Original Article

Variation of fetuin-A in maternal and fetal serum during human parturition

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ABSTRACT

Background: parturition involves multiple signalling pathways and most advances in research underline the importance of fetal development and maturation in the timing of labour, especially the fetal pituitary-adrenal axis. But, there is currently no consensual hypothesis on all the physiological processes responsible for human parturition.

Methods: sixty low-risk pregnant women were enrolled in a prospective cohort. Maternal blood was sampled regularly during consultations each week in last trimester, at delivery and at postnatal consultation. Cord blood was collected at delivery. We used proteomic analysis to identify maternal blood biomarkers of potential interest, focusing on serum proteins from 39 WG in pregnancies to delivery and postpartum.

Results: among 56 peaks we identified variation in the N-terminal part of fetuin-A in maternal serum. Fetuin-A is a natural antagonist of TGF-beta and is able to bind calcium. We found a significant decrease in maternal serum fetuin-A in the days preceding delivery, independently of the mode of delivery. Also, there does not appear to be significant influence of the different fetal parameters (sex, Z-score) on maternal serum variations at delivery.

Conclusion: fetuin-A is described by the literature as a potential biomarker for organ dysfunction and metabolic syndrome disorders. The protein mineral homeostasis would be an interesting pathway to explore during pregnancy and particularly parturition.

Introduction

Human parturition involves multiple interactions and signaling pathways for cervical ripening and uterine contractions. Traditionally, three general contemporaneous theories describe labor initiation: functional loss of pregnancy maintenance factor, synthesis of factors that induce parturition and also signal for parturition beginning [1]. Most advances in research have come from animal models (pregnant sheep, rodents, rhesus monkeys) and underline the importance of fetal development and maturation in the timing of labor. One of the pathways most studied is the fetal pituitary-adrenal (FPA) axis. Maternal levels of free corticotropin-releasing hormone rise with placental mass during gestation and this increase correlates positively with the onset of parturition, probably stimulating fetal adrenal production of dehydroepiandrosterone sulfate and cortisol [2]. The role of the maturation of the fetal hypothalamic-pituitary-adrenal axis has recently been completed by the description of the central action of the nuclear factor kappa B (NF-kB), an important transcription factor, especially in inducing uterine contractions and cervical modifications [3,4]. As mentioned Menon R et al, human parturition seems to be the end of a counting device that measures maturation of the fetal organ systems and the production of hormones and other soluble mediators and that promotes inflammation and orchestrates an immune cascade to propagate signals across different uterine compartments [5].

New techniques, such as proteomic analysis, applied to maternal blood allow the identification of circulating molecules

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of potential interest, particularly serum proteins involved or reflecting parturition. Yuan et al used proteomic techniques to look at variations in small proteins (MW < 10 000 Da) in the placental blood of pregnant women undergoing different modes of delivery [6]. Most changes during labor occurred in proteins related to inflammation and immunomodulatory processes.

The main objectives of our prospective study were first to analyze **longitudinally** maternal serum peptides in low-risk pregnant women, from 39 weeks of gestation (WG) to delivery in physiological conditions, and second to investigate whether one or more of those peptides could be of interest in the days preceding delivery in correlation with observed maternal and fetal clinical parameters.

Materials and methods

### Clinical survey and collection of sera

Sixty low-risk pregnant women enrolled in a prospective cohort after giving written consent (Institutional Review Board (IRB) 00003835) were seen at regular intervals, weekly from 39 WG and then every 48 h at 41 WG in the Department of Obstetrics and Gynecology at the Beaujon teaching hospital (Assistance Publique – Hôpitaux de Paris). Maternal blood was sampled during consultations, at delivery and during a postnatal consultation, 6 to 8 weeks after childbirth. All patients had given birth at the latest by 42 WG. For patients at 41 WG and 5 days, we proposed induction of delivery using prostaglandins according to our usual clinical protocol. To compare fetus, we use a statistical tool based on Z-scores, also known as standardized scores [7]. The Z-score describes the deviation in standard deviation (SD) from the mean. It is recommended by the WHO (World Health Organization) and allows percentiles to be calculated with precision, especially for values close to extremes ($\pm$ 2 SD) [8].

