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Inflammation-related proteins in the blood of extremely low gestational age newborns. The contribution of inflammation to the appearance of developmental regulation

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Abstract

Background—We wanted to assess to what extent concentrations of circulating proteins appear to be developmentally regulated, and to what extent such regulation is influenced by intrauterine inflammation.

Methods—We measured 22 proteins in blood obtained on postnatal days 1, 7, and 14 from 818 children born before the 28th week of gestation for whom we also had information about placenta morphology.

Results—Within the narrow gestational age range of this sample, some protein concentrations increase in blood with increasing gestational age. More commonly, the concentrations of inflammation-related proteins decrease with increasing gestational age. We observed this inverse pattern both in children whose placenta was and was not inflamed.

Conclusions/inferences—Regardless of whether or not the placenta is inflamed, the concentrations of inflammation-related proteins in early blood specimens appear to be developmentally regulated with the most common pattern being a decrease with increasing gestational age.

1. Introduction

The term “developmental regulation” is applied to proteins whose expression or concentration varies with gestational age. Most studies of proteins in preterm newborns have compared the concentrations to those in full term newborns, and shown important differences [1].

The preterm newborn appears to have limited ability to synthesize proteins with anti-inflammatory characteristics [2-5]. In addition, some components of the fetal systemic inflammatory response appear to be considerably more vigorous in very preterm newborns than in children born at older gestational ages. [6-13]. Thus a proinflammatory imbalance of circulating immuno-regulatory proteins can be expected in preterm newborns in response to any inflammatory stimulus.

The likelihood of intra-uterine inflammation is much higher for the fetus destined to be born preterm than the fetus born closer to term [14]. Thus, the protein concentration differences between the preterm and the full term might reflect environmental, obstetrical and neonatal influences, just as readily as developmental regulation [15].

We wanted to see to what extent developmental regulation is apparent in the narrow gestational age range of 23 weeks to 27 weeks and 6 days, and to what extent patterns of developmental regulation in infants whose placenta was inflamed differed from those of infants whose placenta was not inflamed. This information has the potential to inform efforts to reduce the risk of inflammation-associated damage to the brain, lung, bowel, and retina that occur much more commonly among extremely low gestational age newborns than among those born closer to term [16,17]

2. Methods

2.1 The ELGAN Study

The ELGAN study was designed to identify characteristics and exposures that increase the risk of structural and functional neurologic disorders in ELGANs (the acronym for Extremely Low Gestational Age Newborns) [18]. During the years 2002-2004, women who did or might deliver before 28 weeks gestation at one of 14 institutions in 11 cities in 5 states were asked to enroll in the study. The enrollment and consent processes were approved by the individual institutional review boards.

Mothers were approached for consent either upon antenatal admission or shortly after delivery, depending on clinical circumstance and institutional preference. 1249 mothers of 1506 infants consented. Approximately 260 women were either missed or did not consent to participate. The 818 children who comprise the sample for this report had blood specimens collected on two or more days, histologic evaluation of the placenta, and a neurodevelopmental assessment at approximately 24 months post-term equivalent. About 40% of these children had an inflamed placenta (Table 1). A total of 642 children had a blood spot collected on each of the three protocol sampling days.

2.2 Newborn variables

The gestational age estimates were based on a hierarchy of the quality of available information. Most desirable were estimates based on the dates of embryo retrieval or intrauterine insemination or fetal ultrasound before the 14^{th} week (62%). When these were not available, reliance was placed sequentially on a fetal ultrasound at 14 or more weeks (29%), LMP without fetal ultrasound (7%), and gestational age recorded in the log of the neonatal intensive care unit (1%).

Blood spot collection

Drops of blood were collected on (Schleicher & Schuell 903) filter paper on the first postnatal day (range: 1-3 days), the 7th postnatal day (range: 5-8 days), and the 14th postnatal day (range: 12-15 days). All blood was from the remainder after specimens were obtained for clinical indications. Dried blood spots were stored at −70°C in sealed bags with desiccators until processed.

2.3 Elution of proteins from blood spots

For protein elution, 12mm punched biopsies of the frozen blood spots were submerged in 300 μL phosphate buffered saline containing 0.1% Triton X100 (Sigma-Aldrich, St. Louis, MO) and 0.03% Tween-20 (Fisher, Hampton, NH) ,vortexed for 30 seconds, and incubated on a shaker for 1 hour at 4°C. The buffer and biopsy were then transferred over the filter of a SpinX tube (Corning Fisher), centrifuged at 2000 x g and the filtered eluted blood collected. An additional wash of the punch was performed in 100 μL for a final elution volume of 400 μL.

2.4 Protein measurements

In this exploratory, descriptive evaluation, we used an electrochemiluminescence system to measure cytokines, chemokines, adhesion molecules, and proteinases. These proteins were measured in duplicate using the Meso Scale Discovery (MSD) multiplex platform and Sector Imager 2400 (MSD, Gaithersburg, MD), a system that has has been validated by comparisons with traditional ELISA [19,20]. The multiplex assays measuring up to 10 proteins simultaneously were optimized to allow detection of each biomarker within the linearity range of the eluted samples. The MSD Discovery Workbench Software was used to convert relative luminescent units into protein concentrations (pg/mL) using interpolation from several log calibrator curves. Split quality control blood pools tested on each plate showed inter-assay variation of <10-20% for each protein. The total protein concentration in each eluted sample was determined by BCA assay (Thermo Scientific, Rockford, IL) using a multi-label Victor 2 counter (Perkin Elmer, Boston, MA). The measurements of each analyte were then normalized as pg specific protein per mg total protein

