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The relationship between neonatal blood protein profiles and placenta histologic characteristics in extremely low gestation age newborns

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Abstract

Amniotic fluid infection with chorioamnionitis is associated with increased risks of morbidity and mortality in children born prematurely. These risks depend on the presence of a fetal inflammatory response. We measured the concentrations of 25 proteins in the blood of 871 infants born before the 28th week of gestation, and examined their placentas for acute inflammation. Newborns who had inflammatory lesions of the placenta were much more likely than their peers ($p<0.01$) to have elevated blood concentrations of cytokines (IL-1β, IL-6, TNF-alpha), chemokines (IL-8, MIP-1β, RANTES, I-TAC), adhesion molecules (ICAM-1, ICAM-3, E-selectin), matrix metalloproteinases (MMP1, MMP -9), the angiogenic inflammatory factor VEGF and its receptor VEGF-R2 as well as acute phase proteins (SAA and CRP) during first three days after birth. In contrast, newborns with poor placental perfusion had lower levels of inflammatory proteins ($p<0.01$, IL-6, RANTES, ICAM-1, ICAM-3, VCAM-1, E-selectin, MMP-1, MMP-9, MPO, VEGF). An inverse pattern was found between newborn levels of VEGF and its competitive inhibitor VEGF-R1 in both the inflamed and poorly perfused placenta categories. These results confirm the predictive value of placental histology for the presence or absence of elevated inflammatory response in the newborn.

Introduction

Amniotic fluid infection with chorioamnionitis is associated with morbidity and mortality in children born prematurely. (1,2) The risk of injury is associated with the presence of a fetal inflammatory response.(3) We have previously characterized the placentas from a cohort of babies born prior to 28 weeks gestation and shown associations between histologic inflammation and recovery of specific microorganisms,(4,5) pregnancy disorders leading to preterm delivery, (5) as well as ultrasound lesions of the brain and cerebral palsy diagnoses. (6)

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Pregnancy disorders have been divided into those associated with inflammation and those not, largely based on histology signs of inflammation in the placenta.(5) This study assesses a broad range of inflammatory markers (25 cytokines, chemokines, adhesion molecules, tissue remodeling factors and acute phase proteins) in the blood of a large cohort of extremely low gestation age newborns (ELGAN) during the first three days of life to establish the significance of associations with placental inflammatory lesions which may serve as a basis for prevention and improved management of inflammation related morbidity in preterm infants.

Methods

Population and sample collection

Placentas and blood samples were collected as part of a study designed to identify factors that increase risk for structural and functional neurologic disorders in ELGANs (the acronym for Extremely Low Gestational Age Newborns). The details of the population as well as placental collection are described elsewhere.(4) Women delivering before 28 weeks gestation at one of 14 participating institutions were asked to enroll in the study. The enrollment period covered years 2002–2004, 1250 mothers of 1506 infants consented (an estimated 260 mothers were missed or declined to participate),1411 placentas were submitted for pathologic evaluation (totals refer to the number of umbilical cords; i.e. twins are counted as 2 placentas), 871 newborns had both blood samples and placentas available for analysis. The study was conducted in accordance with human subject research guidelines and the Declaration of Helsinki and was approved by Institutional Review Boards at each participating institution.

Drops of blood were collected on Schleicher & Schuell 903 paper (Whatman International Ltd, Florham Park, NJ) on the first postnatal days (range: 1–3 days), allowed to air-dry and stored at −70°C in sealed bags with desiccators until processed. All blood was from the remainder after specimens were obtained for clinical indications.

Placental examination

The procedures for gross and microscopic examination of the placentas as well as the training and inter-pathologist validation have been previously described.(4)

Placental signs of poor perfusion including infarcts and intervillous fibrin, fetal stem vessel thrombosis, and decidual hemorrhage and fibrin deposition were coded as present or absent. Chorionic villi were scored for subjective increase in syncytial knots; few knots are expected prior to the third trimester.

Inflammation of the membranes was described in detail. At the chorionic plate of the disc, acute inflammation was assigned a stage from 0–3 (0 is none, 1 is neutrophils collecting in subchorionic space, 2 is neutrophils into chorionic plate, 3 is neutrophils up to amnionic epithelium). The grade of inflammation at the plate ranged from 1–3 (1 is 1–9 neutrophils, 2 is 10–19 neutrophils, 3 is >20 neutrophils, all recorded at 20×). Inflammation of the free membranes (chorion/decidua) was graded from 0–4 (0 is none, 1 is single focus of 5–10 neutrophils, 2 is several small foci or single focus of >10 neutrophils, 3 is numerous large or confluent foci, 4 is necrotizing).