In a two-step analysis, we first compared longitudinally maternal sera at different terms of gestation and identified the most significant peaks of peptides, and second we analyzed variations of those significant identified peaks and clinical events. Cord blood was also collected at delivery.

Proteomic analysis of maternal serum and peak identification (Graph 1)

Maternal serum proteomic profiles were established at the INSERM U773 laboratory using a serial analyzer SELDI 4000 (Surface-Enhanced Laser Desorption Ionization) followed by analysis by mass spectrometry (PCS4000; Bio-Rad). The analysis generated a molecular profile characterized by an inventory of the molecular masses of proteins. Each peak was characteristic of one protein, the relative abundance of which was reflected by the intensity of the peak. A longitudinal analysis of the proteomic profiles of 33 patient sera using Protein Chip Data Manager (Bio-Rad; array tool Biometric Research Branch, NCI) identified discriminating peaks. Molecular species were identified according to the following stages: selection of the samples where peaks were the most represented, enrichment and partial purification by four different strategies, analysis of the peptide imprint by means of dedicated programs (Profound, Mascot) and databases (Swiss-Prot, NCBI) after sequencing by mass spectrometry MS-MS to achieve identification. The different strategies were:

- Purification by depletion of 14 proteins (albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin fibrinogen, alpha-2-macroglobulin, alpha-1-acid glycoprotein, IgM, apolipoprotein AI, apolipoprotein AII, complement C3 and transthyretin) using an Agilent Human 14 Multiple Affinity Removal System Column (Agilent Technologies, USA), followed by desalting and
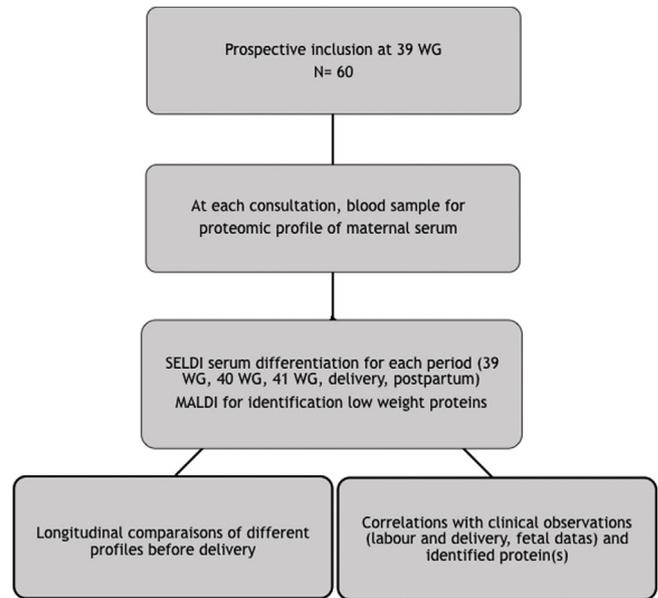
Graph 1. Summary of the proteomic analysis of maternal serums.

concentration by means of a Millipore ZipTip C18 (Millipore, Sigma Aldrich, USA) and fractionation by microLC. Fractions on MALDI-TOF were read and checked to detect peaks of interest.
- Depletion with the Proteoprep® Protein Precipitation Kit (Sigma Aldrich, USA), sample fractionation by microLC and then reading the fractions by MALDI-TOF.
- Depletion with the Proteoprep® Protein Precipitation Kit (Sigma Aldrich, USA) and Profinity IMAC Resin, uncharged (Bio-Rad, USA) to affine to divalent ions, followed by fractionation of the sample by microLC and reading of fractions with MALDI-TOF.
- Deposit samples on Weak Cation Exchange (WCX, Bio-Rad, USA) with different types of eluting solvents, followed by fractionation of the sample by microLC and reading of fractions.

Lower masses (2000 Da) were not taken into account because in this part of the spectrum the matrix interferes with detection. The usual protocol was a first detection with a signal-to-noise ratio of more than 5 and the second with a signal-to-noise ratio of more than 2. Spectral peaks were aligned to fit peaks to the same molecular weights in all profiles. Peaks within a mass window of $\pm$ 0.3% (given by the manufacturer) were considered identical.