The following are the 22 proteins: C-Reactive Protein (CRP), Serum Amyloid A (SAA), Myeloperoxidase (MPO), Interleukin-1β_(IL -1β), Interleukin-6 (IL-6), Interleukin-6 Receptor (IL-6R), Tumor Necrosis Factor-α (TNF-α), Tumor Necrosis Factor Receptor-1 (TNF-R1), Tumor Necrosis Factor Receptor-2 (TNF-R2), Interleukin-8 (IL-8; CXCL8), Monocyte Chemotactic Protein-1 (MCP-1; CCL2) , Macrophage Inflammatory Protein-1β (MIP-1β; CCL4), Regulated upon Activation, Normal T-cell Expressed, and [presumably] Secreted (RANTES; CCL5), Intercellular Adhesion Molecule -1 (ICAM-1; CD54), Intercellular Adhesion Molecule -3 (ICAM-3; CD50), E-selectin (CD62E), Vascular Cell Adhesion Molecule-1 (VCAM-1; CD106), Matrix Metalloproteinase-1 (MMP-1), Matrix Metalloproteinase-9 (MMP-9), Vascular Endothelial Growth Factor (VEGF), Vascular Endothelial Growth Factor Receptor-1(VEGF-R1; Flt-1), Vascular Endothelial Growth Factor Receptor-2 (VEGF-R2; KDR).

2.5 Placenta pathology

In keeping with the guidelines of the 1991 CAP Conference [21], representative sections were taken from all abnormal areas as well as routine sections of the umbilical cord and a membrane roll, and full thickness sections from the center and a paracentral zone of the placental disc. After training to minimize observer variability, study pathologists examined the slides for histologic characteristics listed on a standardized data form they helped create [22,23]. Briefly, at the chorionic plate of the disc, grade 3 acute inflammation was defined as neutrophils up to amnionic epithelium and stage 3 was defined as >20 neutrophils/20x). Grade 3 inflammation

of the external membranes, as well as of the chorion/decidua required numerous large or confluent foci of neutrophils.

Inflammation in the umbilical cord was graded from 0-5. Grade 3 required neutrophils in perivascular Wharton's jelly, grade 4 required panvasculitis and umbilical cord vasculitis extending deep into Wharton's jelly, and grade 5 required a 'Halo lesion' (ring of precipitate in Wharton's jelly encircling each vessel). Neutrophilic infiltration into fetal stem vessels in the chorionic plate required that neutrophils appeared to have migrated towards the amnionic cavity.

2.6 Data analysis

We evaluated the two broad hypotheses. First, the concentrations of inflammation-related proteins in the blood of ELGANs do not vary with gestational age within the narrow gestational range of 23 to 27 6/7 weeks during the first two weeks after birth. Second, the concentrations of these proteins in infants exposed to inflammation (as identified by placenta histology) do not differ from those of infants not so exposed.

We divided the 22 proteins into the categories of cytokines, chemokines, adhesion molecules, and matrix metalloproteinases because each category can each contribute to the traffic of cells out of blood vessels, and into surrounding parenchyma [24]. Each of these categories has the potential to influence how the traffic of cells influences debris removal and repair, or contributes to organ damage [25].

We did not calculate the probability that what we observed is due to chance, first because our goal was to be descriptive, and second, because p values can have little meaning when the three points in time are modeled as linear (when in fact this might not be appropriate) for 22 proteins at 3 gestational ages in the entire sample and in the two strata defined by the presence/absence of placenta inflammation. Rather, following the model of Matoba et al., we present the median concentration of each protein, along with 10^{th} and 90^{th} centiles [1]. We do this first for the entire sample, and then for the strata defined by the presence or absence of histologic inflammation of the placenta (defined as grade 3 and stage 3 inflammation of the chorionic plate, grade 3 inflammation of the chorion/decidua, neutrophilic infiltration of the fetal stem vessels in the chorionic plate, and grade 3 or higher inflammation of the umbilical cord).

3. Results

We present data in a standard format for six groups of proteins. Each cell in the six tables has the median and the 10^{th} and 90^{th} centiles of the respective protein concentration. The top third of each table has protein measurements for the entire sample, the middle third has measurements for children whose placenta was inflamed, while the lower third has measurements for children whose placenta was not inflamed. This format allows us to examine the relationship between fetal inflammation and protein concentrations during the first two postnatal weeks.

We assign the label "inverse developmental regulation" to the predominant pattern of concentrations declining with increasing gestational age.

3.1 Acute phase reactants and MPO (Table 2)

The concentrations of CRP decrease with increasing gestational age on all 3 days in the entire sample. For SAA, such a pattern is seen clearly only on day 1. Characterized by reduced concentration with increasing gestational age, this inverse pattern of developmental regulation is seen for CRP and SAA in the inflamed placenta sub-sample, most consistently on day 14 but not in the non-inflamed placenta sub-sample.

By definition, MPO is not an acute phase reactant because it is not synthesized by the liver. We include it here because its concentration tends to vary in ways similar to CRP and SAA. For example, the concentrations of MPO decrease with increasing gestational age on all 3 days in the entire sample, and on days 7 and 14 in the inflamed placenta sample. Only MPO shows this pattern in the non-inflamed placenta sub-sample.

3.2 Cytokines and their receptors (Table 3)

IL-1β, IL-6, the receptor for IL-6 (IL-6R), TNF-α, and its two receptors (TNF-R1, TNF-R2) all appear to be developmentally regulated in the entire sample on almost all days, and to just a slightly less prominent extent, among children whose placenta was not inflamed. Concentrations of some proteins (e.g., IL-1β , IL-6) tended to decrease with increasing postnatal age, especially in the inflamed placenta subgroup, while the concentrations of others (IL-6R, TNF-α , TNF-R1, TNF-R2) increased, at least in the youngest gestational age group. The day 1 concentrations of only two proteins, IL-1 β and TNF-α, were substantially higher among children whose placenta was inflamed than among children whose placenta was not inflamed.

