Early onset preeclampsia is characterized by altered placental lipid metabolism and a premature increase in circulating FABP4

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Abstract

Preeclampsia is a pregnancy-associated disorder that manifests as a sudden increase in maternal blood pressure accompanied by proteinuria. Because the placenta is a key organ in preeclampsia, we used proteomic and lipidomic analyses to compare placentae from preeclamptic and gestational age matched control pregnancies. Fatty acid binding protein 4 (FABP4), enoyl-CoA dehydrogenase and delta-3,5-delta-2,4-dienoyl-CoA isomerase had altered abundance in preeclamptic placentae compared to controls. FABP4 placental protein and RNA and plasma levels were all increased in early-onset preeclampsia (prior to 28 weeks gestation) compared to controls (6-fold, 3.3-fold and 3.5-fold respectively). After 28 weeks, FABP4 protein in control placenta and plasma increased to the same concentrations as in preeclampsia. Total tetracosapentaenoic acid in preeclamptic placentae was decreased to 0.6 of control levels before 28 weeks. The data indicate a disruption of fatty acid transport and metabolism in the placenta in early onset preeclampsia that is reflected in the maternal plasma.

Introduction

Preeclampsia is a pregnancy associated disorder of unknown etiology and pathogenesis, but impaired placental development resulting in inadequate perfusion and function of the organ is an underlying characteristic of this syndrome[1]. This leads to exaggerated maternal circulatory response in which maternal endothelial cells become hypersensitive to circulating pressors causing vasoconstriction and hypoperfusion of multiple organs[2] that define the systemic inflammation and hypercoagulation that lead to the signs and symptoms of preeclampsia.

In preeclampsia, impaired placental development is caused by the failure of extravillous trophoblasts to convert maternal spiral arteries to high bore, flaccid vessels. The spiral artery remodeling is completed only by the early second trimester followed by a variable lag period of several

weeks of placental insufficiency before the clinical presentation of preeclampsia. Several studies have reported that preeclampsia can occur prior to 20 weeks gestation[3-5], but commonly the disorder presents itself between 22 weeks and term. Because complete removal of the placenta is the only way to reverse the symptoms of preeclampsia[6], it has been hypothesized that the placenta releases some unknown factor(s) into the maternal circulation that initiates the disorder of preeclampsia in the mother. Shed syncytiotrophoblast microparticles[7] and several placental proteins including cytokines[5,8], sFlt1[9] soluble endoglin[10] and PAPP-A[11] are increased in the maternal circulation in preeclampsia and may cause the maternal signs and symptoms of preeclampsia. However, it is not clear which of these proposed factors, if any, are causal to or associated with the development of preeclampsia.

Our approach to identifying placental factors released into the maternal circulation was to carry out unbiased proteomic and lipidomic analysis of the placenta, taking gestational age into account, with the goal of transfer this information the maternal plasma where they would serve as the biomarker(s) for potential diagnosis and/or prognosis. Our rationale was that the altered placental factors would be at higher concentration in the placenta than in the blood, and therefore easier to detect. Proteins and lipids detected in the placenta could then be examined in the maternal circulation in a targeted manner.

We found that the third trimester of gestation is associated with elevated placental fatty acid binding protein 4 (FABP4, aFABP, ALBP, aP2) in both control and preeclamptic pregnancies. However women presenting with early onset preeclampsia (delivery prior to 28 weeks gestation) exhibit a premature increase in placental FABP4 concentration. The changes in placental FABP4 are also reflected in maternal plasma. Further, the premature increase in FABP4 is accompanied by altered placental concentrations of enoyl-CoA hydratase, $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase and a tetracosapentaenoic acid indicating that abnormalities in placental fatty acid transport and metabolism are characteristic of early onset preeclampsia.

