Early pregnancy biomarker discovery study for spontaneous preterm birth

Beernink, Rik H.J.; Schuitemaker, Joost H.N.; Zwertbroek, Eva F.; Scherjon, Sicco A.; Cremers, Thomas I.F.H.

Published in:
Placenta

DOI:
10.1016/j.placenta.2023.06.011

Publication date:
2023

Placenta 139 (2023) 112–119

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Early pregnancy biomarker discovery study for spontaneous preterm birth

Rik H.J. Beernink [a,b,*], Joost H.N. Schuitemaker [b,c], Eva F. Zwertbroek [d], Sicco A. Scherjon [d], Thomas I.F.H. Cremers [a]

[a] Dept. Analytical Biochemistry, University of Groningen, Groningen, the Netherlands
[b] Research & Development, IQ Products BV., Groningen, the Netherlands
[c] Div. of Medical Biology, Dept. of Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands
[d] Dept. of Obstetrics and Gynaecology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands

ARTICLE INFO

Keywords:
Biomarker discovery
Spontaneous preterm birth
Proteomics
ELISA verification

ABSTRACT

(1) Objective: discover new candidate biomarkers for spontaneous preterm birth in early pregnancy samples. When fully clinically validated, early pregnancy biomarkers for sPTB give the possibility to intervene or monitor high-risk pregnancies more intensively through, as example, pelvic exams, ultrasound or sonographic cervical length surveillance.
(2) Study design: Early pregnancy serum samples of eight spontaneous extreme and very preterm birth cases (<32 weeks of gestational age) without any symptoms of preeclampsia and fetal growth restriction and eight uncomplicated pregnancies were analyzed by liquid chromatography mass spectrometry (LC-MS). Thirteen proteins, which were differentially expressed according to the LC-MS data, were subsequently selected for confirmation by enzyme-linked immunosorbent assay (ELISA).
(3) Results: Differential expression of four candidate biomarkers was confirmed by ELISA with decreased early pregnancy levels of gelsolin and fibulin-1 and increased levels of c-reactive protein and complement C5 in the preterm birth group.
(4) Conclusions: The confirmed candidate biomarkers are all to some extent related to inflammatory pathways and/or the complement system. This supports the hypothesis that both play a role in extreme and very preterm birth without any symptoms of preeclampsia and fetal growth restriction. The predictive value of complement C5, c-reactive protein, fibulin-1 and gelsolin should, therefore, be validated in another cohort with early pregnancy samples.

1. Introduction

Preterm birth is the delivery of a fetus before 37 weeks of pregnancy and it is one of the main causes of neonatal mortality and morbidity [1,2]. About 40–45% of the cases are spontaneous preterm labor with intact membranes and in 25–30% of the cases the membranes are ruptured. Both together are seen as spontaneous preterm birth (sPTB) and all other preterm births are mainly medically indicated based on fetal growth restriction (FGR) or maternal complications such as preterm pre-eclampsia (PT-PE) [1]. The causes of sPTB are thought to be related to stress, inflammation, infection, placental abruption or uterine distension [3–5]. The prevalence of preterm birth is globally increasing with incidences ranging from 5 to 9% and the disorder can be subdivided in extreme (<28 weeks of gestational age), very (28–32 weeks) and moderate to late (32–37 weeks) preterm birth [1,6]. Fetal consequences of preterm birth can be mortality, but also serious neonatal morbidity later in life are known such as neurodevelopmental impairments, as cerebral palsy, impaired learning and visual handicaps and increased risk of (non-)infectious respiratory problems [7,8]. Current predictors for sPTB are a short cervical length and maternal risk factors such as a maternal history of sPTB, smoking and increased maternal age [9]. Apart from the placement of a cervical pessary or treatment with progesterone, antibiotics or acetylsalicylic acid treatment, no other therapeutic interventions are available to reduce the risk of sPTB [10–12]. Some of these interventions are preferably started in the first trimester and, therefore, there is a need for predictive early pregnancy biomarkers. Therapies to improve fetal outcome that can be started when there is a threatening preterm labor are magnesium sulfate to protect the fetal brain, steroids to accelerate fetal lung growth and uterine contraction inhibitors to allow these medicines to take effect [13,14].

* Corresponding author. Dept. Analytical Biochemistry, University of Groningen, Groningen, the Netherlands.
E-mail address: r.h.j.beernink@rug.nl (R.H.J. Beernink).

https://doi.org/10.1016/j.placenta.2023.06.011
Received 2 February 2023; Received in revised form 24 April 2023; Accepted 17 June 2023
Available online 19 June 2023

Furthermore, early pregnancy biomarkers can also give new biological insights in the etiology of the disorder and can result in new therapeutic targets.

2. Material and methods

2.1. Study population

This study was approved by the Institutional Review Board of the University Medical Centre Groningen (Medical Ethics Committee (METc) no: 2013-216). As part of this study, demographic and clinical parameters of pregnant women were recorded and a serum sample was collected during a first trimester visit to the health care professional, aliquoted and stored at −80 °C. Because of the various etiologies, sPTB was in this study strictly defined as; labor before 32 weeks in a singleton pregnancy and without any symptoms of PE or FGR. Delivery before 32 weeks is considered as extreme and very prematurity and is responsible for about 20% of the sPTB cases [1]. In total, 8 sPTB cases and 8 control pregnancies were analyzed. Control pregnancies had no co-morbidities, such as systemic lupus erythematosus, diabetes mellitus or anti-phospholipid syndrome, no maternal pregnancy complications such as hypertensive disorders, gestational diabetes mellitus or fetal growth restriction and no peri-/neonatal complications such as sepsis, asphyxia, hypoglycemia, respiratory distress syndrome, intraventricular hemorrhage or necrotizing enterocolitis. Control pregnancies were spontaneously conceived first pregnancies, delivered at term and did not use acetylsalicylic acid or antihypertensive agents.

2.2. Liquid chromatography-tandem mass spectrometry

Before liquid chromatography–mass spectrometry (LC-MS) analysis, serum samples were 1:20 diluted in 100 mM ammonium bicarbonate (ABC) (Sigma Aldrich, Saint Louis, MO), denatured with NuPage LDS-Sample buffer (Thermo Fisher Scientific, San Jose, CA) and loaded on a sodium dodecyl sulfate polyacrylamide gel electrophoresis gel (SDS-PAGE) (Westburg, Leusden, Netherlands). In order to exclude possible bias during sample preparation, a triplicate of both a sPTB and a control sample in combination with a quality control sample were loaded on each gel. After this, gels were stained and cut in uniform gel blocks. These gel blocks were subsequently washed with mixtures of ammonium bicarbonate (ABC) (Sigma Aldrich, Saint Louis, MO) and acetonitrile (ACN) (Biosolve, Valkenswaard, Netherlands) and overnight tryptic digested by Sequencing Grade Modified Trypsin (Promega, Madison, WI) at 37 °C. The next day, the peptides were extracted from the gel and analyzed by an Ultimate 3000 HPLC system (Thermo Fisher Scientific, San Jose, CA) coupled online to a Q Exactive Plus Hybrid Quadrupole-Orbitrap MS with a NanoFlex source (Thermo Fisher Scientific). MS data was acquired through data-dependent acquisition (DDA) and the identification of the spectra was performed with an UniprotKB human proteome UP000005640 (July 2016) by the software PEAKS X+ (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada). After identification and quantification, non-normalized relative LC-MS intensities of all peptides were exported with a False Discovery Rate (FDR) of 0.1% for quality control normalization and statistical analysis. For a full detailed experimental protocol, see supplementary data. Significantly different proteins were further analyzed by Reactome (https://reactome.org) to investigate which biological pathways were altered in sPTB. Enriched pathways with a corrected p-value of <0.01 after Benjamini-Hochberg correction were expected to be involved in the sPTB cases.