3.3 Chemokines (Table 4)

IL-8 is the only chemokine to show the “inverse” pattern of developmental regulation in the entire sample, as well in the both the inflamed and non-inflamed placenta strata. In contrast, RANTES displayed a pattern of increasing concentrations with increasing gestational age on days 7 and 14, in the entire sample, as well in children whose placenta was or was not inflamed. MCP-1 showed the inverse pattern of developmental regulation on days 7 and 14 in the entire sample, the inflamed placenta sample, and non-inflamed placenta sample. The concentrations of MIP-1β consistently displayed the inverse pattern of developmental regulation in the entire sample and in both placenta strata on day 1, and less consistently on day 14.

3.4 Adhesion molecules (Table 5)

ICAM-1 had the inverse pattern of developmental regulation in the entire sample on all days, in the inflamed placenta sub-sample on days 7 and 14, and in the non-inflamed placenta sub-sample on day 14. The blood concentrations on days 1, 7, and 14 of ICAM-3 display an inverse pattern of developmental regulation in the entire sample and both placentas sub-samples. The blood concentrations of E-selectin also display this type of pattern, but less prominently.

The concentrations of VCAM-1 do not seem to be influenced by placenta inflammation.. An inverse pattern is suggested in the non-inflamed placenta sub-sample on day 1, and the opposite type of pattern (increasing concentrations with increasing gestational age) is suggested in blood obtained on day 14.

3.5 Matrix metalloproteinases (Table 6)

MMP-1 has the inverse developmental regulation pattern only on day 1 in the entire sample. More characteristic of this protein is the developmental regulation pattern of increasing concentrations with increasing gestational age, which is seen on days 7 and 14 in the entire sample and the inflamed sub-sample, and on day 14 in the non-inflamed placenta sub-sample. The concentrations of MMP-9 display the inverse pattern of developmental regulation on all days in the entire sample and in the non-inflamed placenta sub-sample, and on days 7 and 14 in the inflamed placenta sub-sample.

3.6 Vascular endothelial growth factor and its receptors (Table 7)

VEGF and its receptors, VEGF-R1 and VEGF-R2, exhibited the inverse pattern of developmental regulation on all days in the entire sample. In the non-inflamed placenta sub-

sample, VEGF-R2 had the inverse pattern on all days, while VEGF and VEGF-R1 showed the pattern on only some days. The day 1 concentrations of VEGF-R1 are modestly higher in the blood of infants whose placenta was not inflamed than in the blood of infants whose placenta was inflamed.

3.7 Summary of Tables 1-7 (Table 8)

Everyone of the proteins showed some evidence of developmental regulation, if only on one day and only in one subsample (VCAM-1). Seven proteins (IL-1beta, TNFalpha, IL-8, MCP-1, ICAM-3, E-SEL, and MMP-9) display the inverse pattern of developmental regulation on at least two of the three days in the entire sample and in both of the subgroups. If the developmental pattern was influenced by placenta inflammation, one would expect to see it in the day 1 specimen. Yet, the developmental regulation pattern often first became evident later in children whose placenta was inflamed. This is exemplified by IL-6, IL-6R, TNF-R1, TNF-R2, CRP, MCP-1, ICAM-1, ICAM-3, E-SEL, MMP-9, VEGF, VEGF-R1, VEGF-R2.

RANTES is the only protein not to show any evidence of the inverse pattern, while SAA is the only protein to show an inverse pattern in the inflamed placenta sample and the a hint of the direct pattern in the un-inflamed placenta sample, and MMP-1 is the only protein to show the inverse pattern early, only to show the direct pattern in later days.

4. Discussion

Six of our findings are worth comment. First, the concentrations of many inflammation-related proteins in the blood of ELGANs decrease with increasing gestational age, a pattern we label “inverse developmental regulation.” Second, this pattern is seen even within the narrow gestational range of 23 to 27 6/7 weeks. Third, the concentrations of these proteins are higher in newborns exposed to inflammation, as documented by placenta histology than in newborns without such documentation. Fourth, although more prominent in inflammation-exposed newborns, this inverse pattern was also seen in newborns who had no documented inflammation exposure. Fifth, the concentrations of some inflammation-related proteins in the blood of ELGANs increase with increasing gestational age. Sixth, the concentrations of VEGF-R1 tend to be higher in newborns not exposed to inflammation than in their peers who were exposed.

4.1 Inverse developmental regulation

We apply the label “inverse developmental regulation” to the decrease in protein concentration that accompanies increasing gestational age. Many proteins involved in structural and physiologic maturation of the fetus are either synthesized by the fetus or the mother/placenta in a pattern that provides adequate amounts of these proteins when needed, causing some of them to rise and others to decline with advancing gestational age [26]. One possible explanation for the overall trend of declining concentrations of immuno-inflammatory mediators with increasing gestational age is that higher values of these proteins in younger gestational ages help protect the fetus/newborn whose deficits in adaptive and innate immune function place them at risk of infections [27,28].

Every category of protein displayed this inverse pattern. Indeed, RANTES is the only one of the 22 proteins measured that at no time had the inverse pattern.

