufacturer. BSA was used as the standard. Replicates were analyzed in a Multiskan EX

microplate reader (Thermo Electron Corp., Vantaa, Finland).

Subjects

With approval from the University of Western Ontario, Health Sciences Research Ethics Board (HSREB) and written informed consent from participants, pregnant women were recruited at St. Joseph's Hospital, London, ON, Canada. Blood samples were collected prior to delivery from mothers who consented to participate in the study. **Table 1A** shows a summary of the characteristics of the patients studied, and the inclusion and exclusion criteria for subject recruitment. Gestational age (GA) was determined by certain last menstrual date of mothers or the first trimester US crown rump length. Congenital infection and chromosomal or congenital anomalies were ruled out. Clinical information abstracted from the antenatal records included standard tests for the evaluation of fetal health and risk factors as part of normal surveillance. The diagnosis of FGR group was based on last trimester estimated fetal weight as determined by fetal US biometry ***(15)*** and calculated using the Hadlock II formula. Birth weight percentiles were calculated based on their respective gender and GA using a standardized growth chart ***(16,17)***. Control group subjects consist of normal pregnancies without medical or obstetric problems and with fetuses within normal growth percentiles (>25th). Measurements less than the 10th percentile for GA at birth were confirmed to be growth restricted **(Table 1B)**. Placental insufficiency was determined by abnormal umbilical artery Doppler ***(18)***.

Maternal Plasma Samples

One to two milliliters of maternal venous blood from FGR and control subjects were collected in EDTA coated tubes. Blood was centrifuged (2000*g*, 10 min at 4°C) and plasma samples were saved in small aliquots at –70°C until analysis.

SDS Polyacrylamide Gel Electrophoresis (1D- and 2D-GE)

All protein separations on 1D and 2D gels were conducted using 1.5 mm 12% SDS polyacrylamide gels, run for 20 min at 80 V followed by approx 1 h 15 min at 120 V. Prestained Broad Range SDS molecular weight (M_r) marker (Bio-Rad Labs, Hercules, CA) was used for estimation of M_r. All relevant reagents for 2D-GE were purchased from Bio-Rad and used according to manufacturer's instructions.

First Dimension Isoelectric Focusing for 2D-GE

The isoelectric focusing (IEF) was performed using PROTEAN IEF cell with ReadyStrip™ immobilized pH gradient (IPG) strips, 7 cm, pH 3.0–10.0 nonlinear (NL), unless otherwise specified. For first dimension separation, maternal plasma (1.5 µL, ~90 µg protein) was made up to 125 µL with rehydration buffer (8 *M* urea, 2% CHAPS, 50 m*M* dithiothreitol [DTT], 0.2% Biolyte pH 3.0–10.0 ampholyte, 0.001% bromophenol blue). Prior to IEF, IPG strips were rehydrated overnight using active or passive rehydration. Proteins were separated using a programmed voltage gradient in steps; S1, 200 V, 100 Vh; S2, 500 V, 250 Vh; S3, 1000 V, 500 Vh; S4, 8000 V, 8000 Vh; S5 500 V (holding step), with rapid ramping and a maximum current of 50 µA/strip throughout. Electrofocused strips were stored at –80°C until used.

Second Dimension Separation for 2D-GE

Focused strips were incubated for 20 min in 2.5 mL equilibration buffer (Tris, pH 8.8 [50 m*M*], urea [6 *M*], SDS [2% w/v], and glycerol [30% v/v] containing DTT [1% w/v]), followed by another 20 min incubation with 2.5 mL equilibration buffer containing iodoacetamide

Table 1A
Study Group Characteristics

Subject characteristics[a]		FGR	Normal pregnancy	*p*-value
Total study subjects		*n* = 28	*n* = 22	
Abnormal Doppler		*n* = 15		
Normal Doppler		*n* = 7		
No Doppler		*n* = 6		
Mean maternal age (range 18 to 37 yr)		25.8 yr (SD = 6.2)	25.9 yr (SD = 4.6)	0.96
GA (range 23 to 40 wk)	>28 wk	25 (89.2%)	15 (68%)	0.084
	≤28 wk	3 (10.7%)	7 (31.8%)	
Mean placental weight (g)	28–36 wk	336.7 (SD = 40.9)	489.18 (SD = 137.1)	0.006
Mode of delivery	Vaginal	14 (50%)	16 (72.7%)	0.1
	Caesarian section	14 (50%)	6 (27.3%)	
Gender of newborn	Male	13 (46.4%)	18 (81.8%)	0.01
	Female	15 (53.6%)	4 (18.2%)	

Table 1B
Distribution of Percentile of the Newborn Based on Gestational Age in FGR Group

FGR Newborn percentile	<28 wk GA (n)	>28 wk GA (n)
<5th	1	6
5th–10th	3	18

[a]Inclusion and exclusion criteria: Singleton pregnancies with growth restricted fetus in North American population were included. The subjects with twins, premature ruptured membranes, abruption placenta, fetal congenital or genetic abnormalities, diabetes, thyroid disorder and chronic hypertensive disorders, chorioamnionitis, preeclampsia smokers, drug users, and subjects with malnutrition were excluded.