2.3. Enzyme-linked immunosorbent assay

The absolute concentrations of the selected proteins were measured by enzyme-linked immunosorbent assay (ELISA). All kits were used according to the supplied protocol. Serum samples were tested undiluted for heparin cofactor II (Abbexa, Cambridge, United Kingdom, abx570183) and C4b-binding protein alpha chain (Abbexa, abx052696). The following dilution factors were used for the other proteins: serum amyloid P-component (Abbexa, abx253561) 1:30, fibronectin (Abcam, Cambridge, United Kingdom, ab108848) 1:100, lumican (Thermo Fisher Scientific, EH310RB) 1:100, complement C3 (Novus Biologicals, Minneapolis, MIN, NBP2-60618) 1:800, kininogen (Thermo Fisher Scientific, EH287RB) 1:1,000, c-reactive protein (R&D Systems, Minneapolis, MIN, DCRP00) 1:2,500, apolipoprotein D (Novus Biologicals, NBP2-69833) 1:25,000, complement factor H (Novus Biologicals, DY4779) 1:25,000, kallistatin (Abcam, ab193714) 1:10,000, prothrombin (Novus Biologicals, NBP2-60590) 1:80,000 and complement C5 (Abcam, ab125963) 1:80,000. The antigen concentration in the samples was calculated by optical density interpolation in the standard curve. All samples were tested in duplicate and intra-assay variations were below 15% for all ELISAs. In four cases the response of heparin cofactor II was not detectable while the samples were already undiluted. These values were set to half the value of the lowest point of the standard curve to make statistical evaluation possible. Next to the unbiased proteomics approach, two well-described markers for other pregnancy related disorders, pregnancy associated plasma protein A (PAPP-A) and placenta growth factor (PlGF), were measured during study enrollment by using PerkinElmer's AutoDelfia. In two other studies by our group focusing on preterm preeclampsia (PT PE) and fetal growth restriction (FGR), differential expression of apolipoprotein D (ApoD) and fibronectin (FINC) were observed and, therefore, these proteins were also included in the ELISA analysis [15] (FGR manuscript in preparation).

2.4. Statistical analysis

Baseline characteristics were expressed as median and interquartile range for continuous variables and categorical variables were expressed as numbers and percentages. Statistical comparison between cases and controls wase performed using Mann-Whitney U for continuous variables and Chi-square tests for categorical variables. After quality control normalization, relative LC-MS intensities of the peptides were logarithmic transformed to obtain a normal distribution and used for statistical comparison. The significantly different peptides were used to see which proteins were differently expressed in sPTB. Only proteins with more than one significant peptide and an average peptide spectrum quality of at least 15 were selected for ELISA confirmation. This peptide spectrum quality indicates the quality of the quantified peptide and is affected by various parameters such as m/z value, retention time difference, signal-to-noise ratios, feature intensity and isotope distribution. By using these parameters in combination with a FDR of 0.1%, the inclusion of false-positive LC-MS results were reduced as much as possible. Absolute protein levels, determined by ELISA, were subsequently compared by Mann-Whitney U test. All statistical analyses were performed using IBM SPSS Statistics (version 26.0; IBM Corporation) and p-values of <0.05 were considered statistically significant.

3. Results

Comparison of the baseline characteristics between control and sPTB pregnancies only revealed significant differences in body mass index (BMI), birth weight and gestational age at delivery (Table 1). Serum samples were collected at an average of 12^{+5} and 13^{+1} weeks for the control and sPTB group, respectively. While a limited number of control pregnancies was available, it was not possible to match for BMI between the groups. In the sPTB group, two birthweight percentiles were missing as no gender was known or the fetus was born too prematurely to determine a birth weight percentile.

3.1. Biomarker discovery

In total, 2466 peptides were identified and quantified during data-analysis by the software PEAKS X+. After statistical analysis, 89 of

Table 1
Baseline characteristics of pregnancies affected by uncomplicated pregnancies (controls) and spontaneous preterm birth (sPTB).

		Controls (n = 8)	sPTB (n = 8)	*p*-value
Maternal age (years)		33 (29–35)	33 (26–36)	0.878[a]
Maternal BMI (kg/m^2)		21.5 (20.4–22.0)	30.4 (25.5–31.2)	<0.01[a]
Gestational age sampling (days)		92 (89–96)	90 (82–95)	0.442[a]
Parity		0 (100%)	0 (100%)	0.584[b]
Gravidity	1	8 (100%)	4 (50%)	
	2	0 (0%)	0 (0%)	
	3	0 (0%)	2 (25%)	
	4	0 (0%)	2 (25%)	
Gender fetus[c]	Female	2 (25.0%)	1 (12.5%)	0.584[b]
	Male	6 (75.0%)	6 (75%)	
Birthweight (g)		3580 (3310–4110)	1383 (1065–1683)	<0.001[a]
Birthweight percentile (%)[c]		61 (13–84)	74 (52–81)	0.755[a]
Gestational age at delivery (days)		287 (285–289)	194 (182–208)	<0.001[a]
Smoking		0 (0%)	1 (12.5%)	0.302[b]
Preterm premature rupture of membranes		0 (0%)	3 (37.5%)	0.055[b]

Data are presented as respectively median and interquartile range for continuous data and numbers and percentages for categorical data.
[a] Analysis by Mann-Whitney *U* test.
[b] Analysis by Chi-square test.
[c] Missing data at baseline; gender fetus n = 1 and birthweight percentile n = 2.

these peptides were significantly different and these peptides originated from 23 proteins (Table 2). To note, each significant peptide from a specific protein showed the same trend in expression; up- or downregulated in sPTB. The Reactome pathway analysis connected the dysregulated proteins in sPTB to 15 pathways with a corrected *p*-value of <0.01 (Table 3). These pathways mainly involved the complement system and fibrinolytic mechanisms.