Results

Patients used in this study

Placental and plasma samples used in this study were obtained with consent from the Perinatal Biobank at St. Joseph's Hospital, London, Ontario. The diagnosis of preeclampsia was made based on the clinical criteria set by the American College of Obstetricians and Gynecologists and the Society of Obstetricians and Gynecologists of Canada: the maternal blood pressure exceeding a systolic pressure of 160 mm Hg and/or a diastolic pressure of 110 mm Hg, and at least one of the secondary inclusion criteria had to be met: proteinuria greater than 3 g in 24 hours or greater than 3+ by dipstick, a platelet count less than $100E^9$/L, intrauterine growth restriction with the EFW below the 5^{th} percentile, oliguria less than 500 ml in 24 hours, cerebral or vascular disturbances, severe edema or epigastric pain. No pregnancies were included in the sampling if they met any of the exclusion criteria: rupture of membrane greater than 18 hours, evidence of chorioamnionitis, fetal congenital or genetic anomalies or polyhydramnios. Control placentae were collected if they exceeded 22 weeks gestation and the pregnancy met neither the inclusion nor exclusion criteria. The characteristics of the patients used in this study are listed in Table 1.

It is now recognized that early onset and late onset preeclampsia are two qualitatively different syndromes[12,13]. The patients in preeclamptic and control groups were split into two groups based on their gestational age of 28 weeks completion.

Differential proteomic analysis of placentas from control and preeclamptic pregnancies

The chorionic villi, the site of interaction between the maternal bloodstream and the fetal part of the placenta, and the source of most of the known fetal exports to the maternal bloodstream, was chosen as the target tissue for proteomic analysis. 10 placentae were analyzed from each sample group: control and preeclamptic of gestation before and after 28 weeks (40 samples). Proteins were extracted

and separated by two dimensional gel electrophoresis (2D-Gel). 2D-Gels were stained and analyzed for spots that differed in intensity between preeclamptic and control placentae which were then excised and identified by LC-MS/MS. The following criteria were used to classify spots as different between control and preeclamptic samples: 1) the difference in protein abundance exceeded +/- 2-fold; 2) the spot of interest was present in all of the gels; and 3) the difference was statistically significant as determined by ANOVA ($p < 0.005$). Nine proteins identified by these criteria differed between control and preeclamptic placentae before 28 weeks gestation, and 3 proteins identified in placentae after 28 weeks gestation. Of the 9 differentially regulated proteins identified in the pre-28 week placentas, three were involved in fatty acid metabolism and transport: fatty acid binding protein 4(FABP4) was increased in preeclampsia by 3.7 fold compared to control levels (figures 1 and 2), while enoyl-CoA hydratase and $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase, were decreased to 0.57 and 0.29 of control values (figure 2). FABP4 is a cytosolic protein that is involved in intracellular fatty acid transport, enoyl-CoA hydratase participates in fatty acid β-oxidation, and $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase is involved in the oxidation of fatty acid with odd-numbered double bonds like oleic or linoleic acid[14]. The other differentially regulated proteins are not presently being disclosed pending further investigation.

Targeted analysis of FABP4 in placenta

FABP4 was chosen for further analysis because of a relatively large increase in abundance in preeclamptic placentas compared to controls prior to 28 weeks (3.7 fold, figure 2), and because of its major role in the symptoms of metabolic syndrome[15,16] and its potential as a biomarker for preeclampsia[17,18]. Western blots were carried out for placental FABP4 protein in control and preeclamptic patients across the range of gestational ages (figure 3, table 2). Placental FABP4 protein increased with increasing gestational age in control patients (Spearman's correlation, rho=0.7, p=5.5E-5). This is in contrast to preeclamptic patients where no correlation between gestational age and FABP4

protein was detected. A 2-way ANOVA confirmed the interaction between disease state and gestational age in predicting FABP4 levels (p=8.0E-4). When the patients were grouped by gestational age (pre- and post-28 weeks), preeclamptic patients had significantly elevated FABP4 in comparison to controls prior to 28 weeks, (table 2, t-test: p=1.2E-7), while no difference was detected after 28 weeks. Taken together, these data suggest that placental FABP4 protein becomes elevated in the course of a normal pregnancy, and that preeclampsia is associated with a premature elevation in placental FABP4.

Placental FABP4 RNA

To confirm the production of FABP4 in the placenta and it's increases with gestational age or preeclampsia, we used RT-PCR to measure placental FABP4 RNA. Prior to 28 weeks gestation, the preeclamptic placentae had a 3.3 fold increase in FABP4 RNA in comparison to controls (table 2, t-test, p=0.035). No correlation between gestational age and FABP4 RNA was detected in either control or preeclamptic patients, nor was there a difference in control FABP4 RNA before or after 28 weeks gestation.