(2.5% w/v). The IPG strips were placed on the second dimensional gel using 0.5% low melting gel agarose in 1X Tris glycine SDS buffer with 0.003% bromophenol blue. Electrophoresis was performed on SDS gels with a narrow (7%) stacking gel. Fixation was for 30 min (10% methanol and 7% acetic acid) followed by staining overnight with SYPRO Ruby gel stain (Bio-Rad). Destaining was done for a minimum of 1.5 h using 10% methanol and 7% acetic acid solution.

Two D-GE Image Processing

The 2D gels were stained with SYPRO Ruby Red and the monochromatic 16-bit digital images were acquired using the Fluorochem 8800 imaging system (Alpha Innotech Corp. San Leandro, CA) employing ultraviolet light excitation with a SYPRO-500 filter integrated with imaging software. Acquisition of all images was performed under controlled conditions with consistent exposure times.

Phoretix 2D Expression Software Analysis

Phoretix 2D Expression 2005 software (Nonlinear Dynamics Ltd., Newcastle Upon Tyne, UK) was used for image analysis of 2D gels using normalization of the spots on replicate gels. Preliminary mapping of plasma protein spots on 2D gels was performed based on the published plasma map (SWISS 2D-GE database available at the

ExPASy (Expert Protein Analysis System) website (http://ca.expasy.org/ch2d).

Protein Identification Using LC-MS/MS

Peptide Sample Preparation

Specific spots containing proteins of interest were picked and manually excised for in-gel digestion. In brief, excised gel spots were transferred to siliconized tubes, destained, and washed. Proteins were reduced, alkylated, and then digested with sequencing grade trypsin (Promega, Madison, WI) overnight at 37°C. After extraction of peptides from the gel, the samples were dried in a vacuum centrifuge and dissolved in 0.2% formic acid ready for LC-MS/MS analysis.

LC-MS/MS

LC-MS/MS analysis was carried out on a Q-TOF Global Ultima mass spectrometer (Waters Micromass® MS; Waters Corp., Milford, MA) coupled with a Waters CapLC. The LC system consisted of a C18 analytical column (75 μm × 15 cm, 5 μm, LC Packings, Amsterdam, the Netherlands) and nano-ESI source. For the standard LC-MS/MS procedure, a gradient (solvent A, 0.2% formic acid in water, B, 0.2% formic acid in acetonotrile, 5% B to 90% B in 60 min) was used to elute the peptides. The data-dependent acquisition function was used to both detect and sequentially perform collision-induced dissociation (CID) on the multiply charged ions that satisfied the selection criteria. The mass spectrometer was operated in positive-ion mode with MS data acquisition range, 300 to 1900 and MS/MS data acquisition range, 50 to 1900. The collision energy (CE) for MS/MS experiments was determined by the charge state and/or by the m/z range of the precursor ion. The CE values were according to standard charge-state recognition and CE files recommended by Micromass (Waters Corp.). The MS to MS/MS switching was allowed for the four most abundant precursors in the survey experiment with a 30-s time period set for MS/MS data acquisition for each peptide. In some cases, a precise list of peptides was set so that specific precursor ions were targeted to undergo tandem MS preferentially. For the MS and MS/MS experiments, the time-of-flight (TOF) instrument was calibrated with an MS/MS spectrum of Glu-fibrinopeptide-b. Data were acquired using MassLynx 4.0 software (Waters Corp.).

Analysis of MS/MS Data

Data was further analyzed using MassLynx 4.0 software (Waters Corp.) and Mascot ***(19)*** for database search on Swiss-Prot. The mass tolerance for precursor and fragment ions was set to 1.2 and 0.1 Da, respectively. Carbamidomethylated cysteine residue was set as a variable modification. All identified peptides during Mascot or Peaks (Bioinformatics Solutions Inc. Waterloo, Canada) searching were verified by manual interpretation of the spectra.

Identification and Qualitative Evaluation of hp Variants by 2D Western Immunoblot Analysis

The identity of hp subtypes/subunits (α1, α2, and β) and qualitative assessment of their expression in select plasma samples was performed by 2D Western immunoblot analysis. An equal volume of maternal plasma (~45 μg protein) from a severe case of FGR and a GA matched control subject were compared using 7-cm IPG strips, pH 4.0–7.0 for 2D-GE. Proteins separated on the gels were transferred onto a PVDF membrane (0.45 μm) (Hoffmann-La Roche Ltd., Basel, Switzerland) using a semi-dry Transblot apparatus (Bio-Rad). The membrane was blocked with 5% non-fat dry milk in TBS (50 m*M* Tris, pH 7.5, 150 m*M* NaCl) for 2 h at room temperature, followed by incubation with the primary polyclonal rabbit anti-human hp antibody that recognizes all hp subtypes (Dako, Glostrup, Denmark)

(1:5000 in TBS containing 1% non-fat dry milk and 0.1% Tween-20) at 4°C for 3 h. Anti-rabbit IgG was used as a secondary antibody in 1:8000 dilution (TBS containing 5% non-fat dry milk) at room temperature for 1 h. The blot was rinsed in TBS containing 0.5% Tween-20, and proteins were visualized using ECL Western Blotting Detection System (GE Healthcare, Piscataway, NJ) according to manufacturer's instructions.