3.2. ELISA verification biomarker discovery data

To exclude possible false-positive results in the ELISA analysis, only proteins with more than one significant peptide and an average spectrum quality above 15 were selected for ELISA verification and this resulted in a selection of fifteen proteins. Due to limited sample material, two proteins had to be excluded and the absolute concentration of thirteen proteins was determined by ELISA (Table 2). For CC3, CFAI, CFH, HEP2 and KAIN no significantly differential expression was observed (Fig. 1A–D). Next to, KNG1, LUM, THRB and SAMP (Fig. 2A–D). In line with our LC-MS data, CRP and CO5 were significantly increased in sPTB: 3566.71 ± 2986.99 versus 8894.69 ± 5594.48 ng/ml for CRP and 117.57 ± 23.54 versus 151.77 ± 17.55 μg/ml for CO5 in the control and sPTB group respectively (Fig. 3A–C). On the contrary, GSN and FBLN1 levels were decreased in sPTB with 24,089.50 ± 3352.45 versus 20,173.74 ± 2838.33 ng/ml for GSN and 21.87 ± 12.15 versus 13.26 ± 4.27 ng/ml for FBLN1 in the control and sPTB group respectively (Fig. 4A–B). Differential early pregnancy expression of both ApoD and FINC was previously observed in two other studies by our group focusing on pregnancies affected by PT-PE and FGR, respectively. In addition, PlGF and PAPP-A are two well-described markers for other pregnancy complication and, therefore, expression levels of these four proteins were also evaluated in this study. ApoD and FINC showed no significant difference and PlGF and PAPP-A were both significantly decreased in early pregnancy samples of sPTB pregnancies with 32.49 ± 16.97 versus 16.00 ± 9.18 pg/ml for PlGF and 4.07 ± 1.27 versus 1.49 ± 0.99 IU/ml for PAPP-A in the control and sPTB group respectively (Fig. 5A–D). For all proteins, the removal of a possible

Table 2
Data of significant differently expressed proteins in spontaneouse preterm birth compared to uncomplicated pregnancies.

Protein	Accession number[a]	Abbreviation	Up-/down-regulated[b]	Significant peptides (total peptides)	Average spectrum quality	Protein coverage of peptides (%)	Selected for ELISA analysis
Alpha-1-acid glycoprotein 1	P02763	A1AG1	↑	1 [4]	23.4	26	
Alpha-2-antiplasmin	P08697	A2AP	↑	1 [7]	14.4	36	
Apolipoprotein L1	P08519	APOA	↑	1 [11]	12.3	3	
C4b-binding protein alpha chain[c]	P04003	C4BP	↑	3 [11]	19.4	30	
Cholinesterase	P06276	CHLE	↑	1 [14]	16.4	37	
Complement C3	P01024	CC3	↑	14 (87)	33.4	68	X
Complement C5	P01031	CO5	↑	7 [59]	17.9	68	X
Complement component C9[c]	P02748	CC9	↑	4 [25]	20.6	44	
Complement factor H	P08603	CFH	↑	5 [15]	24.9	26	X
Complement factor I	P05156	CFAI	↑	3 [7]	22.3	20	X
C-reactive protein	P02741	CRP	↑	2 [6]	17.7	25	X
Fibulin-1	P23142	FBLN1	↓	3 [9]	24.2	10	X
Galectin-3-binding protein	Q08380	LG3BP	↑	3 [15]	11.9	31	
Gelsolin	P06396	GSN	↓	4 [28]	20.6	63	X
Hemopexin	P02790	HPX	↑	1 [8]	10.8	37	
Heparin cofactor 2	P05546	HEP2	↑	5 [17]	22.7	56	X
Kallistatin	P29622	KAIN	↓	4 [19]	26.7	62	X
Kininogen-1	P01042	KNG1	↑	2 [17]	16.1	27	X
Lumican	P51884	LUM	↓	4 [13]	21.8	40	X
Prothrombin	P00734	THRB	↑	3 [11]	26.8	28	X
Serum amyloid P-component	P02743	SAMP	↑	5 [10]	23.6	54	X
Sulfhydryl oxidase 1	O00391	QSOX1	↓	2 [12]	12.3	23	
Vitronectin	P04004	VTNC	↑	1 [12]	10.0	23	

[a] Uniprot Accession number.
[b] Expression level for sPTB compared to control group.
[c] Proteins were not tested due to limited sample material.

Table 3
Altered pathways in sPTB after Reactome analysis of all differentially expressed proteins (p-value <0.01).

Pathway	Reactome identifier	*p*-value	Involved genes
Complement cascade	R-HSA-166658	5.26×10^{-10}	THRB, KNG1, C4BP, CFH, CFAI, VTNC, CRP, CO5 and CC9
Regulation of complement cascade	R-HSA-977606	6.66×10^{-9}	THRB, CC3, C4BP, CFH, CFAI, VTNC, CO5 and CC9
Platelet degranulation	R-HSA-114608	4.60×10^{-6}	KNG1, A1AG1, KAIN, QSOX1, A2AP and LG3BP
Response to elevated platelet cytosolic Ca^{2+}	R-HSA-76005	4.60×10^{-6}	KNG1, A1AG1, KAIN, QSOX1, A2AP and LG3BP
Platelet activation, signaling and aggregation	R-HSA-76002	1.07×10^{-5}	THRB, KNG1, A1AG1, KAIN, QSOX1, A2AP and LG3BP
Innate Immune System	R-HSA-168249	1.07×10^{-5}	THRB, CC3, C4BP, A1AG1, CFH, CFAI, VTNC, CRP, GSN, QSOX1, CO5 and CC9
Regulation of Insulin-like Growth Factor (IGF) transport and uptake by IGF binding Protein	R-HSA-381426	5.15×10^{-5}	THRB, QSOX1, KNG1, CC3 and HEP2
Intrinsic pathway of fibrin clot formation	R-HSA-140837	1.60×10^{-4}	THRB, KNG1 and HEP2
Hemostasis	R-HSA-109582	4.84×10^{-4}	THRB, KNG1, A1AG1, KAIN, HEP2, QSOX1, A2AP and LG3BP
Post-translational protein phosphorylation	R-HSA-8957275	4.97×10^{-4}	QSOX1, KNG1, CC3 and HEP2
Formation of fibrin clot (clotting cascade)	R-HSA-140877	5.09×10^{-4}	THRB, KNG1 and HEP2
Activation of C3 and C5	R-HSA-174577	7.28×10^{-4}	CO5 and CC3
Terminal pathway of complement	R-HSA-166665	8.31×10^{-4}	CO5 and CC9
Immune system	R-HSA-168256	2.74×10^{-3}	THRB, CC3, C4BP, A1AG1, CFH, CFAI, VTNC, CRP, GSN, QSOX1, CO5 and CC9
Peptide ligand-binding receptors	R-HSA-375276	3.45×10^{-3}	THRB, CO5, KNG1, and CC3
Common pathway of fibrin clot (clotting cascade)	R-HSA-375276	5.29×10^{-3}	THRB, CO5, KNG1, and CC3

A1AG1 = Alpha-1-acid glycoprotein 1, CFH = Complement factor H, KAIN = Kallistatin.
A2AP = Alpha-2-antiplasmin, CO5 = Complement C5, KNG1 = Kininogen-1.
C4BP=C4b-binding protein alpha chain, CRP=C-reactive protein, LG3BP = Galectin-3-binding protein.
CC3 = Complement C3, FBLN1 = Fibulin-1, QSOX1 = Sulfhydryl oxidase 1.
CC9 = Complement component C9, GSN = Gelsolin, THRB = Prothrombin.
CFAI = Complement factor I, HEP2 = Heparin cofactor 2, VTNC = Vitronectin.

outlier caused no change in significance and even strengthened the significance in the case of CRP.