Placental total fatty acid profile

Since increased FABP4 protein is induced by fatty acids[19,20], and the decrease in the fatty acid metabolizing enzymes enoyl-CoA hydratase and $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase could disrupt fatty acid metabolism, we examined the fatty acid profile of placentae from control and preeclamptic pregnancies. In addition, an altered fatty acid profile in maternal blood can be a potential diagnostic marker for preeclampsia. FABP4 expression is stimulated by free fatty acids, however, we found no significant differences in free fatty acids in control and preeclamptic placentae (data not shown). Therefore, we examined total placental fatty acids using base-hydrolysis and direct infusion high resolution mass spectrometry (DI-MS) analysis on a 12 T Fourier transform mass spectrometer focusing on very long

chain fatty acids (VLCFA) with carbon chain lengths of 20 or greater. This size range was chosen because previous studies of preeclampsia profiling shorter fatty acids in circulation have had mixed results[21-23].

Total fatty acids were analyzed from 3 preeclamptic and 4 control placentae <28 weeks gestation, and 4 each of control and preeclamptic placentae >28 weeks. 20 fatty acids were identified in each of the tested placentae, and of those, tetracosapentaenoic acid (FA24:5, m/z 357.2799) was significantly decreased in the preeclamptic placentae to 61% of the level in control placentae (0.97± 0.1 vs 1.57 ± 0.03 arbitrary units, t-test: p=0.0004). No significant differences were found between control and preeclamptic placentae after 28 weeks gestation.

Plasma FABP4

To determine if the increase in FABP4 in <28 weeks preeclamptic placentae was reflected in circulation, plasma FABP4 from women with preeclamptic and control pregnancies was assessed using an ELISA assay (Biovendor, figure 4). Prior to 28 weeks gestation, plasma from women with preeclamptic pregnancies contained more FABP4, than from controls (28.2 ± 8.9 vs 8.1 ± 3.1 ng/ml, t-test: p=3.13E-9). After 28 weeks gestation, plasma concentrations of FABP4 in women with preeclamptic and control pregnancies were not different (29.3 and 23.6 ng/mlrespectively). As with placental FABP4 protein, plasma FABP4 was increased in control patients after 28 weeks gestation in comparison to controls before 28 weeks (23.6 ± 9.1 vs 8.1 ± 3.1 ng/ml, t-test: p=2.1E-4). Thus, plasma FABP4 concentrations reflect the FABP4 levels in the placenta, suggesting that the placenta is a major source of circulating FABP4 in women during pregnancy.

Discussion:

The third trimester of a typical pregnancy is a period of hyperlipidemia in the maternal circulation, shows a 300% increase in plasma triglycerides and corresponding increases in very low density lipoproteins (VLDL) levels and enrichment of triglycerides in low density lipoproteins (LDL), a 50% increase in cholesterol and a 10-20% increase in free fatty acids. We have found that along with hyperlipidemia, the maternal circulation also showed an increase of FABP4 (2.9-fold to 23.6 ng/ml). In addition, in cases of early onset preeclampsia (<28 weeks gestation), we found that the maternal plasma exhibited a premature increase in FABP4 in comparison to controls (28.2 vs 8.1 ng/ml). In early onset preeclampsia, the placenta also exhibited a premature increase in FABP4 protein and RNA levels in comparison to control pregnancies, and a disruption of fatty acid metabolism characterized by decreased tetracosapentaenoic acid and decreased levels of enzymes responsible for unsaturated fatty acid metabolism.

FABP4, a cytosolic protein that plays an key role in insulin resistance[15], also promotes the production of inflammatory cytokines in macrophages[24] suggesting that it may play a causative role in the systemic inflammation associated with preeclampsia. This is consistent with the presence of elevated circulating FABP4 in other inflammatory states like metabolic syndrome[25-27] and gestational[28] and type 2 diabetes[29,30].