Identification and Semiquantitative Evaluation of hp Subtypes Using 1D Western Immunoblot Analysis

One-dimensional Western immunoblot analysis of maternal plasma was performed on five representative samples from FGR and three from the control group. The FGR subjects were the most severe cases with <3rd percentile newborn birth weight for GA, whereas controls were of appropriate for gestational age. The dilutions of the polyclonal rabbit, anti-human hp antibody (Dako) and the conditions for immunoblotting were the same as described earlier for 2D immunoblots. The plasma samples in equal volumes (~5 μg protein) were pretreated with 2-mercaptoethanol (final concentration 1%) and subjected to 1D-GE. The immunoblot consisted of 0.1 μg standard purified hp (GE Healthcare) as a reference/positive control. Optical densitometric analysis of bands corresponding to the three hp subtypes (α1, α2, and β) in respective bands on the 1D immunoblot was performed using Phoretix 1D software. The background subtraction, selection of lanes analyzed and the detection of specific bands for software quantification were all done manually. The quantification of 1D immunoblots was performed by densitometry using Phoretix 1D advanced v5.10 software (Nonlinear Dynamics Ltd.).

Two D-GE Image Pocessing and Phoretix 2D Expression Software Analysis

For quantification of intensities of the spots on 2D gels for the specified proteins, Phoretix 2D expression software was used to analyze replicate gels under identical conditions. The software includes automated spot detection, gel matching, background correction, and data normalization. For analysis in this study however, spots of interest were manually matched because of the complexity of the gel images. The background intensity for individual spots was based on the intensity in a radius that extended 45 pixels in all directions (mode of non-spot). The spot volume was denoted by the sum pixel intensity within a spot minus the background. Individual spot volumes were divided by the sum total of all spot volumes on a particular gel and multiplied by a constant (100). "Normalization of spot volumes" was carried out to correct for random error as well as inherent total protein variation, and was used for statistical comparison.

Statistical Analysis

Chi-square test was used to compare categorical variables between the FGR and control groups, or the Fisher's exact test where appropriate. The independent samples *t*-test was used to compare mean differences in continuous variables between the two groups. The Pearson correlation coefficient assessed the relationship between two continuous variables and scatter plots were produced to test for linearity. Statistical significance was determined at $p \leq 0.05$. Because the sample sizes based on GA grouping described in **Table 1A** were low in the respective groups, and the observations in each group did not follow a normal distribution, we employed a nonparametric procedure using the Mann-Whitney test.

Results

Clinical Data

The clinical characteristics of the subjects in this study are described in **Table 1A**,**B**. Pregnancies were categorized into two groups namely <28 wk or ≥28 wk gestation. The

controls were of appropriate for GA. Based on the uterine artery Doppler velocimetry >50% of subjects were diagnosed with placental insufficiency, and the majority were in the ≥28 wk GA group. The placental weights of FGR pregnancies (336.7 ± 40.9 g) were significantly lower than those of controls (489.2 ± 137.1 g) ($p = 0.006$).

Proteomic Analysis of Maternal Plasma From FGR and Normal Pregnancies Using 2D-GE

Initial evaluative studies showed that IEF with active rehydration provided a better resolution of proteins as compared to passive rehydration (data not shown). In addition, an improved resolution of proteins was attained on the 2D gels using IPG strips, pH 3.0–10.0 NL compared to linear that is currently not depicted. All subsequent 2D-GE analyses were thus performed using IPG strips pH 3.0–10.0 NL. A representative comparison of protein spots on 2D-GE maps of maternal plasma from GA matched FGR and control subjects is shown in **Fig. 1A (i)** and **(ii)**, respectively. Although IPG strips, pH 3.0–10.0 NL provided better resolution of plasma proteins, the 7-cm strips did not optimally separate all abundant plasma proteins, specifically, in the higher M_r range. High abundant proteins, such as albumin and IgG, obscured the identification and quantification of minor proteins in and around this zone. The current protocol however, allowed successful discrimination of protein quantities between control and FGR samples, specifically in the lower M_r region. Only well-separated and defined spots on the 2D gel images, quantifiable by software analysis, were further characterized. From the gel images, a set of five major visible spots in the lower M_r range, were prominent, labeled as 1–5 **(Fig. 1 [ii])**. Comparison with the plasma map (EXPASY) indicated spots 1–3 as hp α2 chain variants, spot 4 as transthyretin (TTR), and spot 5 as retinol binding protein (RBP).