4. Discussion

The differentially expressed proteins after the untargeted discovery analysis were mainly involved in the complement system and coagulation. The complement system is, among other systems, strongly associated with our immune and coagulation system. Dysregulation of both systems is associated with several pathways in the development of sPTB [16–19]. After untargeted analyses, candidate biomarkers should preferably be confirmed in a targeted analysis, such as ELISA, and thirteen proteins were, therefore, selected for ELISA confirmation. In total, differential expression of four proteins was confirmed by ELISA and these proteins were mainly related to inflammatory pathways: gelsolin (GSN), fibulin-1 (FBLN1), c-reactive protein (CRP) and complement C5 (CO5). Possible reasons why the other selected proteins were not confirmed by ELISA could be that the used ELISA assays were targeted against the full native protein, while differential expression was only present for a specific isoform - or the other way around - or the used LC-MS intensities of the peptides did not reflect the actual concentration of the protein.

GSN is a calcium dependent regulator of the actin cytoskeleton and next to this cellular form, there is a secretory isoform [20]. Decreased levels of this secretory form are linked to various pathological (inflammatory) conditions such as preeclampsia (PE), sepsis, active live injury, Alzheimer's disease, fibrosis and arthritis [21–26]. Circulating GSN is responsible for the removal of extracellular actin originating from damaged tissue and by doing this, it prevents an inflammatory reaction. Further, studies also showed that the actin scavenging system, including GSN, is altered during PE in the late second and third trimester. At that moment, PE is also characterized as a pro-inflammatory state [24,27]. Altered GSN levels are not yet commonly associated with sPTB, but it is known that an inflammation and/or failure of maternal tolerance to the semi-allogeneic fetus represents a large proportion of the sPTB cases without any symptoms of PE and FGR [1,18,28].

The complement system, which is part of the innate immune response, mediates various steps in inflammatory responses and is crucial for a successful pregnancy [29,30]. While the defense mechanism to protect the mother and fetus against infectious agents needs to maintain, the immune system also needs to accept the semi allogenic fetus and this delicate balance during pregnancy can be disrupted [31,32]. From implantation till early second trimester a pregnancy is a strong inflammatory condition, as the fetus needs to break the uterus' lining, damage the endometrial tissue to invade and replace cells during spiral artery remodeling [33]. In all these processes, an inflammatory environment is required for an adequate repair and removal of cellular debris and an imbalance in this condition is associated with sPTB [17,18]. This dysregulation in the immune response and mainly in the complement system is also observed in our data with confirmed increased early pregnancy levels of CRP and CO5 in sPTB pregnancies by ELISA.

The protein CRP is a multifunctional component of the acute phase response and is synthesized by the liver in response to, among other factors, interleukin-6 of activated macrophages and T cells during infection and tissue injury [34]. The protein regulates the classical complement pathway through activating complement component 1q

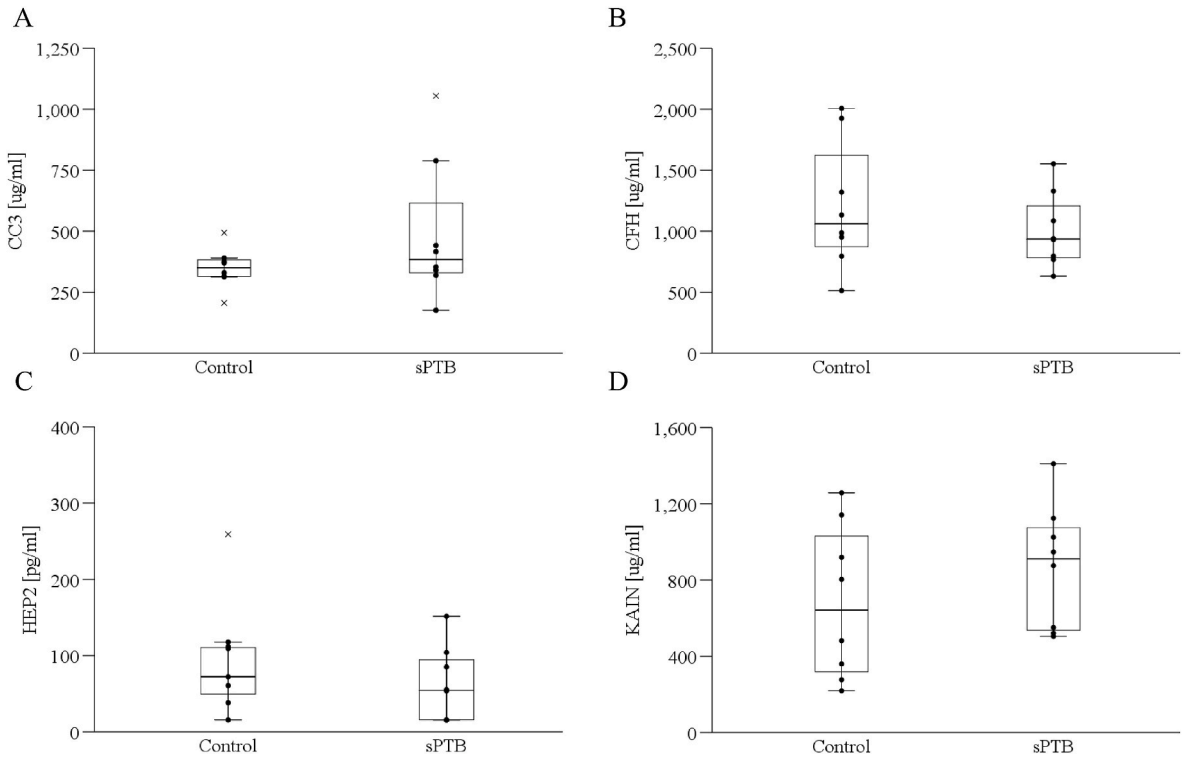

Fig. 1. Boxplots of uncomplicated (control) pregnancies and spontaneous preterm birth (sPTB) pregnancies. Boxplot of complement C3 (CC3) [A], complement factor H (CFH) [B], heparin cofactor 2 (HEP2) [C] and kallistatin (KAIN) [D] concentrations in uncomplicated (Control) and spontaneous preterm birth (sPTB) pregnancies. For each of the groups the median is shown as horizontal line in the interquartile range box with minimum and maximum whiskers. Each individual data point is shown as dot and outliers are presented as cross.