Increased maternal circulating FABP4 levels have recently been reported in preeclampsia[17,18]. In this study we have qualified that report by demonstrating that placental and circulating FABP4 were increased in both normal and preeclamptic pregnancies in the third trimester, and that elevated FABP4 relative to controls was observed only in early onset preeclampsia. The dependence of FABP4 levels on gestational age in control pregnancies has not been noted previously[17,18], possibly because the range of gestational ages for control patients used: 30.4 ± 2.1 in Fasshauer et al (mean ± SD) and 32 (29-40) (median (range)) in Shangguan et al only encompassed part of the age range used in this study. In

addition, the failure to group patients by gestational age in those studies may be reflected in the smaller changes in circulating FABP4 they report: 1.7 fold in Fasshauer et al and 1.9 fold in Shangguan et al compared to 3.5 fold reported here. The increase of FABP4 concentrations only in early onset preeclampsia suggests that this protein may serve as a potential predictor of this more severe form of preeclampsia, which is associated with low for gestational age birthweight[13,31] and a 20-fold increase in maternal mortality[32] although it accounts only for 5-20% of all cases of preeclampsia.

Our findings indicate that the FABP4 that we identified in the placenta by 2D gel and western blot originated in the placenta, and was not due to contamination with blood. Firstly, the placental FABP4 protein data are supported by RT-PCR data showing an increase in preeclamptic placentae prior to 28 weeks (table 2). Secondly, the 2D gels show FABP4 as a significant spot (figure 1) although this technique does not have sufficient dynamic range to visualize proteins of such low abundance in blood, in contrast to other methods such as immunoblotting or ELISA. FABP4 is well known to be expressed in adipocytes and macrophages, but it has been shown previously to be expressed in the placenta[33] and in primary cultured trophoblasts[34]. Since the plasma FABP4 levels mirrored the changes in placental FABP4 protein and RNA in that they were increased in early onset preeclampsia and with advanced gestational age, it is likely that the placenta is the source of the increase in circulating FABP4, although release by adipocytes cannot be ruled out[25,35,36].

Our data also revealed that a disruption of fatty acid metabolism accompanied the premature elevation of FABP4 in the placenta of patients with early onset preeclampsia. Dyslipedemia is a hallmark of preeclampsia, with a 100% increase in triglyceride levels and a 50% increase in free fatty acid in maternal circulation in comparison to the hyperlipidemia of normal pregnancies[37,38]. We found evidence of disrupted fatty acid metabolism in preeclamptic placentae, where enoyl-CoA hydratase and $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase, enzymes involved in fatty acid metabolism, were both decreased compared to controls, as well as tetracosapentaenoic acid. Significant metabolism of fatty acids occurs

in the placenta[39,40] and genetic deficiencies in fetal fatty acid metabolism can cause preeclampsia-related pregnancy complications[41]. Therefore, it is likely that the disruption of fatty acid metabolism observed in our study plays a causative role in preeclampsia. Enoyl-CoA hydratase catalyzes the hydrolysis of the double bond of trans-Δ^2-enoyl-CoA to form 3-hydroxylacyl-CoA in the fatty acid β-oxidation cycle. $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase catalyzes the second step of the alternative pathway for oxidation of unsaturated fatty acids with odd numbered doubled bonds, converting trans-Δ^3-cis-Δ^5-dienoyl-CoA to trans-$\Delta^{2,4}$-dienoyl-CoA[14]. Fatty acids with odd numbered double bonds (e.g. oleic or linoleic acid) can be metabolized by either the classical pathway or the alternative pathway. Interestingly, since enoyl-CoA hydratase is the first enzyme in the classical pathway and $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase is a member of the alternative pathway for metabolism of odd numbered unsaturations[42], both pathways for this metabolic process were downregulated in early onset preeclampsia. This could disrupt oxidation of odd-numbered unsaturated fatty acids (e.g. linoleic and oleic acids) and lead to the accumulation of trans-Δ^2-cis-Δ^5-dienoyl-CoA intermediates which may act as inhibitors of β-oxidation[43].

We also examined placental fatty acids to look for species that may interact with the identified proteins of interest. For example, an increase in PPARγ agonists like linoleic acid, prostaglandin J2 or oxidized fatty acids would have been consistent with the induction of FABP4 through peroxisome proliferator-activated receptor gamma (PPARγ)[36,44,45] but no known PPARγ agonists were found to be altered. However a decrease was detected in tetracosapentaenoic acid in preeclamptic placentas in comparison to controls before 28 weeks. Our analysis doesn't allow us to determine the regiochemistry of the fatty acids identified. A form of tetracosapentaenoic acid, C24:5n-3 is an intermediate in the synthesis of docosahexaenoic acid (C22:5n-3)[46], an important polyunsaturated fatty acid implicated in several physiological and pathological processes. Further, C24:5n-3 conversion to docosahexaenoic acid requires transport from the endoplasmic reticulum to the peroxisome, which, in hepatic tissue, is