The fetal inflammatory response was gauged by inflammation in the umbilical cord, which was graded from 0–5 (0 is none, 1 is neutrophils within the inner third of one umbilical vessel, 2 is neutrophils within the inner third of at least two umbilical vessels or through the wall of one vessel, 3 is neutrophils in perivascular Wharton's jelly, 4 is inflammation extending deep into Wharton's jelly, 5 is 'Halo lesion'; ring of precipitate in Wharton's jelly

encircling each vessel). Neutrophilic and eosinophilic infiltration into fetal stem vessels in the chorionic plate was also noted as present or absent.

Blood spot elution and protein analysis

All blood spots were from drops of blood that remained in the needle or syringe after specimens were obtained for clinical indications. The earliest specimen was the one analyzed. 67 % were obtained on day 1, 33% were obtained on day 2, and 0.23% on day 3. Dried blood spots were stored at −70degC in sealed bags until processed. All blood spots were processed for elution and protein analysis in a central laboratory (Laboratory of Genital Tract Biology, Brigham and Women's Hospital) using standardized operational procedures as described below. For protein elution, the frozen dried blood spots (DBS) were punched using 12 mm disposable biopsy AcuPunch (Acuderm, Inc, Fort Lauderdale, FL). The punched paper specimen was submerged in 300 μL PBS-based buffer containing 0.1% Triton X100 (Sigma-Aldrich, St. Louis, MO) and 0.03% Tween-20 (Fisher, Hampton, NH) vortexes for 30 sec and incubated on a shaker for 1h at 4°C. The punched paper along with the buffer were then transferred over the filter of a SpinX tube (Corning Fisher), centrifuged at 2000 × g followed by collection of the filtered eluted blood. An additional wash of the paper punch was performed in 100 μL for a final elution volume of 400 μL. The eluted blood samples were aliquoted and stored frozen at −70°C in bar-coded air-tight microtubes (USA Scientific, Orlando FL).

Proteins were measured in duplicate using the Meso Scale Discovery (MSD) multiplex platform and Sector Imager 2400 (MSD, Gaithersburg, MD). This electrochemiluminescence (ECL) detection system has been validated by comparisons with traditional ELISA. (7,8) The ECL multiplex assays, measuring up to 10 proteins simultaneously, were optimized to allow detection of each biomarker within the linearity concentration range of the eluted samples. The MSD Discovery Workbench Software was used to convert relative luminescent units into protein concentrations 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) and the measurements of each inflammatory marker were normalized to mg total protein.

We measured the following 25 proteins: 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), Monocyte Chemotactic Protein-4 (MCP-4; CCL13), Macrophage Inflammatory Protein-1β (MIP-1β; CCL4), Regulated upon Activation, Normal T-cell Expressed, and [presumably] Secreted (RANTES; CCL5), Interferon-inducible T cell Alpha-Chemoattractant (I-TAC; CXCL11), Intercellular Adhesion Molecule-1 (ICAM-1; CD54), Intercellular Adhesion Molecule-3 (ICAM-3; CD50), Vascular Cell Adhesion Molecule-1 (VCAM-1; CD106), E-selectin (CD62E), Matrix Metalloproteinase-1 (MMP-1), Matrix Metalloproteinase-9 (MMP-9), C-Reactive Protein (CRP), Serum Amyloid A (SAA), Myeloperoxidase (MPO), Vascular Endothelial Growth Factor (VEGF), Vascular Endothelial Growth Factor Receptor-1(VEGF-R1; Flt-1), Vascular Endothelial Growth Factor Receptor-2 (VEGF-R2; KDR), Insulin Growth Factor Binding Protein-1 (IGFBP-1). This choice of proteins provided a broad and redundant representation of all categories of inflammatory mediators e.g. cytokines and their receptors, chemokines, adhesion molecules, tissue remodeling factors and acute phase proteins (Table 1) while the numbers of biomarkers in each group was determined by the reliable simultaneously detection in three platforms of similar dynamic ranges.

Data analysis

We evaluated the generalized null hypothesis that the risk of a blood protein concentration in the highest quartile for gestational age was not associated with any histologic characteristic of the placentas.