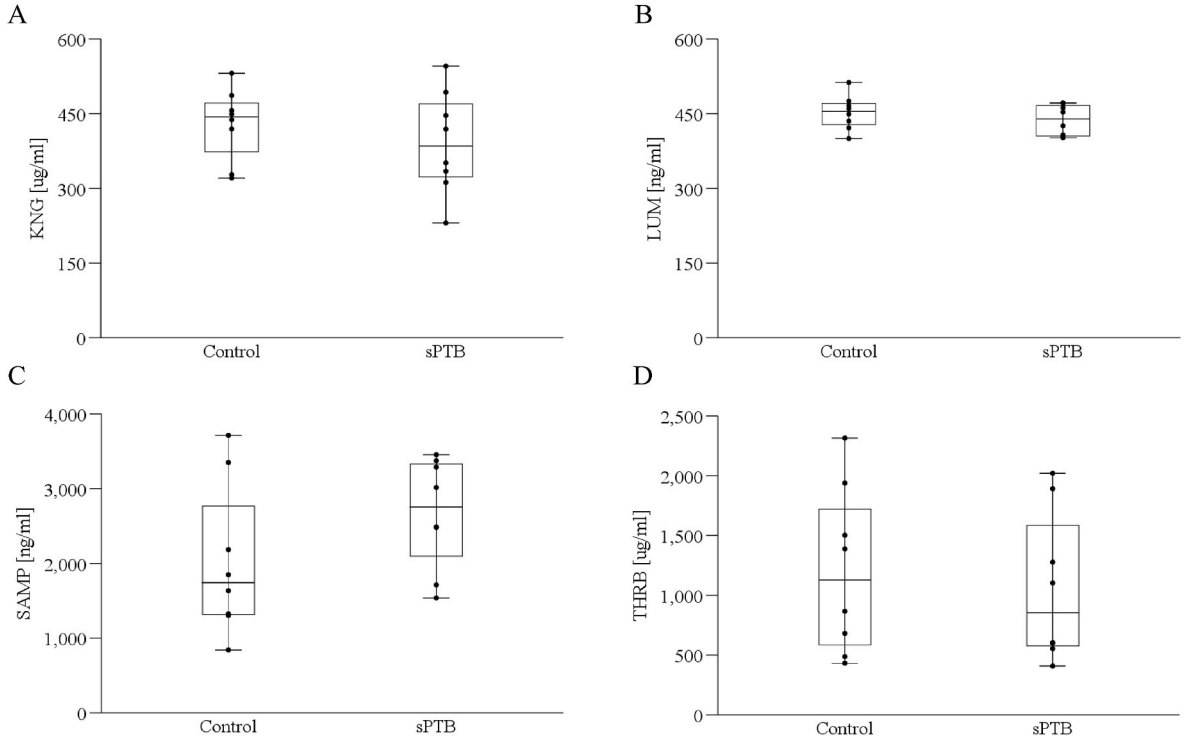

Fig. 2. Boxplots of uncomplicated (control) pregnancies and spontaneous preterm birth (sPTB) pregnancies. Boxplot of kininogen-1 (KNG1) [A], lumican (LUM) [B], serum amyloid P-component (SAMP) [C] and prothrombin (THRB) [D] concentrations in uncomplicated (Control) and spontaneous preterm birth (sPTB) pregnancies. For each of the groups the median is shown as horizontal line in the interquartile range box with minimum and maximum whiskers. Each individual data point is shown as dot.

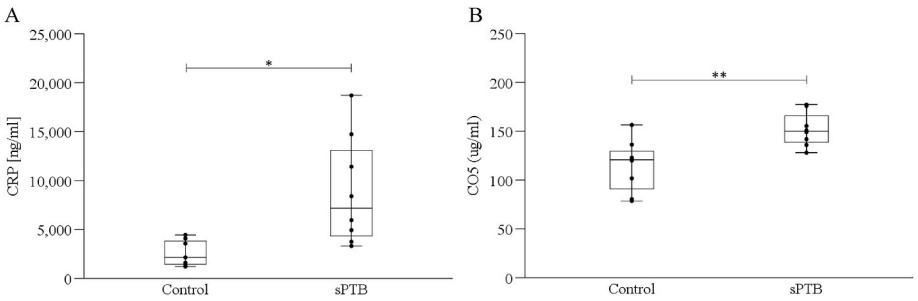

Fig. 3. Boxplots of uncomplicated (control) pregnancies and spontaneous preterm birth (sPTB) pregnancies. Boxplot of c-reactive protein (CRP) [A] and complement C5 (CO5) [B] concentrations in uncomplicated (Control) and spontaneous preterm birth (sPTB) pregnancies. For each of the groups the median is shown as horizontal line in the interquartile range box with minimum and maximum whiskers. Each individual data point is shown as dot. * *p*-value <0.05; ** *p*-value <0.01.

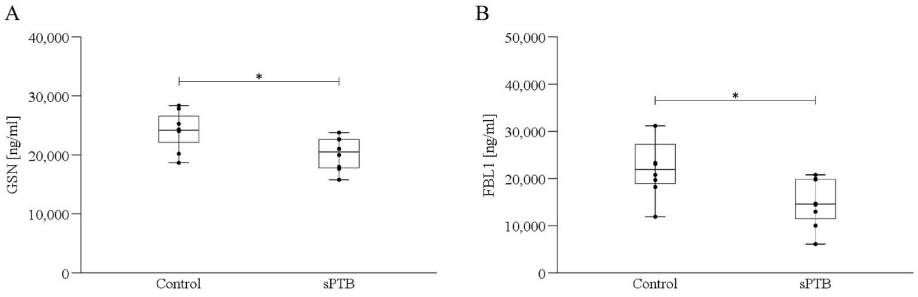

Fig. 4. Boxplots of uncomplicated (control) pregnancies and spontaneous preterm birth (sPTB) pregnancies. Boxplot of gelsolin (GSN) [A] and fibulin-1 (FBLN1) [B] in uncomplicated (Control) and spontaneous preterm birth (sPTB) pregnancies. For each of the groups the median is shown as horizontal line in the interquartile range box with minimum and maximum whiskers. Each individual data point is shown as dot. * *p*-value <0.05; ** *p*-value <0.01.

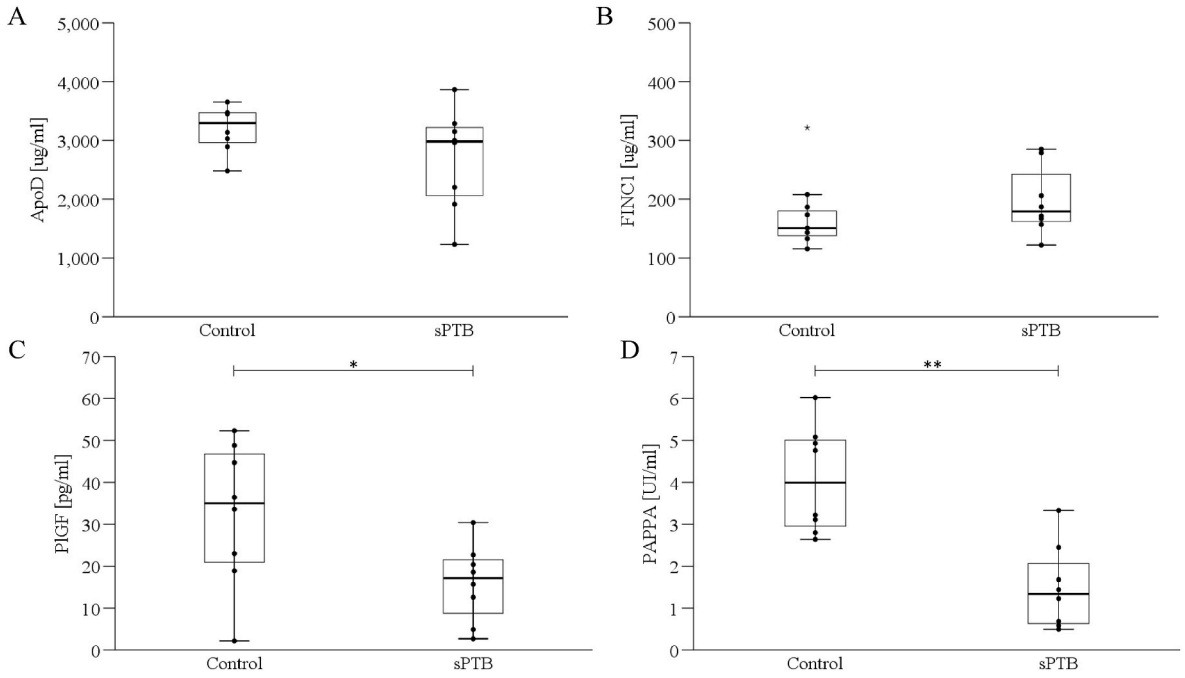

Fig. 5. Boxplots of uncomplicated (control) pregnancies and spontaneous preterm birth (sPTB) pregnancies. Boxplot of apolipoprotein D (ApoD) [A], fibronectin (FINC) [B], placental growth factor (PlGF) [C] and pregnancy associated plasma protein A (PAPP-A) [D] concentrations in uncomplicated (Control) and spontaneous preterm birth (sPTB) pregnancies. For each of the groups the median is shown as horizontal line in the interquartile range box with minimum and maximum whiskers. Each individual data point is shown as dot and outliers are presented as cross. * *p*-value <0.05; ** *p*-value <0.01.