Peaks detected with the software ProteinChip Data Manager Client 3.5 were included in the analysis if the coefficient of standardization was between 0.5 and 2.5. Mann-Whitney tests were performed using the BRB-ArrayTools software. Standard analyses were done with GraphPad Prism 7.0 (Windows version GraphPad Software, California, USA). A 2-day period was taken into account, because for the initial inclusions from 39 WG the terms were calculated from ultrasound exams done early in pregnancy.

A 1 μL sample was removed from every fraction, mixed with 5-mg/mL cyano-4-hydroxycinnamic acid in ACN/water/TFA (50/50/0.1) and analyzed by MALDI-MS on a MALDI-TOF/TOF Autoflex III in positive linear mode. Fractions containing an *m/z* relationship of interest lower than 4000 Da were analyzed in reflectron mode. The MS/MS spectrum obtained was submitted to the local search engine Mascot 2.3.0 (Matrix Science), using a tolerance for the precursor of 0.6 Da, an MS/MS tolerance of 0.5 Da, with the oxidation of methionine specified as a variable modification and no indicated enzyme.

Immunoassay of maternal serum

Sera were stored in PerinatCollection (ANR-10-EQPX-0010) until assay. Fetuin-A was quantified using an immunoassay (ab119594 Abcam ®).
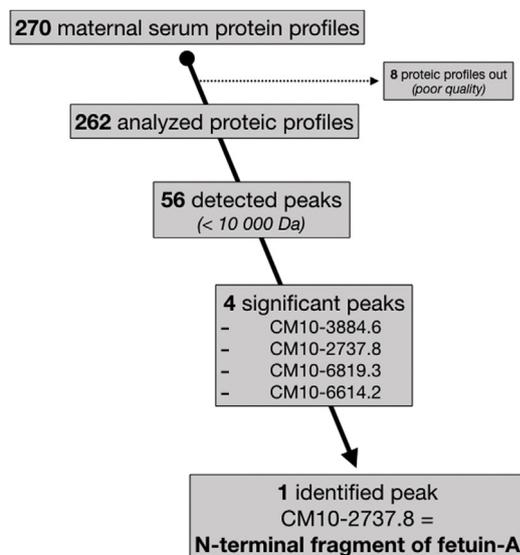
Results

After standardization of mass spectrometry analysis, 8 of 270 profiles were not retained. To check reproducibility and estimate variability in the tests, a serum control was randomly deposited both pins. The inter-chip coefficient of variability of the intensities of the peaks present in 100% of spectra was calculated from the controls. We had a 25% relative standard deviation for the small masses, which complies the manufacturer's standards. Fifty-six peaks were detected for the profiles of masses under 10 000 Da (Graph 2).

For identification of the peaks of interest according to term, we defined a cohort for which the time between delivery and inclusion (39 W G) was the longest, so as to have a longitudinal follow-up of these patients. Clinical data of our initial cohort are described in Table 1. Twenty-two patients (36.7%) were kept for at least ten days between inclusion and parturition, corresponding to the median in the initial cohort.

Under 10 000 Da, four peaks were observed: 3884.6 Da, 2737.8 Da, 6819.3 Da and 6614.2 Da. For the peak CM10-2737.8, after fragmentation of the peptide (TVVQPSVGAAAGPVVPPCP-GRIRHFKV) the Mascot search pointed to the N-terminal fragment of α-2-HS glycoprotein (AHSG, fetuin-A). Fetuin-A is a 55–59 kDa phosphorylated glycoprotein of two polypeptide chains. This N-terminal fragment has no known function; other fragments correspond to a carrier of crystals of apatite and a TGF-β binding site [9].

The second peak, CM10-3884.6, was highlighted in the linear analysis by MALDI, but unfortunately we were unable to fractionate it. Fiedler et al reported that this peak corresponds to a doubly charged fragment of platelet factor 4 (PF4, CXCL4), which is found in granules of platelets and could be released during centrifugation of maternal serum samples as a bias [10].

The peaks at 6819.3 Da and 6614.2 Da were two components of apolipoprotein C1, a lipoprotein produced primarily in the liver and found in plasma. It has an important role in the exchange of esterified cholesterol between lipoproteins [11].

Table 1
Maternal and fetal characteristics (N = 60).