mediated by liver FABP[47]. Thus it is plausible that the increased FABP4 in the preeclamptic placenta results in a decrease in tetracosapentaenoic acid levels by facilitating its metabolism. Previous studies of circulating lipid species have not identified any strong fatty acid indicators of preeclampsia; major 16-18 carbon fatty acids are increased in the same proportion as the overall fatty acid increase[21-23] and arachidonic acid may or may not[48] be increased more than other fatty acids. Further will be undertaken to develop a targeted method to quantify VLCFA, particularly tetracosapentaenoic acid in plasma as a potential biomarker for preeclampsia.

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***Table 1*:** Characteristics of patients used in this study.

Category	**< 28 weeks**		**> 28 weeks**	
	Control (n = 17)	PE (n = 16)	Control (n = 18)	PE (n = 16)
Non-twin Pregnancy (%)	82.35% +/- 39.30%	100%	83.33% +/- 38.35%	93.75% +/- 25%
Age (yrs)	24.76 +/- 5.84	28.5 +/- 5.88	27.71 +/- 5.75	26.13 +/- 5.55
First Pregnancy (%)	52.94% +/- 51.45%	62.5% +/- 50%	61.11% +/- 50.16%	62.5% +/- 50%
Gestational Age (wks)	26.07 +/- 1.54	25.98 +/- 1.3	33.23 +/- 1.95	33.68 +/- 2.07%
Severe PE (%)	N/A	75% +/- 44.72%	N/A	87.5% +/- 34.16%
PE + HELLP (%)	N/A	18.75% +/- 40.31%	N/A	12.5% +/- 34.16%
Antenatal Steroids (%)	70.59% +/- 46.97%	81.25% +/- 40.31%	55.56% +/- 51.13%	68.75% +/- 47.87%
Birth Weight* (g)	921.41 +/- 217.63	680.75 +/- 174.69	2247.4 +/- 568.70	1848.31 +/- 581.33
Placenta Weight** (g)	311.79 +/- 133.55	214.75 +/- 57.93	559.4 +/- 162.98	414.93 +/- 159.47***
Caesarean Section (%)	47.01% +/- 51.45%	62.5% +/- 50%	50% +/- 51.45%	68.75% +/- 47.87%

Value +/- Standard Deviation

* In twins pregnancy, the weight of baby A is chosen to calculate the mean

** The mean placental weight is for non-twin pregnancies only

*** The mean placenta weight of singleton pregnancy n = 14. One placenta weight is unknown.

Table 2. FABP4 protein and RNA levels in control or preeclamptic placentas.

	Western blot band density (arbitrary units)		RNA amount (ng/ng 18S RNA)	
	Ctrl	PE	Ctrl	PE
Under 28 weeks	619 (± 427[a], n=11)	3481 (± 1464, n=15)*	5.8 (± 6.5, n=5)	19.1 (± 7.8, n=4) †
Over 28 weeks	2434 (± 956, n=16)‡	3144 (± 1258, n=15)	9.3 (± 9.1, n=5)	10.3 (± 6.6, n=3)

[a] values are ± standard deviation

* different from control by t-test of square root-transformed data, p=1.5E-6

‡ different from under 28 weeks by t-test of square root-transformed data, p=1.9E-6