The protein concentrations varied with gestational age at delivery and therefore for the purposes of statistical analysis the samples were divided into 3 groups defined by gestational age (23–24, 25–26, 27 weeks). We dichotomized the distribution of each protein's concentration into the highest quartile and the lower three quartiles based on the rationale that the most extreme levels of inflammatory mediators would be most biologically relevant. To control the variation of protein concentrations with gestational age at delivery, we dichotomized each protein's concentration separately in each of the 3 gestational age groups (23–24, 25–26, 27 weeks).

Our unit of measurement is the odds ratio (and 99% confidence interval) that newborns whose placenta had each histologic finding were more or less likely to have a protein measurement in the top quartile as compared to newborns without that placental characteristic. We selected the 99% confidence interval rather than the conventional 95% confidence interval because we wanted to modify our analyses for multiple comparisons (25 proteins and 7 histologic characteristics), while not appreciably increasing the risk of a type 1 (false negative) error. Consequently, only p-values <0.01 were considered statistically significant. (9)

The odds ratios and 99% confidence intervals for Tables 2 and 3 were calculated with logistic regression equations, and for Table 4 with multinomial (also known as polytomous or polychotomous) logistic regression models. For each placental variable we report the odds ratio of having the concentration of each blood protein in the highest quartile for gestational age after adjustment for gestational age.

Results

Incidence of chorionic and umbilical cord inflammation versus poor perfusion of ELGAN placentas

We observed intense inflammation of the chorionic plate in 18% of placentas, of the cord in 15%, and of the chorionic plate vessels in 23%. Inflammation of the chorionic plate, membranes and umbilical cord tended to occur preferentially in the presence of each other. Infarct and increased syncytial knots also tended to occur with one another, but these two groups of lesions occurred in the same placenta only about 10% of the time. Morphologic features associated with poor utero-placental perfusion including infarcts, increased syncytial knots, and decidual hemorrhage suggesting abruption were seen in 15%, 18% and 16% of placentas respectively. The frequency of perfusion related lesions was inversely related to those associated with inflammation. Younger gestational age favored inflammation (Table 1), especially when the inflammation was intense. A subset of 10 placentas showed isolated umbilical cord inflammation. We identified cord inflammation in 3% of placentas that had no chorionic inflammation and in 8% that did not have chorionic plate vasculitis. (10)

Association between inflammatory biomarkers in ELGAN blood and placental inflammation

Although the incidence of inflammation in the placenta declined with increasing gestational age (23–24 weeks: 85/170=50%; 25–26 weeks: 161/367=44%; 27 weeks: 74/262=28%), the newborn protein concentrations associated with placental inflammation declined minimally

with age at birth. The majority of the inflammation-related proteins adjusted by gestational age were elevated in newborns whose placenta had moderate to severe inflammation (Table 2–3). Among these proteins were cytokines (IL-1β, IL-6, TNF-α), cytokine receptors (IL-6R, TNF-R1, TNF-R2), chemokines (IL-8, MIP-1β, RANTES, I-TAC), adhesion molecules (ICAM-1, ICAM-3, VCAM-1, E-selectin), matrix metalloproteinases (MMP-1, MMP-9), systemic inflammation markers (CRP, SAA, MPO), an angiogenic protein (VEGF), and one of its receptors (VEGF-R2). Blood proteins showed a dose-response pattern to inflammation that was best seen with increasing stage of neutrophils infiltration at the chorionic plate. Data for moderate to severe inflammation in the membranes and vasculitis of the fetal stem vessel are not shown, but were also strongly associated with elevated blood proteins. Infants whose placenta had histologic inflammation were at reduced risk of having an elevation of two proteins with angiogenic properties (VEGF-R1 and IGFBP-1). An almost inverse pattern was characteristic of infants whose placenta had syncytial knots or infarcts (Table 4).

The highest quartile concentrations of MPO, a marker of neutrophil activation, and the acute phase proteins CRP and SAA were significantly associated with inflammation of the chorionic plate or membranes regardless of the involvement of the umbilical cord (Table 5). The 10 Infants whose placentas had isolated umbilical cord inflammation were also at increased risk of elevated concentrations of these three proteins, but the increase was not statistically significant.