The median concentrations of inflammation-related proteins among children whose placenta was inflamed were only modestly higher than those of infants whose placenta was not inflamed. Elsewhere, we reported that an inflammation-related protein concentration in the top quartile on day-1 was considerably more common among newborns whose placenta was inflamed than

if the placenta did not show appreciable inflammation [Hecht, in preparation]. This pattern of a smaller shift of the median than of the 75th centile indicates a disproportionate increase in concentrations at the high end.

4.2 Narrow gestational range of this study

In most previous studies of protein concentrations in preterm newborns, term newborns serve**d** as the comparison group. We, however, have evaluated protein concentrations within a narrow gestational age range, 23 to 27 6/7 weeks. We are reluctant to extrapolate the significance of our findings beyond this narrow range.

4.3 Higher concentrations when placenta inflamed

The concentrations of inflammation-related proteins are higher in the blood of newborns whose placenta had histologic inflammation than in newborns whose placenta had no or reduced degrees of inflammation. This supports the hypothesis that placenta inflammation and a fetal inflammatory response are biologically linked. Both might share a common stimulus, or the stimulus might promote placenta inflammation, which, in turn, provokes a response evident in the fetal/newborn circulation.

We defined inflammation as at least grade 3 inflammation of the chorionic plate, the chorion/decidua, or the umbilical cord, as well as neutrophilic infiltration of the fetal stem vessels that give rise to the umbilical cord blood vessels. Although these are not equivalent lesions and our grouping them might have resulted in some heterogeneity, we are able to show that the fetal response is influenced to some extent by placenta inflammation.

We expected that the influence of processes related to placenta inflammation would be evident in blood collected on day 1. Yet, more often than not, the inverse pattern of developmental regulation first became evident after day 1 in children whose placenta was inflamed. This observation raises the possibility that some of the influence of intrauterine inflammation is delayed or increases after birth.

4.4 Inverse developmental regulation seen even when placenta not inflamed

Although more prominent in inflammation-exposed newborns, the inverse pattern was also seen in newborns who had no documented inflammation exposure. This finding can be interpreted in two ways. First, if these newborns were truly not exposed to inflammatory stimuli *in utero*, then the inverse pattern could be seen as characteristic of the fetus. Second, if these newborns were exposed to inflammatory stimuli *in utero* but their placentas did not become inflamed, then the inverse pattern might reflect more frequent or stronger inflammatory stimuli among the gestationally youngest.

4.5 The concentrations of some proteins increase with increasing gestational age

The concentrations of some of the proteins, exemplified by SAA among infants whose placenta was not inflamed, by RANTES (regardless of the presence or absence of placenta inflammation), and MMP-1, increase with increasing gestational age. This non-inverse pattern is typical of many proteins that we did not measure [29,30].

4.6 The concentrations of some proteins increase with increasing postnatal age

With advancing postnatal age, the concentrations of some proteins increased dramatically among infants whose placenta was not inflamed. E-selectin is the most prominent example, although other proteins also displayed this phenomenon (e.g., MMP-9, IL-6R, TNF-α, ICAM-1, and VEGF). The magnitude of these elevations raises the possibility that some of what we see represents responses to environmental stimuli rather than to endogenous signals.

4.7 VEGF-R1 concentrations and preeclampsia

The concentrations of VEGF-R1 tend to be higher in newborns not exposed to inflammation than in their peers who were exposed. Although, increased expression of VEGF-R1 is found in the placentas of infants born to preeclamptic women [31], and in the placentas of severely growth-restricted newborns [32], to our knowledge only one study has found elevated concentrations of VEGF-R1 in the blood of preterm infants born to preeclamptic women [33].

We have found an inverse relationship in the placenta between histologic characteristics of preeclampsia and those of inflammation [34,35]. On the other hand, some investigators suggest that preeclampsia and inflammation are linked [36,37]. Nevertheless, our data encourage us to continue to divide pregnancy disorders that lead to preterm delivery into those associated with inflammation, and those not.[34,35]

4.8 Sources of the proteins measured

The material available for measurement was the eluting solution after punched specimens of dried blood spots were soaked for an hour. As such, the proteins are not only those in the serum, but those released from cells in the circulation. We have normalized our protein measurements to the total protein concentration, but have not normalized them for WBC/dl. We urge caution in comparing our measurements to those obtained from serum or plasma collected directly from the newborn and not stored as a dried blood spot.

4.9 The future

In preterm newborns, inflammation-related proteins are either involved in, or markers of processes leading to damage in the lung [38], brain [39], bowel [40] and retina [41]. Consequently, a better characterization of what increases the concentrations of these proteins is likely to help identify ways to prevent such organ damage in the most vulnerable.

In summary, in a sample of preterm infants born within the narrow gestational range of 23 to 27 6/7 weeks, blood concentrations of most of the inflammation-related proteins we measured decrease with increasing gestational age, a pattern we label “inverse developmental regulation.” For many of these proteins, this pattern was seen regardless of whether or not the placenta was inflamed, although placenta inflammation was associated with higher blood levels.