† different from control, p=0.035

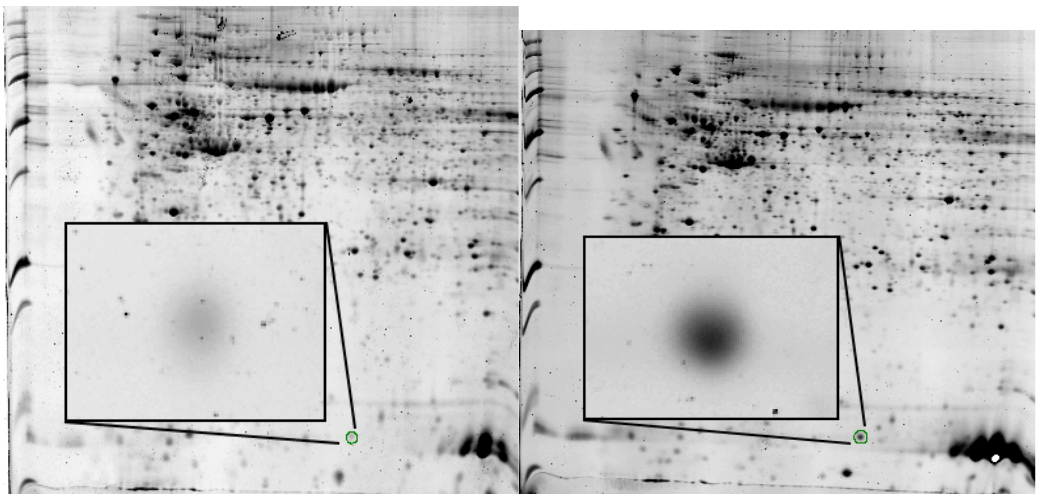

Figure 1. Representative 2D gels indicate that FABP4 (inset) is increased in a preeclamptic placenta (right) in comparison to a control sample (left).

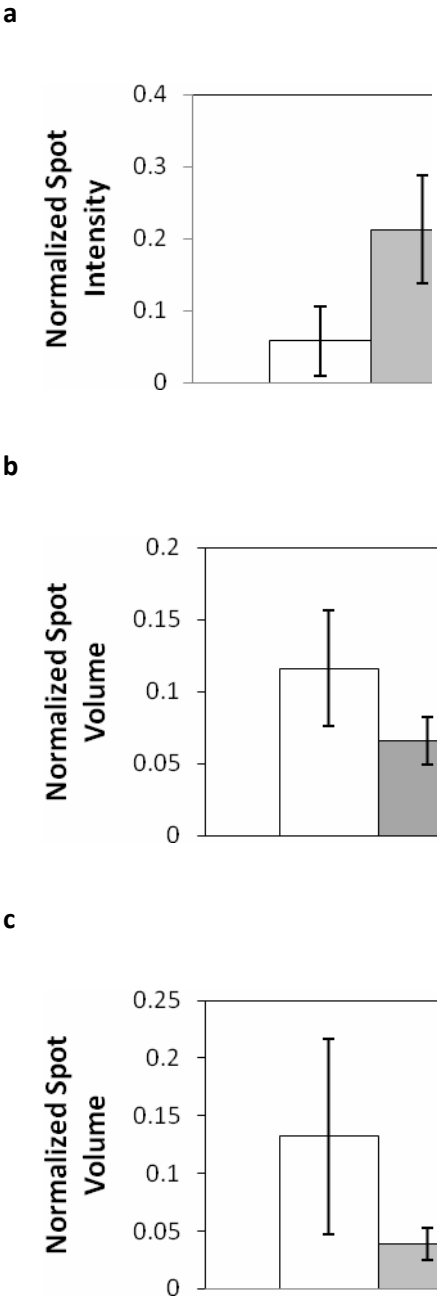

Figure 2. The average normalized intensities from replicate 2D gels indicate that (**a**) FABP4 is upregulated in the placenta from preeclamptic pregnancies (grey bars) in comparison to controls (white bars), while (**b**) enoyl-CoA hydratase and (**c**) $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase are decreased. n=10 for all samples and error bars represent standard deviations.