The hp α2 in plasma from FGR pregnancies showed three different patterns of expression. In the first pattern, hp spots 1, 2, and 3 were near to, or below, the threshold of detection, as shown in **Fig. 1B (i)**, that was seen in 29% of FGR pregnancies. This pattern in controls was detected in 33% of pregnancies. In the second pattern, all three hp spots (1, 2, and 3) were of relatively high intensity, as shown in **Fig. 1B (ii)**, was most commonly observed in controls (52% of pregnancies) and present in only in a small number (13%) of FGR pregnancies. In the third pattern, only spot 1 was reduced in intensity, as shown in **Fig. 1B (iii)**, was most prevalent (67%) in the FGR pregnancies, and was not commonly seen in the controls (14% of pregnancies).

Shown in **Fig. 1C** is the correlation of the same protein between two gels run under identical conditions. The R-squared for the linear plot was 0.86 suggesting relatively low intergel variability.

Identification of Proteins

The identities of spots 1, 2, and 3 as variants of hp α2 chain, and spot 4 as TTR were made by LC MS/MS followed by Mascot searches **(Table 2)**. Although a low coverage (3–11%) for hp α2 variants and (28%) for TTR were obtained**,** the high quality of MS/MS spectra **(Fig. 2)** established the identity of these proteins in the select spots. No significant hits for other proteins were found in the region of these spots.

Hp α2 variant proteins identified by MS were confirmed by 2D immunoblot analysis **(Fig. 3A [i])**. Plasma from FGR and control subject showed variable intensities of protein species with M_r 16, 18, and 42 kDa, corresponding to α chains (α1 and α2) and a β chain of hp, respectively **(Fig. 3A [i, ii])**. Both hp α1 and α2 variants were reduced significantly in FGR **(Fig. 3A [i])** compared to controls **(Fig. 3A [ii])**. Immunoblotting corroborated the 2D-GE data with SYPRO Ruby stained gels **(Fig. 1A [i, ii])**. Additional spots (**a**, **b**, and **c**) were detected