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Table 1

Sample description. The numbers in each cell are of children for whom measurements were made of proteins in a blood spot obtained on the day that heads each column

		Number of children with placenta and proteins		
Sample	**GA**	**Day 1**	**Day 7**	**Day 14**
Entire	23-24	170	165	150
	25-26	367	369	340
	27	262	274	236
Inflamed placenta sub-sample	23-24	85	84	74
	25-26	161	165	151
	27	74	75	63
Non-inflamed placenta sub-sample	23-24	85	81	76
	25-26	206	204	189
	27	188	199	173
Total N		799	808	726

Table 2

Median (10^{th}, 90^{th} centiles) for 3 proteins (C-reactive protein, serum amyloid A and Myeloperoxidase) on 3 different days presented separately for 3 gestational age categories in the entire sample and then in samples stratified by whether or not the placenta was inflamed. Protein concentrations are pg/mg total protein

Protein		Median (10^{th} , 90^{th} centile)		
	GA	Day 1	Day 7	Day 14
Total sample				
CRP $\times 10^{-2}$	23-24	11 (1, 243)	11 (2, 103)	20 (2, 279)
	25-26	8 (1, 225)	11 (2, 105)	16 (2, 218)
	27	6 (1, 119)	7 (1, 79)	10 (1, 144)
SAA $\times 10^{-2}$	23-24	22 (1, 808)	7 (1, 223)	12 (2, 269)
	25-26	17 (2, 758)	8 (2, 109)	11 (3, 225)
	27	12 (1, 496)	7 (2, 91)	11 (3, 209)
MPO $\times 10^{-3}$	23-24	67 (18, ****)	86 929, 658)	109 (41, 4353)
	25-26	65 (18, 939)	69 (25, 214)	90 (35, 182)
	27	56 (16, 197)	43 (17, 146)	68 (22, 208)
Inflamed placenta				
CRP $\times 10^{-2}$	23-24	29 (1, 466)	15 (3, 100)	29 (3, 284)
	25-26	33 (2, 401)	15 (3, 86)	13 (2, 203)
	27	13 (1, 426)	6 (1, 58)	6 (1, 64)
SAA $\times 10^{-2}$	23-24	89 (3, 1066)	10 (2, 240)	14 (4, 201)
	25-26	99 (3, 1109)	7 (2, 90)	9 (3, 377)
	27	50 (2, 1185)	6 (2, 53)	7 (2, 61)
MPO $\times 10^{-3}$	23-24	86 (23, ****)	136 (53, ****)	114 (55, ****)
	25-26	96 (25, ***)	98 (43, 354)	82 (38, 171)
	27	86 (23, 351)	81 (32, ****)	60 (26, 153)
Non-inflamed placenta				
CRP $\times 10^{-2}$	23-24	5 (1, 117)	8 (1, 105)	14 (2, 264)
	25-26	3 (1, 85)	10 (2, 112)	19 (2, 229)
	27	4 (1, 63)	8 (1, 93)	14 (2, 204)
SAA $\times 10^{-2}$	23-24	5 (1, 323)	5 (2, 195)	11 (2, 274)
	25-26	6 (1, 183)	8 (2, 110)	11 (3, 113)
	27	9 (1, 149)	8 (2, 110)	14 (3, 298)
MPO $\times 10^{-3}$	23-24	55 (15, 264)	65 (23, 155)	108 (37, 332)
	25-26	50 (15, 146)	48 (20, 142)	98 (34, 190)
	27	45 (12, 143)	35 (15, 99)	72 (21, 218)

**** above the detectable range

Table 3

Median (10^{th}, 90^{th} centiles) for selected cytokines and their receptors on 3 different days by gestational age category in the entire sample and in strata defined by the presence or absence of histologic inflammation. Protein concentrations are pg/mg total protein

Protein		Median (10^{th} , 90^{th} centile)		
	GA	Day 1	Day 7	Day 14
Entire sample				
IL-1β × 10^3	23-24	72 (0, 463)	64 (0, 231)	75 (0, 228)
	25-26	48 (0, 234)	45 (0, 148)	55 (0, 180)
	27	37 (0, 218)	30 (0, 166)	48 (0, 175)
IL-6 × 10^3	23-24	28 (6, 698)	12 (5, 44)	14 (5, 40)
	25-26	23 (5, 421)	11 (4, 35)	11 (4, 46)
	27	16 (5, 153)	7 (2, 28)	7 (3, 24)
IL-6R	23-24	69 (28, 154)	118 (54, 216)	133 (58, 259)
	25-26	70 (32, 143)	114 (63, 200)	124 (63, 211)
	27	64 (29, 114)	105 (56, 179)	116 (61, 197)
TNF-α × 10^3	23-24	69 (11, 240)	81 (25, 189)	102 (40, 240)
	25-26	54 (17, 180)	61 (22, 142)	74 (29, 188)
	27	42 (11, 126)	44 (16, 119)	57 (18, 141)
TNF-R1	23-24	15 (6, 40)	21 (10, 47)	26 (12, 59)
	25-26	12 (5, 34)	16 (8, 34)	19 (8, 40)
	27	10 (4, 24)	12 (6, 25)	14 (7, 30)
TNF-R2	23-24	19 (7, 46)	27 (13, 60)	37 (17, 81)
	25-26	17 (7, 42)	25 (12, 50)	30 (14, 63)
	27	13 (6, 34)	19 (10, 39)	23 (12, 50)
Inflamed placenta				
IL-1β × 10^3	23-24	104 (14, 584)	80 (0, 300)	82 (22, 242)
	25-26	85 (6, 368)	50 (0, 160)	53 (0, 175)
	27	74 (0, 375)	57 (0, 266)	44 (0, 134)
IL-6 × 10^3	23-24	39 (6, 3446)	14 (5, 43)	15 (5, 54)
	25-26	48 (7, 1270)	11 (4, 31)	10 (4, 38)
	27	22 (5, 652)	8 (3, 35)	6 (1, 20)
IL-6R	23-24	68 (27,148)	133 (61. 244)	137 (67, 258)
	25-26	81 (32, 163)	128 (73, 211)	122 (73, 204)
	27	74 (30, 149)	117 (63, 206)	111 (55, 170)
TNF-α × 10^3	23-24	85 (25, 318)	87 (14, 184)	118 (39, 290)
	25-26	73 (24, 237)	68 (21, 150)	63 (27, 166)
	27	57 (10, 171)	49 (15, 190)	48 (7, 112)
TNF-R1	23-24	18 (8, 42)	26 (11, 54)	31 (13, 70)
	25-26	18 (8, 46)	19 (8, 38)	18 (9, 38)