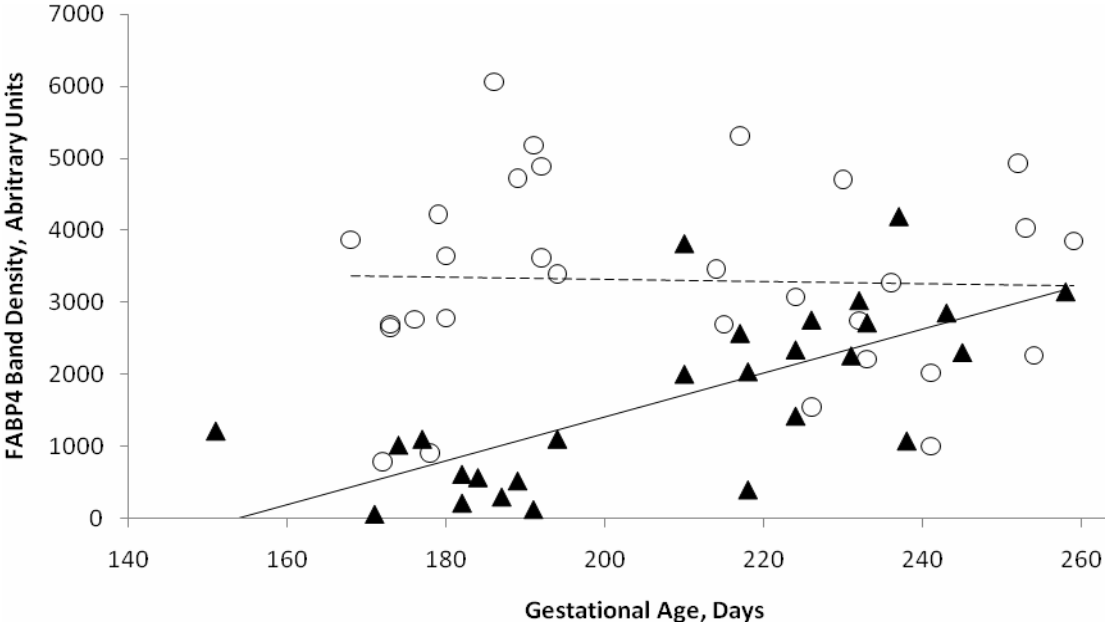

Figure 3. Placental FABP4 protein increases with gestational age in control patients, but not in preeclamptic patients. Western blots were used to determine the FABP4 protein concentrations in preeclamptic placentas (open circles) or in control placentas (filled triangles), normalized to actin. The solid line indicates the best fit for control placentas ($y=3.1x - 469.6$, $R^2=0.49$) and the dashed line indicates the best fit for preeclamptic placentas ($y=-0.1x +361.0$, $R^2=0.00$).

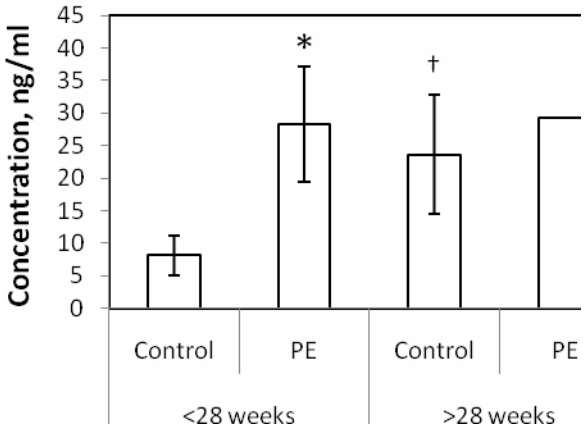

Figure 4. FABP4 protein plasma concentration as determined by ELISA. Plasma taken from women with preeclamptic or control pregnancies was subject to FABP4 analysis by ELISA assay. * indicates significantly different from control (p=3.1E-9, t-test), † indicates different from <28 weeks (p=2.1E-4, t-test) and error bars indicate the standard deviation. n=12 for control <28 weeks, n=17 for preeclamptic <28 weeks, n=4 for control >28 weeks, and n=2 for preeclamptic >28 weeks.

Materials and Methods

Sample Collection of the Chorionic Villous (CV)

Both preeclamptic and control placenta CV samples were collected immediately after delivery. 12 individual 1cm x 1cm tissue samples per placenta were excised using a grid. The basal plate decidua and the chorionic plate were surgically removed and each sample was snap frozen in liquid nitrogen. All twelve samples from each placenta were pooled and stored at -80°C.

Protein Extraction for 2D-Gel Electrophoresis

Pooled frozen CV was ground to a powder in liquid nitrogen. To 200 µg of powdered CV was added 435µL of PE extraction buffer [4% CHAPS, 50mM ammonium bicarbonate, 20mM DTT 20µL/mL Ettan protease inhibitor mix (GE Healthcare Bio-Sciences Corp. Piscataway, NJ), 5mM EDTA]. Protein extraction proceeded on ice with aspiration through a 30.5 gauge needle followed by addition of urea and thiourea to a final concentration of 7M urea, 2M thiourea. Samples were subsequently dialyzed in Slide-A-Lyzers (Pierce, Rockford, IL) against 1mM EDTA for 48 h. Protein concentrations of the dialyzed samples were determined by Bradford Assay (Bio-Rad, Hercules, CA).