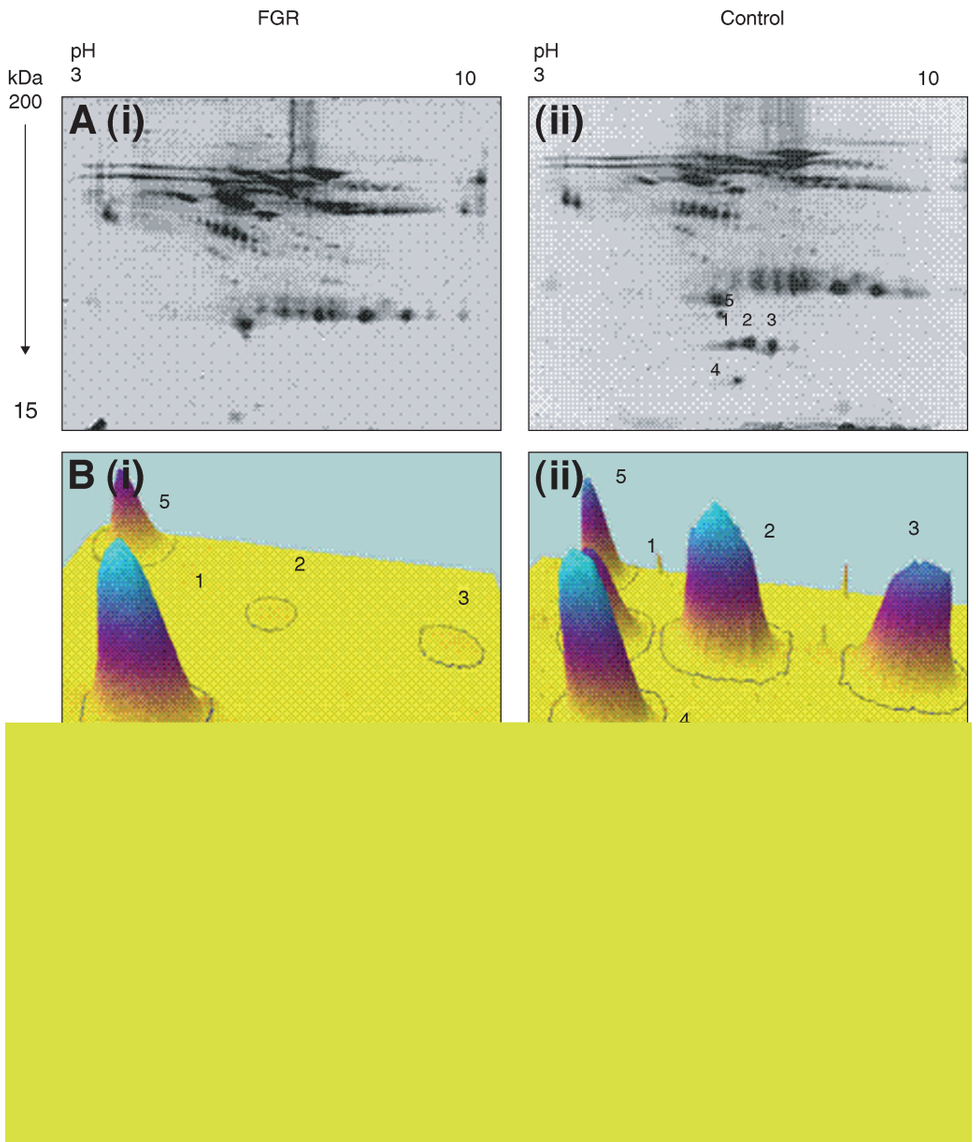

Fig. 1. **(A)** Representation of comparison of 2D-GE maps using maternal plasma from FGR **(i)** and control **(ii)** subjects with matched GA. For FGR gels **(i)** spots 1, 2, and 3 (hp α2 variants) were absent as compared with the control **(ii)**. Spot 4 (TTR) was moderately increased in FGR subjects while spot 5 (RBP) showed no detectable change in its expression. **(B)** A magnified, maximally contrasted three-dimensional rendering of 2D images for spots 1–5. The three different patterns primarily observed are represented **(B [i, ii, and iii])**. **(C)** Comparison of matched spots with normalized volumes detected on two 2D-GE maps of an identical maternal plasma sample prepared on separate occasions. The R squared for the trend-line was 0.86.

compared to 2D-GE. As shown in **Fig, 3A (ii)**, spot **(a)** corresponded to the fourth variant of hp α2, whereas spots **(b)** and **(c)** corresponded to two extra variants of hp α1 in the control plasma. The additional spots **(a)**, **(b)**, and **(c)** seen in the controls were absent in the FGR.

Table 2
Summary of Software Spot Quantation of 2-D Gels and MS/MS Identification on Specific Protein Spots From Maternal Plasma From FGR and Control Pregnancies.

Spot #	Protein ID	Observed		Coverage (%)	MS/MS ion & sequence tag		Swiss-Prot Mascot score	Mean volume (SD)		P value
		pI	Mr (κDa)		*m/z* (charge) a.a.	Sequence		FGR (*n*=28)	Control (*n*=22)	
1	hp α2	5.4	18	6	720.33 (2+) 119-131 448.22 (2+) 154-161	TEGDGVYTLNNEK NPANPVQR	145	0.12 (0.16)	0.26 (0.18)	0.006
2	hp α2	5.8	18	11	720.33 (2+) 119-131 448.22 (2+) 154-161 656.31 (2+) 60-71 581.76 (2+) 142-151	TEGDGVYTLNNEK NPANPVQR TEGDGVYTLNDK LPECEAVCGK	165	0.52 (0.55)	0.65 (0.47)	0.285
3	hp α2	6.1	18	3	656.31 (2+) 60-71	TEGDGVYTLNDK	85	0.29 (0.33)	0.38 (0.26)	0.450
4	TTR	5.6	15	28	336.70 (2+) 36-41 683.88 (2+) 42-54 787.40 (3+) 125-146	VLDAVR GSPAINVAVHVFR YTIAALLSPYSYST TAVVTNPK	156	0.63 (0.21)	0.44 (0.17)	0.001
5	RBP	5.2	20	*	*	*	*	0.24 (0.12)	0.19 (0.09)	0.157

*not identified.

Semiquantitative Evaluation of hp Variants Using 1D Immunoblotting

The specificity of the polyclonal antibody provided additional information on all hp subtypes. The analysis of plasma samples from FGR and control subjects **(Fig. 3B)** using anti-human hp antibody showed variable intensities of three bands with M_r 16, 18, and 42, corresponding to hp α1, α2, and β subtypes, respectively (**Fig. 3B**, lanes 1–8). The hp standard purified from pooled human plasma shown in lane 9 **(Fig. 3B)** as a positive control, exhibited high intensity of 18 and 42 kDa bands but a fainter band at 16 kDa.

Of the five samples representing the maternal plasma from FGR subjects in this study, three revealed hp α2 to be absent in FGR (**Fig. 3B**; lanes 2, 3, and 5). The band corresponding to hp α1 however, was absent in two out of five FGR samples (**Fig. 3B**; lane 1 and 4), whereas the intensity of this variant was relatively reduced in the remaining three samples (**Fig. 3B**; lanes 2, 3, and 5). Although the representative samples from controls (lanes 6–8) showed varied signals, the intensities for both hp α2 and hp α1 bands were relatively higher than the FGR group. Quantitation of mean hp band intensity using optical densitometry led to determine the reduction in hp. A 25.6% reduction was observed for hp β, 31.3% for hp α1 and 93.0% for hp α2 subtypes in FGR compared to the control. The data demonstrated a