Protein		Median (10^{th} , 90^{th} centile)		
	GA	Day 1	Day 7	Day 14
	27	14 (6, 37)	12 (7, 31)	12 (6, 27)
TNF-R2	23-24	22 (10, 53)	32 (13, 65)	40 (18, 85)
	25-26	23 (11, 54)	27 (13, 62)	32 (15, 64)
	27	21 (8, 47)	18 (10, 38)	19 (11, 36)
Non-inflamed placenta				
IL-1β $\times 10^3$	23-24	46 (0, 276)	52 (0, 172)	69 (0, 216)
	25-26	27 (0, 129)	41 (0, 145)	56 (0, 185)
	27	27 (0, 130)	24 (0, 112)	49 (0, 182)
IL-6 $\times 10^3$	23-24	26 (6, 310)	10 (4, 59)	13 (5, 29)
	25-26	19 (4, 114)	11 (4, 38)	11 (4, 48)
	27	15 (4, 105)	7 (2, 26)	8 (3, 27)
IL-6R	23-24	71 (29, 156)	103 (51, 189)	130 (54, 259)
	25-26	63 (33, 116)	102 (57, 184)	127 (61, 214)
	27	60 (28, 104)	100 (54, 165)	119 (63, 199)
TNF-α $\times 10^3$	23-24	53 (9, 175)	76 (30, 195)	83 (41, 196)
	25-26	41 (11, 115)	57 (22, 129)	81 (31, 215)
	27	36 (11, 105)	43 (16, 99)	58 (21, 160)
TNF-R1	23-24	14 (4, 36)	17 (9, 40)	25 (12, 52)
	25-26	9 (5, 24)	14 (8, 31)	20 (8, 42)
	27	8 (4, 21)	12 (6, 23)	16 (7, 31)
TNF-R2	23-24	17 (6, 36)	22 (12, 52)	34 (16, 74)
	25-26	12 (7, 29)	23 (11, 48)	28 (14, 61)
	27	11 (6, 25)	19 (11, 39)	26 (12, 52)

Table 4

Median (10^{th}, 90^{th} centiles) for selected chemokines on 3 different days by gestational age category in the entire sample and in strata defined by the presence or absence of histologic inflammation. Protein concentrations are pg/mg total protein

Protein		Median (10^{th} , 90^{th} centile)		
	GA	Day 1	Day 7	Day 14
Entire sample				
IL-8 (CXCL8) × 10	23-24	51 (12, 265)	61 (25, 179)	82 (28, 248)
	25-26	40 (12, 171)	42 (16, 116)	48 (19, 156)
	27	28 (9, 101)	22 (9, 81)	30 (11, 97)
MCP-1	23-24	113 (25, 678)	88 (34, 234)	102 (52, 242)
	25-26	92 (25, 536)	74 (33, 183)	88 (40, 203)
	27	93 (24, 583)	66 (29, 169)	77 (40, 155)
MIP-1β × 10	23-24	22 (2, 61)	13 (0, 47)	29 (3, 83)
	25-26	19 (0, 53)	12 (0, 39)	24 (2, 70)
	27	15 (0, 39)	13 (0, 38)	24 (3, 76)
RANTES $\times 10^{-1}$	23-24	38 (16, 83)	33 (13, 69)	54 (13, 102)
	25-26	41 (14, 86)	43 (17, 90)	59 (23, 119)
	27	34 (13, 75)	45 (15, 91)	69 (22, 129)
Inflamed placenta				
IL-8 (CXCL8) × 10	23-24	70 (23, 534)	69 (36, 205)	91 (38, 290)
	25-26	59 (20, 218)	49 (20, 120)	41 (18, 116)
	27	39 (12, 216)	30 (10, 128)	24 (10, 67)
MCP-1	23-24	84 (18, 623)	87 (29, 258)	98 (48, 221)
	25-26	87 (24, 781)	66 (30, 146)	86 (42, 180)
	27	65 (24, 669)	49 (22, 132)	70 (32, 116)
MIP-1β × 10	23-24	28 (8, 75)	18 (0, 69)	32 (6, 89)
	25-26	27 (7, 67)	16 (0, 45)	25 (5, 72)
	27	23 (0, 50)	18 (0, 51)	24 (2, 64)
RANTES $\times 10^{-1}$	23-24	40 (16, 90)	34 (17, 68)	57 (20, 116)
	25-26	46 (15,103)	47 (19, 97)	60 (21, 131)
	27	37 (18, 81)	55 (24, 95)	70 (27, 134)
Non-inflamed placenta				
IL-8 (CXCL8) × 10	23-24	36 (10, 149)	53 (22, 162)	72 (25, 242)
	25-26	31 (9, 108)	37 (14, 106)	55 (21, 197)
	27	24 (8, 88)	21 (9, 55)	31 (12, 136)
MCP-1	23-24	143 (37, 678)	91 (33, 226)	106 (65, 245)
	25-26	96 (25, 429)	87 (35, 224)	94 (37, 234)
	27	106 (24, 570)	74 (36, 184)	82 (43, 167)
MIP-1β × 10	23-24	17 (0, 44)	10 (0, 32)	28 (0, 80)