2D-Gel Electrophoresis.

Differential protein expression between control and preeclamptic placentae was quantified by 2-dimensional gel electrophoresis using the Ettan IPGphor II isoelectric focusing apparatus (GE Healthcare) for the first dimension and the the PROTEAN mini-gel system (Bio-Rad, Hercules, CA) for the second dimension. Gels were stained with Sypro Ruby (Molecular Probes, Eugene OR) and imaged on a ProXPRESS Proteomic Imaging System (Perkin-Elmer, Boston, MA). Phoretix 2D Expressions gel documentation software (Non-Linear Dynamics, Newcastle UK) was used for spot quantification. Spots of interest were excised and identified using tryptic digestion and LC-MS/MS on a Micromass Q-tof

Ultima Global (Waters, Milford, MA) using standard methods. More detail on the 2D-gels and protein identification can be found in the supplementary methods.

FABP4 Analysis in Placental Samples by Immunoblotting.

To each pooled CV tissue sample 1ml of extraction buffer (2% SDS, 50mM Tris pH 6.8) was added for each 500 mg of CV tissue being extracted. The CV was homogenized using a PowerGen 700 tissue homogenizer (Fisher Scientific) followed by protein extraction in a boiling water bath for 10 minutes and aspiration through a 30.5 gauge needle. The samples were spun at 15,000g for 15 minutes at 15°C and the supernatant was aliquoted and stored at -80°C. Protein concentration was determined using a Bradford assay (Bio-Rad). 10 μg of each placental protein extract was taken for immunoblotting using rabbit anti-FABP4 (Cayman, Ann Arbor, MI). Actin was used as a loading control. More details can be found in the supplementary methods.

FABP4 Quantification in Plasma

Plasma was collected from patients prior to delivery. The FABP4 concentration was assayed using the human adipocyte fatty acid binding protein ELISA assay from Biovendor (Czech Republic) according to the manufacturers instructions.

Quantitative RT-PCR.

RNA was extracted from placenta samples and analyzed using a Taqman® RT-PCR kit (Applied Biosystems, Carlsbad, CA) according to the manufacturers instructions. FAPB4 RNA signal for each sample was normalized to a dilution series of pooled RNA, and then normalized to 18S RNA levels.

Lipid Analysis Using High Resolution Direct Infusion Mass Spectrometry.

Lipids were extracted from placenta samples using a variation of the Bligh-Dyer method[49] and hydrolyzed by heating in KOH followed by re-extraction, lyophilization and resuspension in in 1 ml of chloroform/methanol/water (7:2:1) with 0.1% NH_4OH (details in supplementary methods). Total fatty acids were analyzed using a Varian 920 triple quadrupole 12 Tesla Fourier transform (FT) mass

spectrometer (Varian, Palo Alto, CA). Samples were introduced into the mass spectrometer using flow injection at a flow rate of 0.5 ul/min and ionized in negative mode by electrospray ionization from a stainless steel needle (Proxeon, Cambridge MA). The inline triple-quadrupole was set to scan between m/z 330 and 600 with a resolution of ± m/z 30 to fill the FT-cell with ions of this mass range for maximum sensitivity and mass accuracy in the presence of more abundant shorter chain fatty acids. Transients were converted, using FT-Doc software version 9.1.20 (Varian), to m/z ratios which were compared to a list of possible lipid species generated from the Lipid MAPS database[50] with an error tolerance of ± 0.5 ppm. The ion intensities were normalized to the total ion intensity for each sample. Each sample was analyzed in duplicate, and the relative intensities were averaged for each pair.

Statistical Analysis

Statistical analysis of gel spot densities was carried out using Phoretix 2D Expressions gel documentation software (Non-Linear Dynamics). All other statistical analysis was carried out using R (Team, R.D.C. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, 2007). Prior to any t-tests, equality of variances was tested with an F-test and normality was tested using a Kolmogorov-Smirnoff test. The placental FABP4 protein and plasma FABP4 data were square root transformed prior to t-tests to equalize the variance. Placental free fatty acids were not tested for normality or variance because of the small number of samples. All reported values are mean ± standard deviation.

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