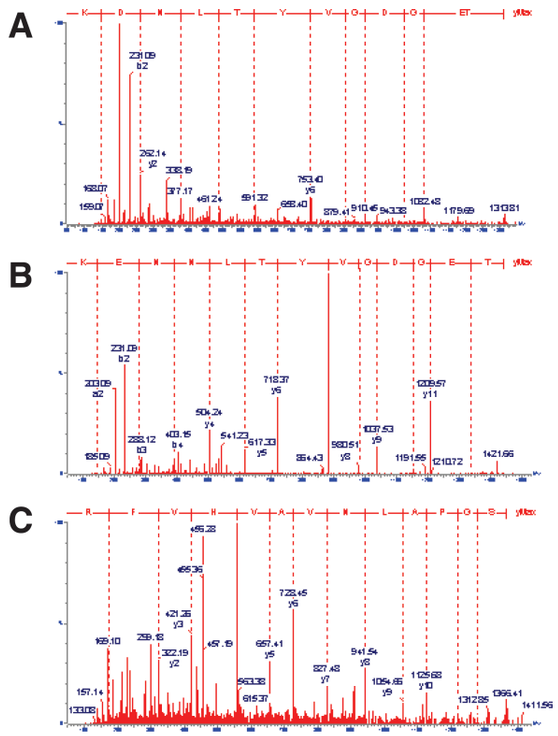

Fig. 2. LC-MS/MS spectra for the identification of the tryptic peptides sequences of hp α2 variants and TTR. MS/MS spectra of ion at m/z 656.31 (+2) **(A)** and 720.33 (2+) **(B)** from spot 1, 2, and 3 corresponding to hp α2; and ion at m/z 683.88 (+2) **(C)** from spot 5 corresponding to TTR.

moderate or inconclusive reduction of hp β and hp α1 expression, nonetheless, a distinct reduction in hp α2 in FGR.

Image Analysis Quantification of hp α2 Variants

Statistical evaluation of normalized spot volumes for spots 1–3, (hp α2 variants 1 to 3), spot 4 (TTR), and spot 5 (RBP) on 2D gels as represented in **Fig. 1A (i, ii)** in FGR and control subjects is shown in **Table 2**. We used RBP as a quantitative control for a better indication of validity of evaluation of spots on the gels. The mean spot intensity for hp variant 1 was significantly reduced in FGR compared to controls (p = 0.006), whereas TTR was higher in FGR compared to controls (p = 0.001). To further analyze the distribution of hp variant 1 between control and FGR, the distribution of normalized volumes was plotted based on their z score, *low* (<0), *medium* (0–1 to 1) and *high* (normal) (>1). **Figure 4** illustrates the intensity of hp α2 variant 1 to be *high* or *medium* in the majority (59%) of controls, in comparison to only 28% of FGR subjects. As shown in **Fig. 4**, the distribution of hp α2 variant 1 volumes for FGR were negatively skewed, with 72% of hp α2 variant 1 volumes being >1 SD below the overall mean compared to 40% in control.

Furthermore, we investigated the relationship between uterine artery Doppler flow velocimetry and the frequency of reduced hp α2 variant 1 in FGR accompanied by abnormal Doppler. Hp spot 1 expressed as normalized volumes were *low* to *medium* (53.3% with *low* and 27% with *medium*) in 80% of FGR accompanied by abnormal Doppler, as compared to only 20% of FGR with normal Doppler.

Discussion

Our results suggest that 2D-GE combined with LC-MS/MS is a feasible approach to identify changes in relatively abundant low molecular weight proteins in unfractionated plasma. Although 2D-GE analysis of whole plasma is a challenging and time consuming approach for comprehensive protein analysis, it allowed us to discover semiquantitative changes in the proteome of FGR pregnancies. New powerful image analysis software was crucial in the initial identification of changes in the plasma proteome and led us to determine that a specific variant of hp α2 chain was reduced in the maternal plasma of FGR, but not of controls.

FGR is associated with increased mortality of the fetus and increased morbidity in the neonatal period and childhood, as well as in adulthood ***(4)***. Ultrasound fetal biometry is used for the estimation of fetal weight in FGR