Maternal Characteristics	
- Age (years, Median, min – max)	30.5 y (19 – 41)
- Parity	2 (1 – 6)
- BMI at end of pregnancy (Median, min – max)	28 (22.3 – 41.6)
- GA at delivery (WG, median, min-max)	40.6 WG (39.3 – 42)
- Vaginal delivery (%)	91.66% (5 C-sections)
- Induction of parturition (%)	23.33%
Fetal Characteristics	
- birth weight (grams, mean ±SD)	3441g ± 399
- Apgar 10/10	100%

To investigate the peak CM10-2737.8, identified as a fragment of fetuin-A, we measured maternal serum fetuin-A (mSF-A) using an ELISA in our entire cohort, even in cord blood. The kinetics of this fragment in the days before the delivery made us continues the investigations. Levels ranged from 15.3 ± 6.46 ng/mL to 10.6 ± 4.24 ng/mL at delivery. We established that there was a significant decrease in the days preceding delivery (Graph 3). At delivery, there was no observed difference between induced or natural parturition and vaginal delivery or cesarean section.

At delivery, despite we observed in our cohort significant higher Z-scores for males than females (Table 2, p = 0.026), there was not difference for concentrations of fetuin-A in blood cord between males and females. Serum concentrations of fetuin-A were distributed according to fetal trophicity. Despite this, there is no significant difference in serum concentrations of Fetuin-A on either side of the placenta at birth. The mean level of fetuin-A in

Graph 2. Results of the proteomic analysis.
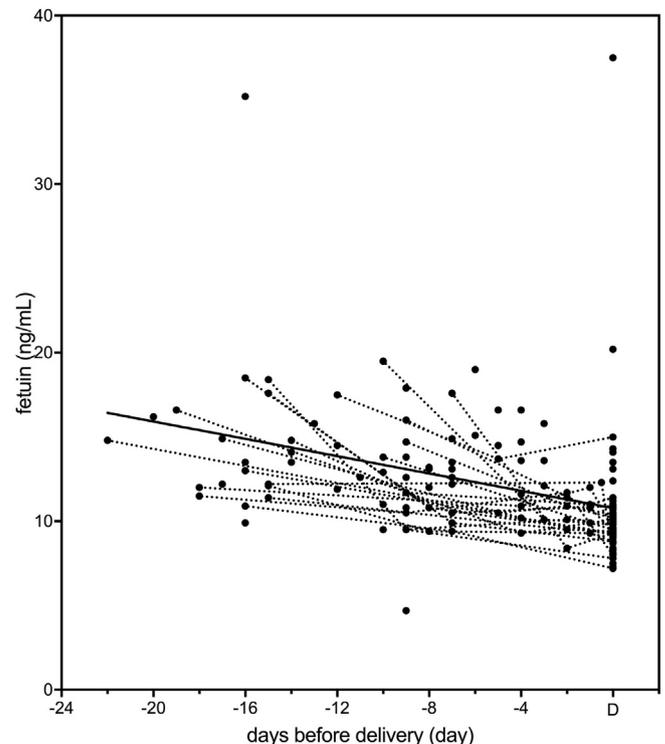
Graph 3. maternal fetuin-A concentration few days before and at delivery (r2= 0142; p< 00,001).

Table 2
Fetal Z-scores and fetuin-A at delivery, both side of the placenta.

	−18 ± 2d [-20, -16d]	−13 ± 2d [-15, -11d]	−8 ± 2d [-10, -6d]	−3 ± 2d [-5, -1d]	Delivery	p	Cord blood	p	Post partum	p
FETUIN-A (ng/mL)	15.3 ± 6.46 (9.9-35.2)	14.3 ± 2.37 (11.4-18.4)	12.7 ± 3.21 (4.7-19.5)	11.8 ± 2.28 (8.4-16.6)	10.6 ± 4.24 (7.2-37.5)	< 0.0002	11.4 ± 2.57 (7.3-18.1)	0.33	13.7 ± 3.23 (8.5-21.3)	0.003

fetal cord blood was only 1.15 times that in maternal blood, with no significant correlation ($r2 = 0.0053$, $p = 0.674$) (7.3–18.1 ng/mL).

At postpartum, more than six weeks after delivery mean maternal fetuin-A level was higher than at delivery (13.7 ± 3.23 ng/mL vs $10.6 ± 4.24$ ng/mL, $p = 0.003$).