(C1q) which will result in phagocytosis after multiple interactions between several complement factors. Various studies already showed that CRP could possibly be used as a biomarker for the prediction of sPTB [35,36], but also for other disorders such as PE, FGR, gestational diabetes and neonatal complications [37–39]. In general, CRP levels are already increased during pregnancy compared to non-pregnant individuals as early as 4 weeks of gestation and this emphasizes the importance of this factor during pregnancy [40].

CO5 is cleaved by complement C3b into C5a and C5b in the alternative complement pathway. C5a can attract and activate inflammatory cells and C5b binds other complement factors to initiate the formation of the membrane attack complex (MAC) [29]. Increased levels of both C5a and C5b are already associated in literature with PE and PTB [29,41]. CFAI is another mediator in the complement system and it can inactivate C3b and C4b by cleaving their peptide bonds [42]. Other important cofactors in this reaction are, among others, CFH and C4BP, which were also significantly different in our proteomics data and for both, increased serum levels were observed in sPTB. Unfortunately, it was not possible to confirm these observations for C4BP due to limited material and CFH showed no difference in the ELISA data. Surprisingly, differential expression of another candidate biomarker with many significant different peptides and a role in the complement system, CC3, was not confirmed in the ELISA analysis. To rule out that only a specific isoform of this protein is differentially expressed, a targeted LC-MS analysis focusing on, for example, the five peptides with the largest statistical difference or fold change can be set up in future experiments.

FBLN1 is the only confirmed candidate biomarker which is not directly associated with the immune system. In general, the fibulin family consists of extracellular matrix proteins involved in tissue remodeling and, more specifically, FBLN1 has a role in fibrin formation, merges with fibrinogen in the blood and can interact with fibronectin [43]. Interestingly, two isoforms of FBLN1 are only expressed by the placenta and FBLN1 levels significantly increase during pregnancy [44,45]. Studies also showed that FBLN1 alters through the menstrual cycle and it might be a mediator of progesterone activity during endometrial receptivity [46–48]. Low progesterone levels are associated with labor and the steroid hormone is also known as a strong modulator of the immune system during pregnancy [28,49]. Moreover, high-risk sPTB pregnancies benefit from early administration of progesterone to prevent preterm labor [50]. Our observed decreased FBLN1 levels in sPTB are in line with studies that showed decreased FBLN1, FBLN3 and FBLN5 levels in the weak zones of the fetal membranes just before rupture. Furthermore, FBLN1 levels were also observed to be decreased during the complete pregnancy in PPROM pregnancies [45,51]. Since there is a statistical difference in BMI between the groups, the correlation between the confirmed candidate biomarkers and BMI was also determined and there was a significant Pearson's correlation for BMI with CRP (R^2 = 0.591, p-value <0.05) and GSN (R^2 = −0.684, *p*-value <0.01).

Next to the unbiased proteomics approach, a more biased approach was used by including the proteins ApoD, FINC, PlGF and PAPP-A in the ELISA analysis. All four proteins were already associated with preterm PE or FGR by us and other studies [15,52–54]. Significantly decreased levels were observed for both PlGF and PAPP-A in sPTB pregnancies and no difference was observed for ApoD and FINC. PlGF is a member of the vascular endothelial growth factor sub-family and is predominantly expressed in the placenta. Further, it plays a role in angiogenesis and is, most likely, an indicative factor for abnormal placentation [55]. Decreased PlGF levels are already widely associated with the manifestation of (PT) PE and FGR and it is suggested that this is related to a failure of the differentiation, invasion and remodeling of the uterine spiral arteries in these disorders [55–57]. Abnormal remodeling will result in inadequate perfusion, stress in the intervillous space, hypoxia and malnutrition. This will eventually result in apoptosis and release of inflammatory factors at the end of pregnancy. The latter can cause systemic endothelial dysfunction and organ failure with the clinical symptoms of PE as result [57]. PAPP-A, also called pappalysin-1, is primarily synthesized in the syncytiotrophoblast and is a member of the insulin-like growth factor (IGF) family [58]. The protease regulates growth-promoting processes through cleavage of a specific subset of the IGF binding proteins, which will result in the release of bioactive IGFs [52]. The role of PAPP-A in PT-PE and FGR is, most likely, more related to its influence on reprogramming energy metabolism and trophoblast cells instead of angiogenesis, as in the case of PlGF [59]. Low levels of PAPP-A result in lower levels of free bioactive IGFs, which have a role in endometrial receptivity, placental formation and fetal growth [60–62].

5. Conclusion

First, differential expression of 23 proteins was observed in the untargeted biomarker discovery study and differential expression of four proteins was confirmed by ELISA. These proteins are mainly (in)directly related to inflammatory pathways and the complement system. This supports the hypothesis that an excessive inflammation and/or a failure of maternal tolerance to the semi-allogeneic fetus play a role in extreme and very preterm birth cases without any symptoms of PE and FGR. Second, decreased ApoD and FINC levels were observed in preterm PE and FGR pregnancies in previous studies, but not in the sPTB pregnancies. Although the sample size of this study was small due to limited sPTB cases, this observation may indicate that sPTB pregnancies without any symptoms of PE or FGR is caused through a different pathophysiology than FGR and PE. In addition, differential expression of ApoD and FINC might be specifically indicative for the development of, respectively, PT PE and FGR. When fully clinically validated, early pregnancy biomarkers for sPTB give the possibility to interevene or monitor high-risk pregnancies more intensively through, as example, pelvic exams, ultrasound or sonographic cervical length surveillance. Furthermore, biomarkers can also give new biological insights in the pathophysiology of the disorder with possible new therapeutic targets as result. The predictive value of CO5, CRP, FBLN1 and GSN should, therefore, be validated in another cohort.

Author contributions

Conceptualization, RHJB, TIFH and EFZ; methodology, RHJB and TIFH; software, RHJB; validation, RHJB and JS; formal analysis, RHJB; investigation, RHJB and JS; resources, EFZ; data curation, EFZ and RHJB; writing—original draft preparation, RHJB; writing—review and editing, JS, EFZ, SAS and TIFH; visualization, RHJB; supervision, SAS and TIFH; project administration, EFZ and RHJB. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

Authors RHJB and JHNS are employed by IQ Products. JHNS and TIFH are shareholders of IQ Products and the remaining authors report no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2023.06.011.