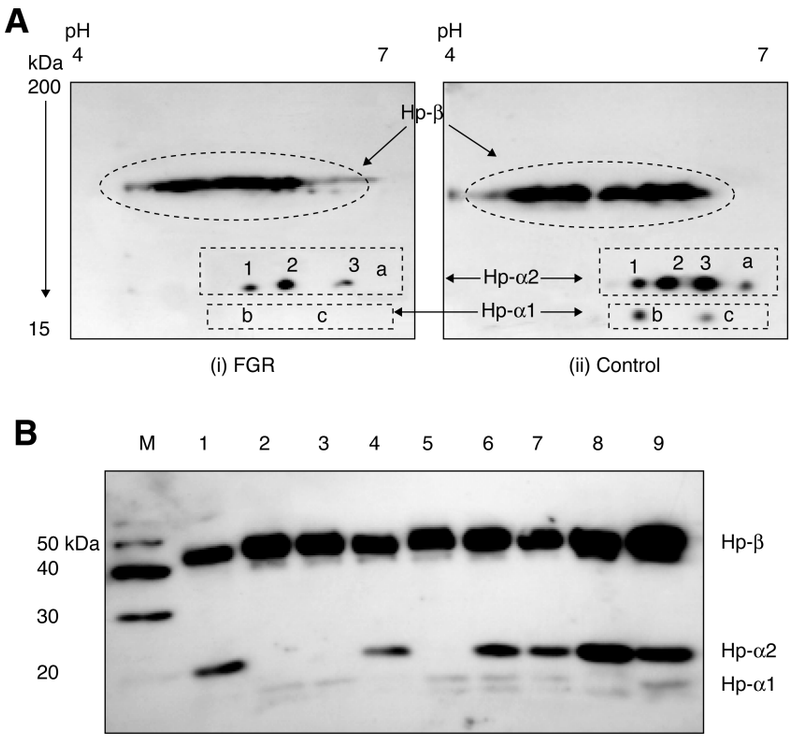

Fig. 3. **(A)** A comparative 2D Western immunoblot analysis using equal amount of maternal plasma protein from a FGR **(i)** and a GA matched control **(ii)**. Differentially expressed spots were detected and are identified numerically. **Figure 3A (i)** shows significantly reduced spots 1, 2, and 3 corresponding to hp α2 variants as compared to the control **(ii)**. An additional spot, denoted as **(a)** was detected in the control **(ii)** that represents the fourth variant of hp α2 (plasma map, EXPASY). Spots **(b)** and **(c)**, corresponding to hp α1 variants, were only detected in the control **(ii)** sample. **(B)** Semiquantitative evaluation of hp variants in maternal plasma using 1D immunoblot analysis. Representative maternal plasma samples from FGR (n = 5) and control (n = 3) subjects were selected. Lane "M" consists of M_r marker. Lanes 1–5 are maternal plasma from FGR subjects with low birth weight newborns (<5th percentile for GA); lanes 6–8 from the control subjects with normal birth weight infants. Equal amount of total protein was loaded in lanes 1–8. Lane 9 consists of 0.1 μg pure hp (Sigma) used as a positive control. Sample in lanes 4 and in 8 were common between 1D and 2D **(A)** immunoblot analysis.

fetuses ***(15–18,20)***, and umbilical artery Doppler ***(18)*** is used to distinguish FGR fetuses owing to uteroplacental insufficiency. Biophysical profile assessment is also used to determine fetal health and to provide indications for delivery. However, these tests reflect the condition of the fetus at the time of assessment and do not indicate the pathology or progression of the disease process that underlay FGR. Additional tests are, therefore, necessary to determine pathological FGR and its potential pathophysiology.

FGR is a multifactorial disease with diverse etiology and pathophysiology ***(2)***, thus changes in the proteome may not be universal. Correspondingly, to minimize heterogeneity and the effects of confounding factors in this study, the exclusion criteria used in the current selection of subjects ensured requisite health and lifestyle factors that also include

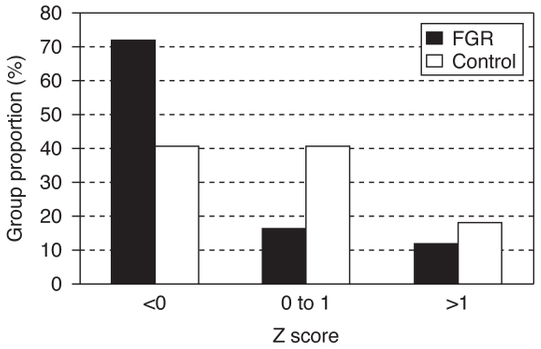

Fig. 4. Quantitative distribution of significantly different hp α2 variant I expression in maternal plasma from FGR and control subjects.

malnutrition. All subjects in this study were confirmed cases of FGR and the majority of them were a result of uteroplacental insufficiency ***(21)***. Because delivery was delayed if intervention was not necessary ***(2)***, most of the FGR pregnancies were ≥28 wk gestation. Poor placental growth has been shown to limit concurrent development of the fetus ***(21)*** and as expected, our data shows the placental weight of FGR pregnancies to be significantly smaller than the matching controls. These results concur with the significance of abnormal placental development in FGR pregnancies ***(22)***.

Based on their relationship to growth and development of the fetus, several peptides have been proposed as potential biomarkers ***(23)***. Prealbumin has been suggested to be associated with fetal defects and pregnancy complications ***(24)***. Leptin may reflect a generalized response to hypoxic stimuli ***(25)***, and free β-hCG and PAPP-A levels have been associated with general fetal abnormality ***(26)***. Elevated α-fetoprotein levels in the amniotic fluid through amniocentesis have been suggested to be of potential use in the detection of FGR ***(27)***, however, its association with adverse pregnancy outcome has not been confirmed ***(28)***. Novel biomarkers that may be more reliable and specific in their diagnostic and prognostic value in FGR remain to be identified.