Protein		Median (10^{th} , 90^{th} centile)		
	GA	Day 1	Day 7	Day 14
	25-26	15 (0, 44)	9 (0, 35)	23 (2, 64)
	27	12 (0, 33)	12 (0, 33)	24 (3, 89)
RANTES $\times 10^{-1}$	23-24	35 (15, 78)	32 (12, 71)	51 (12, 90)
	25-26	37 (14, 75)	40 (16, 82)	57 (25, 117)
	27	33 (12, 67)	42 (14, 89)	67 (20, 129)

Table 5

Median (10^{th}, 90^{th} centiles) for selected adhesion molecules on 3 different days presented for 3 gestational age categories in the entire sample and in strata defined by the presence or absence of histologic inflammation. Protein concentrations are pg/mg total protein

Protein		Median (10^{th} , 90^{th} centile)		
	GA	Day 1	Day 7	Day 14
Entire sample				
ICAM-1 $\times 10^{-1}$	23-24	40 (18, 90)	75 (44, 177)	170 (82, 327)
	25-26	39 (19, 81)	78 (41, 153)	139 (62, 304)
	27	32 (16, 73)	71 (36, 130)	112 (50, 222)
ICAM-3	23-24	12 (3, 47)	22 (5, 69)	31 (9, 80)
	25-26	11 (3, 45)	17 (6, 53)	24 (8, 53)
	27	8 (3, 26)	13 (4, 42)	19 (4, 51)
E-SEL1 $\times 10^{-1}$	23-24	11 (3, 31)	25 (10, 59)	34 (14, 67)
	25-26	11 (4, 30)	23 (10, 46)	32 (14, 63)
	27	8 (3, 25)	16 (8, 36)	25 (11, 50)
VCAM-1 $\times 10^{-2}$	23-24	22 (11, 38)	26 (15, 40)	26 (14, 41)
	25-26	21 (12, 34)	26 (15. 41)	28 (16, 41)
	27	18 (10, 31)	25 (15, 41)	28 (17, 46)
Inflamed placenta				
ICAM-1 $\times 10^{-1}$	23-24	48 (22, 93)	81 (45, 222)	181 (96, 342)
	25-26	50 (26, 99)	80 (40, 143)	13 (63, 253)
	27	44 (18, 88)	63 (32, 114)	9 (44, 185)
ICAM-3	23-24	14 (4, 63)	31 (9, 84)	32 (11, 75)
	25-26	16 (4, 76)	25 (9, 71)	23 (8, 52)
	27	13 (4, 58)	23 (7, 67)	19 (4, 48)
E-SEL1 $\times 10^{-1}$	23-24	15 (6, 40)	30 (12, 65)	38 (15, 80)
	25-26	18 (7, 36)	26 (12, 56)	32 (15, 62)
	27	15 (5, 34)	19 (9, 46)	26 (11, 50)
VCAM-1 $\times 10^{-2}$	23-24	23 (13, 39)	26 (17, 41)	27 (16, 45)
	25-26	23 (13, 36)	28 (16, 43)	29 (18, 41)
	27	19 (10, 31)	26 (15, 37)	28 (17, 42)
Non-inflamed placenta				
ICAM-1 $\times 10^{-1}$	23-24	31 (17, 86)	70 (40, 141)	159 (77, 322)
	25-26	31 (17,60)	77 (44, 157)	150 (62, 334)
	27	29 (16, 63)	73 (37, 137)	120 (50, 246)
ICAM-3	23-24	10 (3, 38)	18 (4, 41)	27 (8, 82)
	25-26	9 (3, 26)	13 (5, 36)	24 (8, 55)
	27	7 (2, 20)	11 (3, 28)	19 (5, 52)
E-SEL1 $\times 10^{-1}$	23-24	8 (2, 22)	21 (8, 47)	33 (13, 60)

Protein		Median (10^{th} , 90^{th} centile)		
	GA	Day 1	Day 7	Day 14
Entire sample				
	25-26	7 (3, 20)	21 (9, 40)	31 (12, 64)
	27	7 (3, 16)	16 (8, 33)	24 (11, 50)
VCAM-1 $\times 10^{-2}$	23-24	22 (10, 36)	24 (12, 37)	25 (13, 40)
	25-26	20 (12, 33)	24 (15, 39)	27 (15, 41)
	27	18 (10, 32)	25 (14, 43)	29 (16, 48)

Table 6

Median (10^{th}, 90^{th} centiles) for 2 matrix metalloproteins (MMP-1 and MMP-9) on 3 different days presented separately for 3 gestational age categories in the entire sample and then in samples stratified by whether or not the placenta was inflamed. Protein concentrations are pg/mg total protein

		Median (10^{th}, 90^{th} centile)		
Protein	**GA**	**Day 1**	**Day 7**	**Day 14**
Entire sample				
MMP-1 $\times 10^{-1}$	23-24	66 (18, 171)	52 (17, 105)	65 (21, 174)
	25-26	59 (20, 150)	53 (24, 148)	68 (27, 204)
	27	57 (19, 148)	69 (20, 173)	90 (26, 250)
MMP-9 $\times 10^{-2}$	23-24	93 (25, 317)	173 (55, 659)	197 (68, 567)
	25-26	81 (26, 322)	141 (51, 430)	162 (66, 352)
	27	76 (23, 304)	97 (36, 299)	126 (47, 294)
Inflamed placenta				
MMP-1 $\times 10^{-1}$	23-24	66 (23, 174)	47 (22, 103)	66 (25, 183)
	25-26	75 (27, 175)	58 (27, 169)	73 (29, 239)
	27	70 (19, 197)	73 (18, 166)	98 (28, 278)
MMP-9 $\times 10^{-2}$	23-24	106 (25, 382)	306 (93, 855)	196 (99, 638)
	25-26	110 (28, 480)	219 (91, 589)	159 (65, 309)
	27	126 (26, 589)	190 (88, 547)	112 (50, 285)
Non-inflamed placenta				
MMP-1 $\times 10^{-1}$	23-24	64 (14, 167)	58 (14, 111)	60 (18, 142)
	25-26	46 (16, 114)	49 (23, 123)	64 (25, 187)
	27	51 (19, 131)	66 (20, 181)	83 (25, 241)
MMP-9 $\times 10^{-2}$	23-24	82 (25, 209)	114 (41, 405)	197 (60, 534)
	25-26	71 (26, 219)	95 (37, 302)	164 (68, 408)
	27	65 (22, 171)	79 (33, 172)	131 (45, 297)