Association between inflammatory biomarkers in ELGAN blood and histologic signs of poor placental perfusion

In contrast to newborn with inflamed placentas, newborns with signs of poor placental perfusion expressed either no risk or significantly lower risk of elevated inflammatory proteins in their blood (Table 4). They were less likely to have higher levels of IL-6, RANTES, ICAM-1, ICAM-3, VCAM-1, E-selectin, MMP-1, MMP-9 and MPO ($p<0.01$) if both infarcts and increased syncytial knots were observed. The presence of decidual hemorrhage and fibrin deposition was neutral in term of risk for elevated inflammatory proteins. VEGF was decreased only in the newborns with syncytial knots in the placenta and occurred at the background of increased VEGF-R1, again confirming the inverse relationships between these two biomarkers seen albeit in the opposite direction in the presence of inflammation. VEGF-R2 and IGFBP-1 showed also negative association with poor placental perfusion in contrast to the positive association observed with inflammation.

Discussion

We have shown an association between placental inflammation and a fetal inflammatory response that is consistent with previous studies of term and pre-term births.(11) Previous work has shown similar associations, most commonly for IL-6 and IL-8,(12,13) as well as IL-1β, TNF-α and MMP-9, and other chemokines, regulatory proteins, and growth factors. (14–16) The range of biomarkers analyzed in this study is one of the largest simultaneously assessed in published reports and represents all categories of inflammatory mediators.

Placental inflammatory infiltrates include both maternal and fetal contributions. Chorionic plate infiltration is viewed as histologic evidence of a maternal response, (17,18) while inflammation of umbilical cord vessels and fetal stem vessels in the chorionic plate, so called fetal vasculitis, are the histologic hallmarks of a fetal inflammatory response. (4,11,13,19) Neonatal morbidity has tended to be better predicted by fetal vasculitis than by maternal inflammation at the chorionic plate. (11,19)

However, we describe a strong association between histologic markers of both maternal and fetal inflammation and systemic inflammatory response in the newborn. The intensity of placental inflammation is thought to evolve in a sequence developing from chorionic plate inflammation to fetal vasculitis at the chorionic plate to cord inflammation.(18) The intensity may reflect the duration and extent of infection.(20) This sequence implies that maternal response precedes fetal response. Consistent with this model, we observe that most cord inflammation occurs in combination with high grade/stage plate inflammation. In addition, we see a dose response relationship between maternal fetal plate inflammation and blood proteins (tables 2–3).

Despite the model, an accelerated fetal response may sometimes occur. A subset of placentas showed isolated umbilical cord inflammation. Similar findings have been observed in 5–8% of preterm and 17% of term placentas.(21) Infants with cord-only inflammation tended to have elevated concentrations of blood acute phase reactants. Although the maternal response is generally thought to precede the fetal response, this subset of cases suggests that an accelerated fetal response is possible. We have previously reported that some microorganisms including Actinomyces sp., Group B, Group D, and alpha-hemolytic Streptococci are more likely to promote fetal vasculitis than high-grade chorionic plate inflammation.(4)

This study also shows that the inflammatory response is not merely a local neutrophil mediated process in the placenta, but is systemic. The fetus is exposed to intra-amniotic infection at three interfaces; the subamniotic tissue of the placental disc and cord, in the lungs, and in the GI tract through oral intake of amniotic fluid. With placental histologic inflammation, we see increased levels of circulating factors that reflect neutrophil activation (MPO), and endothelial activation allowing chemotaxis and leukocyte migration (*e.g.*, MMP-9, E-selectin, VCAM-1, ICAM-1, and ICAM-3). We also see a systemic response in the acute phase reactants produced by the liver (SAA, CRP). In sepsis, circulating blood cells and vascular cells produce TNF-α and IL-1β leading to activation of nuclear factor-kappa B (NF-κB) and subsequent production of IL-6, IL-8, and IFNγ which mediate systemic effects including acute-phase reactants such as CRP and SAA as well as MMP-9. TNF-α and IL-1β can also induce premature labor.(22)

Some of the vulnerability of the brain, lung, bowel, and eye in extremely low gestational age newborns has been attributed to their propensity to respond to inflammatory stimuli more vigorously than infants born at term.(12) Consequently, our finding such strong links between circulating proteins and evidence of a putative stimulus raises the possibility that these circulating proteins might be intermediates between the inflammatory stimulus and organ damage.