Proteomic approaches offer new opportunities for potential biomarker discovery in pregnancy disorders ***(14)***. When searching for diagnostic markers in plasma, depleting most abundant protein(s) may be valuable, however, it could also lead to variability and nonspecific protein loss ***(29)***. Although whole plasma analysis renders the possibility of detecting the desired biomarkers challenging ***(30,31)***, it nonetheless mitigates the complications associated with plasma prefractionation ***(32)***. Protein expression profiling using direct MS may be superior for identifying a larger number of total proteins ***(33)***, however, because of the lack of complete amino acid sequence coverage for a protein using either MALDI-MS or LC-MS/MS, there has been limited success in using MS based approach to identify the isoform and posttranslational modification in plasma ***(34)***. 2D gels on the other hand, generate different spots that correspond to a change in overall protein charge and/or molecular weight. In the current study, this technique has allowed the identification of hp α2 as a differentially expressed protein in the plasma proteome, which was then confirmed by immunoblotting. This study was feasible because of the high abundance and adequately separate detectable spots for hp which were discriminated by software analysis. These spots have previously been recognized as structurally different species of hp α2 chain ***(11,35,36)***. Immunoblotting revealed additional hp α2 and hp α1 variants in the control plasma, which has been reported previously ***(35)***. Subsequent studies in a larger FGR population using an immunological assay with higher sensitivity and specificity will be crucial in determining the clinical utility of hp α2. Hp is a positive acute phase protein ***(37)*** that is variably expressed in maternal plasma during the course of pregnancy ***(38)***. Increased expression of serum hp α2 has been detected in ovarian cancer and is used as a biomarker for screening this disease ***(39,40)***. The specific

reduction in hp α2 variant, and possibly of hp α1 protein chain, may not reflect a generalized positive acute phase response in the FGR.

Our data is the first to demonstrate consistently, the absence and/or suppression of only a specific variant of hp α2 that migrates in the most acidic location of the three variants as identified on 2D gels. The differences in isoelectric points (pI) of hp α2 variants may be because of its posttranslational modification, such as deamidation of asparagines ***(41,42)***. Although the variability in intensity of hp α2 variant 1 has been linked with a carboxypeptidase that leads to different turnover rates of this variant in blood ***(35)***, the pathophysiologic basis for the suppression of specific hp variants in FGR is still unknown. The rates of deamidation of human proteins have been suggestive of a biologically relevant phenomenon that serves as molecular timer of biological events ***(43)***. It is possible that differences that lead to the separation or comigration of hp α2 variants in FGR may be attributed to subtle structural modifications of the protein that occurs during the disease process. The site and the cause/effect phenomenon for this modification in FGR is not known, however, it is likely that the placenta plays an important role in this process. Changes in posttranslational processing and not in the synthesis or secretion of hp have been shown in colon cancer ***(44)***.

Hp is an important protein in reproduction ***(45)*** and has been shown to be involved in the early signaling process during preimplantation ***(46)***. Hp is present in uterus and its role in endometrium has been suggested to be in protecting the fetus from a maternal allograft-like immune response ***(47)***. Hp is involved in HELLP syndrome, which is linked with hemolysis ***(48–50)***. It also suggested to be involved in placental angiogenesis ***(51–53)***. Because placental insufficiency is the major common factor in FGR, it is logical to suggest the role of hp in placental vessel remodeling. Given the contribution of hp to placental development ***(47)***, it will be important to determine if hp is a marker of placental insufficiency.

Clinically relevant biomarkers may however be of either causal or associative in nature ***(54)***. Based on our current findings, we have initiated a study to elucidate the mechanistic basis for hp suppression in the pathophysiology of FGR using human hepatoma cells as hepatic synthetic machinery in vitro ***(55,56)***. Our on-going study shows that hp biosynthesis in hepatoma cells is altered in a similar manner by hypoxia (unpublished data), a condition that is commonly associated with FGR.

In this study, we present evidence that expression of a specific variant of hp α2, that is a high abundance protein, is unique in FGR patients. This is the first report in our search toward discovery of biomarker(s) in FGR. However, for detection of larger number of plasma proteins, specifically of low molecular weight and low abundance, we propose to employ our recently established prefractionation strategy in a follow up study to further elucidate the FGR proteome. Furthermore, identification of proteins in the plasma by 2D-GE followed by MS and the high throughput 2D LC-MS has demonstrated that the majority of the identified protein set was unique to each method ***(57)*** therefore, for a comprehensive coverage, as suggested by Choi et al. it will be ideal to apply the two methods to achieve optimal results for the analysis of the plasma proteome.

The maternal blood samples in this study were collected from pregnant women just before delivery. The results from this study are consistent among the subjects, who in our assessment represent the clinical population with the problem, and, therefore, strongly show that we have been successful in identifying a potential biomarker in maternal plasma indicative of a late manifestation of FGR. It is recognized that a differential expression does not positively identify any protein as a biomarker of FGR, fulfillment of other criteria such as a

larger sample size, collection of maternal plasma prior to birth at different gestational ages and multifactorial analyses, will be essential before hp α2 could be used in a clinical setting. The fact that the change in hp α2 correlates with the Doppler outcomes suggests it can be a potential diagnostic and/or prognostic marker. If changes of hp precede the clinical diagnosis of FGR and placental insufficiency, inclusion of this protein in routine antenatal estimation of fetal weight and/or umbilical artery Doppler examination could be useful clinically.

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