References

[1] R.L. Goldenberg, J.F. Culhane, J.D. Iams, R. Romero, Epidemiology and causes of preterm birth, Lancet 371 (9606) (2008) 75–84.
[2] J.P. Vogel, S. Chawanpaiboon, A.B. Moller, K. Watananirun, M. Bonet, P. Lumbiganon, The global epidemiology of preterm birth, Best Pract. Res. Clin. Obstet. Gynaecol. 52 (2018) 3–12.
[3] A. Staneva, F. Bogossian, M. Pritchard, A. Wittkowski, The effects of maternal depression, anxiety, and perceived stress during pregnancy on preterm birth: a systematic review, Women Birth 28 (3) (2015) 179–193.
[4] K.L. Downes, K.L. Grantz, E.D. Shenassa, Maternal, labor, delivery, and perinatal outcomes associated with placental abruption: a systematic review, Am. J. Perinatol. 34 (10) (2017) 935–957.
[5] T.K. Morgan, Role of the placenta in preterm birth: a review, Am. J. Perinatol. 33 (3) (2016) 258–266.
[6] S. Beck, D. Wojdyla, L. Say, A.P. Betran, M. Merialdi, J.H. Requejo, et al., The worldwide incidence of preterm birth: a systematic review of maternal mortality and morbidity, Bull. World Health Organ. 88 (1) (2010) 31–38.
[7] M.A. Ream, L. Lehwald, Neurologic consequences of preterm birth, Curr. Neurol. Neurosci. Rep. 18 (8) (2018) 48.