Discussion

Our study by SELDI confirms that several maternal serum proteins fluctuate three weeks before delivery, especially proteins involved in inflammatory and immunotolerance processes. Using MALDI-TOF technology, we identified the N-terminal fragment of fetuin-A. Fetuin-A is a complex multifunctional protein expressed in numerous tissues such as bone before and after birth and is essentially secreted by hepatocytes in adult life and placenta during pregnancy [12]. Fetuin-A is a natural antagonist of TGF-beta and is able to bind calcium [13]. It circulates at high levels in fetal blood and then declines in childhood and in adulthood, when it fluctuates widely under the influence of many factors (genetic, diet, exercise, age) [14].

Fetuin-A circulates in the blood and contributes to different physiological and pathological processes: calcification in many tissues such as in bone and tooth mineralization, insulin resistance and dyslipidemia leading to obesity and cardiovascular diseases, acute and subchronic inflammation. Also, low levels of fetuin-A are associated with vascular calcification and inflammation [15–17]. Siegel-Axel et al found that fetuin-A influences the expression of proinflammatory and angiogenic proteins via toll-like receptor 4 (TLR4) pathway in perivascular fat cells cultured *in vitro* [18]. The TLR4 pathway culminates in activation of the transcription factor NF-kB, which controls the expression of an array of inflammatory cytokine genes [19]. NF-kB leads numerous proinflammatory cytokines that during parturition and the withdrawal of progesterone allow the contraction of the myometrium via estrogens and prostaglandins.

Kalabay et al described an increase of mSF-A determined by radial immunodiffusion in the second part of a normal pregnancy [20]. Gomez et al observed *in vitro* that fetuin-A reduced extravillous cytotrophoblast viability and invasion in the first trimester and particularly reduced expression of insulin receptor substrate-1 and of tyrosine phosphorylated insulin receptor substrate-1 [21]. In a prospective cohort of 309 women in preterm labor with suspected intraamniotic infection, Pereira et al identified 23 peptide masses that discriminated preterm labor from spontaneous preterm birth [22]. Also, 48 of 52 proteins were classified into different functional pathways involved in cases of preterm labor: complement/coagulation cascade, inflammation/immune response, fetal or placental development, extracellular matrix proteins. Particularly, fetuin-A, classified in the inflammation response or in fetoplacental development, showed a negative fold change (-1.22, $p < 0.0001$) and was associated with preterm birth. Yuan et al reported that the signal transduction of parturition has pathways that vary among modes of delivery and that the hepatocyte nuclear factor 1 homeobox A (HNF1A) is likely to play a key role [6]. Induction of these pathways during labor was characterized by the appearance of several inflammation-related proteins, as fetuin-A. In our study, we did not observe significant variation in fetuin-A induced by the mode of delivery but spontaneously few days before delivery.

On the fetal side, Iliodromiti et al studied fetuin-A in amniotic fluid in Down Syndrom fetus in the second trimester and founded reduced levels than euploid fetus (5,3 ng/mL vs 6,8 ng/mL, $p = 0008$) [23]. They suggested an association with altered metabolic pathways and could be potentially associated with features like growth restriction or impaired osteogenesis. In our study there is no difference between macrosomic and fetal growth restriction fetus, or male and female fetus.

Fetuin-A has an important role in mineralization biology. Schafer et al. described its affinity for calcium phosphate minerals like hydroxyapatite and in fetuin-A knockout mice [24,25]. In fetal side, concentrations of fetuin-A were decreased in amniotic fluid of idiopathic preterm premature rupture of the membranes (PPROM) [26]. Taken together, the serum variation of fetuin-A on both the fetal and maternal side and the proteins involved in the calciprotein particles mechanism would be an interesting avenue to explore in parturition or in some diseases during pregnancy like diabetes or preeclampsia.

Conclusion

At the beginning of parturition, there is mobilization of proteins, such as fetuin-A, that are likely to be involved in inflammatory and immune tolerance processes and maternal adaptation to pregnancy. Fetuin-A may play a role between the pro-inflammatory pathway NF-kB and the transcription factor HNF1A, which is involved in the expression of several liver-specific genes. The literature describes fetuin-A as a potential biomarker for organ dysfunction and metabolic syndrome disorders. The protein mineral homeostasis would be an interesting pathway to explore during human parturition.

Conflict of interest

The authors report no conflict of interest.

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