Table 7

Median (10^{th}, 90^{th} centiles) for vascular endothelial growth factor (VEGF) and its receptors on 3 different days presented for 3 gestational age categories in the entire sample and in strata defined by the presence or absence of histologic inflammation. Protein concentrations are pg/mg total protein

Protein		Median (10^{th} , 90^{th} centile)		
	GA	Day 1	Day 7	Day 14
Entire sample				
VEGF × 10	23-24	60 (0, 191)	88 (25, 218)	112 (29, 215)
	25-26	57 (0 180)	87 (14, 181)	101 (33, 203)
	27	40 (0, 139)	62 (11, 153)	89 (31, 182)
VEGF-R1	23-24	18 (3, 70)	7 (0, 24)	9 (0, 24)
	25-26	14 (3, 57)	6 (0, 22)	7 (0, 22)
	27	13 (1, 81)	5 (0, 17)	7 (0, 17)
VEGF-R2	23-24	56 (27, 94)	62 (37, 106)	84 (41, 138)
	25-26	54 (29, 93)	61 (36, 106)	77 (45, 134)
	27	45 (23, 82)	58 (31, 95)	74 (42, 119)
Inflamed placenta				
VEGF × 10	23-24	78 (18, 225)	120 (54, 235)	122 (44, 229)
	25-26	89 (14, 236)	108 (37, 221)	97 (36, 194)
	27	76 (20, 234)	100 (35, 211)	91 (27, 183)
VEGF-R1	23-24	16 (3, 62)	8 (0, 31)	8 (0, 25)
	25-26	12 (1, 44)	7 (0, 23)	7 (0, 22)
	27	11 (1, 65)	5 (0, 54)	7 (0, 29)
VEGF-R2	23-24	60 (28, 95)	64 (41, 108)	89 (52, 150)
	25-26	60 (34, 101)	64 (37, 116)	79 (45, 131)
	27	51 (22, 108)	62 (30, 85)	67 (35, 108)
Non-inflamed placenta				
VEGF × 10	23-24	39 (0, 136)	60 (11, 144)	108 (23, 193)
	25-26	41 (0, 145)	68 (0, 153)	104 (32, 210)
	27	31 (0, 98)	54 (8, 130)	88 (31, 182
VEGF-R1	23-24	21 (5, 76)	6 (0, 18)	9 (0, 23)
	25-26	18 (4, 76)	6 (0, 20)	6 (0, 21)
	27	14 (2, 90)	4 (0, 16)	6 (0, 17)
VEGF-R2	23-24	53 (26, 94)	60 (33, 93)	79 (36, 124)
	25-26	47 (28, 81)	59 (35, 101)	76 (46, 140)
	27	44 (23, 78)	57 (31, 99)	77 (44, 121)

Table 8

Summary table of the pattern of protein concentrations seen among the three gestational age categories. The inverse pattern of developmental regulation (concentrations tend to decrease with increasing gestational age) is identified with ∇, while the direct pattern of developmental regulation (concentrations tend to increase with increasing gestational age) is identified with ▲. The absence of a symbol in a cell indicates that the variation with gestational age did not fit either of these patterns

		Total sample			Placenta define sub-samples					
Category	**Protein**				**Inflamed**			**Non-inflamed**		
	Day	1	7	14	1	7	14	1	7	14
Cytokines and receptors	IL-1beta	∇	∇	∇	∇		∇	∇	∇	∇
	IL-6	∇	∇	∇		∇	∇	∇		∇
	IL-6R	∇	∇	∇		∇	∇	∇		∇
	TNFalpha	∇	∇	∇	∇	∇	∇	∇	∇	∇
	TNF-R1	∇	∇	∇		∇	∇		∇	∇
	TNF-R2	∇	∇	∇		∇	∇			∇
Systemic inflammation	CRP	∇	∇	∇			∇			
	SAA	∇					∇			▲
Chemokines	IL-8(CXCL8)	∇	∇	∇	∇	∇	∇	∇	∇	∇
	MCP-1 (CCL2)		∇	∇		∇	∇		∇	∇
	MIP-1β (CCL4)	∇			∇			∇	∇	∇
	RANTES(CCL5)		▲	▲		▲	▲		▲	▲
Adhesion molecules	ICAM-1 (CD54)			∇		∇	∇			∇
	ICAM-3 (CD50)		∇	∇		∇	∇		∇	∇
	E-SEL (CD62E)	∇	∇	∇		∇	∇	∇	∇	∇
	VCAM-1 (CD106)								∇	
Metallo-proteinases	MMP-1	∇	▲	▲		▲	▲	∇	▲	▲
	MMP-9	∇	∇	∇		∇	∇	∇	∇	∇
Angiogenic proteins	VEGF	∇	∇	∇		∇	∇			∇
	VEGF-R1	∇	∇	∇			∇	∇		
	VEGF-R2	∇	∇	∇			∇	∇	∇	