[8] M.J. Platt, Outcomes in preterm infants, Publ. Health 128 (5) (2014) 399–403.
[9] E. Greco, R. Gupta, A. Syngelaki, L.C. Poon, K.H. Nicolaides, First-trimester screening for spontaneous preterm delivery with maternal characteristics and cervical length, Fetal Diagn. Ther. 31 (3) (2012) 154–161.
[10] E.B. da Fonseca, R. Damião, D.A. Moreira, Preterm birth prevention, Best Pract. Res. Clin. Obstet. Gynaecol. 69 (2020) 40–49.
[11] K.M. Griggs, D.A. Hrelic, N. Williams, M. McEwen-Campbell, R. Cypher, Preterm labor and birth: a clinical review, MCN Am. J. Matern./Child Nurs. 45 (6) (2020) 328–337.
[12] M.K. Hoffman, S.S. Goudar, B.S. Kodkany, M. Metgud, M. Somannavar, J. Okitawutshu, et al., Low-dose aspirin for the prevention of preterm delivery in nulliparous women with a singleton pregnancy (ASPIRIN): a randomised, double-blind, placebo-controlled trial, Lancet 395 (10220) (2020) 285–293.
[13] K.F. Brookfield, A. Vinson, Magnesium sulfate use for fetal neuroprotection, Curr. Opin. Obstet. Gynecol. 31 (2) (2019) 110–115.
[14] C.A. Crowther, P.F. Middleton, D. Wilkinson, P. Ashwood, R. Haslam, M.S.G. the, Magnesium sulphate at 30 to 34 weeks' gestational age: neuroprotection trial (MAGENTA) - study protocol, BMC Pregnancy Childbirth 13 (1) (2013) 91.
[15] R.H.J. Beernink, E.F. Zwertbroek, J.H.N. Schuitemaker, T. Cremers, S.A. Scherjon, First trimester serum biomarker discovery study for early onset, preterm onset and preeclampsia at term, Placenta 128 (2022) 39–48.
[16] A. Keren-Politansky, T. Breizman, B. Brenner, G. Sarig, A. Drugan, The coagulation profile of preterm delivery, Thromb. Res. 133 (4) (2014) 585–589.
[17] M.E. Brien, I. Boufaied, N. Bernard, J.C. Forest, Y. Giguere, S. Girard, Specific inflammatory profile in each pregnancy complication: a comparative study, Am. J. Reprod. Immunol. 84 (6) (2020), e13316.
[18] M. Cappelletti, S. Della Bella, E. Ferrazzi, D. Mavilio, S. Divanovic, Inflammation and preterm birth, J. Leukoc. Biol. 99 (1) (2016) 67–78.
[19] K. Vered, B.-W. Ruth, R. Tal, M. Moshe, E. Offer, The role of the coagulation system in preterm parturition, in: E. Offer (Ed.), Preterm Birth, IntechOpen, Rijeka, 2013. Ch. 3.
[20] E. Piktel, I. Levental, B. Durnaś, P.A. Janmey, R. Bucki, Plasma gelsolin: indicator of inflammation and its potential as a diagnostic tool and therapeutic target, Int. J. Mol. Sci. 19 (9) (2018).
[21] H. Halis, T. Gunes, S. Korkut, B. Saraymen, A. Şen, O. Bastug, et al., In the diagnosis of neonatal sepsis importance of gelsolin and relationship with mortality and morbidity, Med. Hypotheses 94 (2016) 77–80.
[22] P.S. Lee, S.R. Patel, D.C. Christiani, E. Bajwa, T.P. Stossel, A.B. Waxman, Plasma gelsolin depletion and circulating actin in sepsis: a pilot study, PLoS One 3 (11) (2008), e3712.
[23] N.A. Nadkarni, A. Rajakumar, N. Mokhashi, S.D. Burke, S. Rana, S. Salahuddin, et al., Gelsolin is an endogenous inhibitor of syncytiotrophoblast extracellular vesicle shedding in pregnancy, Pregnancy Hypertens 6 (4) (2016) 333–339.
[24] D.S. Tannetta, C.W. Redman, I.L. Sargent, Investigation of the actin scavenging system in pre-eclampsia, Eur. J. Obstet. Gynecol. Reprod. Biol. 172 (2014) 32–35.
[25] T.M. Osborn, M. Verdrengh, T.P. Stossel, A. Tarkowski, M. Bokarewa, Decreased levels of the gelsolin plasma isoform in patients with rheumatoid arthritis, Arthritis Res. Ther. 10 (5) (2008) R117.
[26] M.J. DiNubile, Plasma gelsolin as a biomarker of inflammation, Arthritis Res. Ther. 10 (6) (2008) 124.
[27] W.M. Lee, R.M. Galbraith, The extracellular actin-scavenger system and actin toxicity, N. Engl. J. Med. 326 (20) (1992) 1335–1341.
[28] E.S. Green, P.C. Arck, Pathogenesis of preterm birth: bidirectional inflammation in mother and fetus, Semin. Immunopathol. 42 (4) (2020) 413–429.
[29] G. Girardi, J.J. Lingo, S.D. Fleming, J.F. Regal, Essential role of complement in pregnancy: from implantation to parturition and beyond, Front. Immunol. 11 (2020) 1681.
[30] M.M. Markiewski, J.D. Lambris, The role of complement in inflammatory diseases from behind the scenes into the spotlight, Am. J. Pathol. 171 (3) (2007) 715–727.
[31] Z. Derzsy, Z. Prohászka, J. Rigó Jr., G. Füst, A. Molvarec, Activation of the complement system in normal pregnancy and preeclampsia, Mol. Immunol. 47 (7–8) (2010) 1500–1506.
[32] B. Abu-Raya, C. Michalski, M. Sadarangani, P.M. Lavoie, Maternal immunological adaptation during normal pregnancy, Front. Immunol. 11 (2020), 575197.
[33] G. Mor, I. Cardenas, The immune system in pregnancy: a unique complexity, Am. J. Reprod. Immunol. 63 (6) (2010) 425–433.
[34] N.R. Sproston, J.J. Ashworth, Role of C-reactive protein at sites of inflammation and infection, Front. Immunol. 9 (2018) 754.
[35] B.M. Najat Nakishbandy, S.A. Barawi, Level of C - reactive protein as an indicator for prognosis of premature uterine contractions, J Prenat Med 8 (1–2) (2014) 25–30.
[36] S. Huang, J. Tian, C. Liu, Y. Long, D. Cao, L. Wei, et al., Elevated C-reactive protein and complement C3 levels are associated with preterm birth: a nested case-control study in Chinese women, BMC Pregnancy Childbirth 20 (1) (2020) 131.
[37] G.D. Ernst, L.L. de Jonge, A. Hofman, J. Lindemans, H. Russcher, E.A. Steegers, et al., C-reactive protein levels in early pregnancy, fetal growth patterns, and the risk for neonatal complications: the Generation R Study, Am. J. Obstet. Gynecol. 205 (2) (2011), 132.e1-12.
[38] A. Amirian, F.A. Rahnemaei, F. Abdi, Role of C-reactive Protein(CRP) or high-sensitivity CRP in predicting gestational diabetes Mellitus:Systematic review, Diabetes Metabol. Syndr. 14 (3) (2020) 229–236.
[39] F. Rebelo, M.M. Schlüssel, J.S. Vaz, A.B. Franco-Sena, T.J. Pinto, F.I. Bastos, et al., C-reactive protein and later preeclampsia: systematic review and meta-analysis taking into account the weight status, J. Hypertens. 31 (1) (2013) 16–26.
[40] G.P. Sacks, L. Seyani, S. Lavery, G. Trew, Maternal C-reactive protein levels are raised at 4 weeks gestation, Hum. Reprod. 19 (4) (2004) 1025–1030.
[41] J.F. Regal, J.S. Gilbert, R.M. Burwick, The complement system and adverse pregnancy outcomes, Mol. Immunol. 67 (1) (2015) 56–70.
[42] K.E. Lintner, Y.L. Wu, Y. Yang, C.H. Spencer, G. Hauptmann, L.A. Hebert, et al., Early components of the complement classical activation pathway in human systemic autoimmune diseases, Front. Immunol. 7 (2016) 36.
[43] R. Timpl, T. Sasaki, G. Kostka, M.-L. Chu, Fibulins: a versatile family of extracellular matrix proteins, Nat. Rev. Mol. Cell Biol. 4 (6) (2003) 479–489.
[44] H. Tran, M. Mattei, S. Godyna, W.S. Argraves, Human fibulin-1D: molecular cloning, expression and similarity with S1-5 protein, a new member of the fibulin gene family, Matrix Biol. 15 (7) (1997) 479–493.
[45] A.B. Orvik, M.R. Andersen, L. Pedersen, C. Ritz, S. Stender, P.B. Szecsi, Plasma fibulin-1 levels during pregnancy and delivery: a longitudinal observational study, BMC Pregnancy Childbirth 21 (1) (2021) 629.
[46] B. Haendler, H. Yamanouchi, B.A. Lessey, K. Chwalisz, H. Hess-Stumpp, Cycle-dependent endometrial expression and hormonal regulation of the fibulin-1 gene, Mol. Reprod. Dev. 68 (3) (2004) 279–287.
[47] T. Nakamoto, H. Okada, T. Nakajima, A. Ikuta, K. Yasuda, H. Kanzaki, Progesterone induces the fibulin-1 expression in human endometrial stromal cells, Hum. Reprod. 20 (6) (2005) 1447–1455.
[48] H. Okada, T. Tsuzuki, H. Shindoh, A. Nishigaki, K. Yasuda, H. Kanzaki, Regulation of decidualization and angiogenesis in the human endometrium: mini review, J. Obstet. Gynaecol. Res. 40 (5) (2014) 1180–1187.
[49] M.P. Piccinni, R. Raghupathy, S. Saito, J. Szekeres-Bartho, Cytokines, hormones and cellular regulatory mechanisms favoring successful reproduction, Front. Immunol. 12 (2021), 717808.
[50] J.E. Norman, Progesterone and preterm birth, Int. J. Gynaecol. Obstet. 150 (1) (2020) 24–30.
[51] R.M. Moore, R.W. Redline, D. Kumar, B.M. Mercer, J.M. Mansour, E. Yohannes, et al., Differential expression of fibulin family proteins in the para-cervical weak zone and other areas of human fetal membranes, Placenta 30 (4) (2009) 335–341.
[52] I. Kirkegaard, N. Uldbjerg, C. Oxvig, Biology of pregnancy-associated plasma protein-A in relation to prenatal diagnostics: an overview, Acta Obstet. Gynecol. Scand. 89 (9) (2010) 1118–1125.
[53] E.A. Huhn, I. Hoffmann, B. Martinez De Tejada, S. Lange, K.M. Sage, C.T. Roberts, et al., Maternal serum glycosylated fibronectin as a short-term predictor of preeclampsia: a prospective cohort study, BMC Pregnancy Childbirth 20 (1) (2020) 128.
[54] T. Gredmark, B. Bergman, L. Hellström, Total fibronectin in maternal plasma as a predictor for preeclampsia, Gynecol. Obstet. Invest. 47 (2) (1999) 89–94.
[55] K. Chau, A. Hennessy, A. Makris, Placental growth factor and pre-eclampsia, J. Hum. Hypertens. 31 (12) (2017) 782–786.
[56] R.L. Zur, J.C. Kingdom, W.T. Parks, S.R. Hobson, The placental basis of fetal growth restriction, Obstet. Gynecol. Clin. N. Am. 47 (1) (2020) 81–98.
[57] D. Hayes Ryan, F.P. McCarthy, K. O'Donoghue, L.C. Kenny, Placental growth factor: a review of literature and future applications, Pregnancy Hyperten. 14 (2018) 260–264.
[58] C. Oxvig, The role of PAPP-A in the IGF system: location, location, location, J. Cell Commun. Sign. 9 (2) (2015) 177–187.
[59] H.C. Bartels, J.D. Postle, P. Downey, D.J. Brennan, Placenta accreta spectrum: a review of pathology, molecular biology, and biomarkers, Dis. Markers (2018), 1507674, 2018.
[60] M. Yu, J. Wang, S. Liu, X. Wang, Q. Yan, Novel function of pregnancy-associated plasma protein A: promotes endometrium receptivity by up-regulating N-fucosylation, Sci. Rep. 7 (1) (2017) 5315.
[61] D.J. Fowler, K.H. Nicolaides, J.P. Miell, Insulin-like growth factor binding protein-1 (IGFBP-1): a multifunctional role in the human female reproductive tract, Hum. Reprod. Update 6 (5) (2000) 495–504.
[62] C.J. Bowman, R.D. Streck, R.E. Chapin, Maternal-placental insulin-like growth factor (IGF) signaling and its importance to normal embryo-fetal development, Birth Defects Res. B Dev. Reprod. Toxicol. 89 (4) (2010) 339–349.