Because our study utilized whole blood lysates, our analyses are based on measuring both the soluble and cell-bound forms of membrane receptors (*e.g.* E-selectin, VCAM-1, ICAM-1, ICAM-3, TNF-R1 and 2, IL-6R, and VEGF-R1 and 2). Circulating forms of VCAM-1, E-selectin, and ICAM-1 have been detected in plasma and are elevated during systemic inflammatory conditions as well as on endothelial cells. The origins of circulating VCAM-1, E-selectin, and ICAM-1 are unclear, but they may arise from shedding or proteolytic cleavage from endothelial cells.(23–25) Similarly, other membranous proteins (*e.g.*, TNF-α receptors) undergo shedding through the actions of MMPs and thus increased levels of these receptors may reflect MMP upregulation in addition to upregulation of the specific receptor genes. (26)

The concentrations of a few protein biomarkers (*e.g.*, IGFBP-1 and VEGF-R1), decreased with placental inflammation and increased with syncytial knots and infarcts, which are

presumably histologic indicators of vascular insufficiency. The concentrations of IGFBP-1, one of the binding proteins that control serum levels of insulin-like growth factor, and VEGF-R1, the soluble fms-like tyrosine kinase-1,(27) are abnormally high in women who have preeclampsia, a syndrome associated with poor placental vascularization and fetal growth restriction. *In-vitro* IGFBP-1 can be induced by chronic hypoxia.(28) Thus, an increased level might be expected with histologic characteristics attributed to vascular insufficiency. The low levels of IGFBP-1 seen with histologic inflammation may be explained by increased degradation by MMP-9 and other MMPs that were not measured in this study since this phenomenon has been observed in inflamed amniotic fluid. (29)

Newborns whose placenta had infarcts and increased knots were unlikely to show increased concentrations of inflammatory proteins. This is consistent with previous observations that vascular and inflammatory characteristics of the placenta tend not to occur together.(5,30)

Vascular endothelial growth factor (VEGF) promotes blood vessel formation and endothelial maintenance when bound to the second of its circulating receptors, VEGF-R2 (KDR/Flk-1). VEGF is upregulated by proinflammatory activation downstream from IL-1β and TNF-α signaling.(31) In keeping with this, we found that VEGF concentrations were elevated in newborns whose placenta had both maternal and fetal inflammation and lower in newborns whose placenta did not have inflammation, but did have syncytial knots and or infarcts.

The first VEGF receptor, VEGF-R1 (Flt-1), appears to function as a competitive inhibitor, minimizing the physiologic capability of VEGF bound to it. VEGF-R1 in the maternal circulation, likely produced by placental trophoblast, is elevated in preeclampsia. (12) VEGF-R1 in the newborn has not been extensively reported. We were not surprised to see elevated concentrations of day-1 VEGF-R1 in newborns whose placenta had infarcts and increased syncytial knots since both these histologic lesions are common in preeclampsia. However, decreased VEGF-R1 associated with placental inflammation was a new finding and may be a result of physiologic degradation of VEGF-R1 by matrix metaloproteinases. This is a normal regulatory function of MMP that increases the bioavailability of VEGF for endothelial cells.(32) It is possible that inflammation in the placenta or systemic activation of MMP may have a similar but non-localized effect.

Our not finding any significant relationship between decidual hemorrhage (i.e. abruption) and any protein in the newborn's blood might truly indicate no relationship. On the other hand, abruption is seen among preterm deliveries associated with both severe chorioamnionitis(33) and preeclampsia.(34) Perhaps this heterogeneity diminished our ability to identify a relationship between inflammation and decidual hemorrhage

The major strengths of our study are the large number of proteins uniformly measured at one time, the large number of infants born before the 28th week of gestation, selection of our sample on the basis of gestational age rather than birth weight, and recording of all histologic findings in a uniform manner after efforts to reduce observer variability. A limitation of our study is the small number of newborns who had isolated umbilical cord inflammation. We were also, as in all observational studies, unable to distinguish between causation and association as explanations for what we found.

In summary, we found a strong inflammatory signal in the blood of newborns delivered before the 28th week of gestation whose placenta had moderately severe inflammation of the chorionic plate alone, severe inflammation of both chorionic plate and umbilical cord inflammation, or severe inflammation of just umbilical cord alone. Our findings suggest a need for placental examination in all ELGAN with emphasis on a detailed description of the pattern of inflammation since the presence of placental inflammation predicted increased

odds ratios of newborn inflammatory response within the first three days of life regardless of gestation age. Histologic placental inflammation, especially when of high stage in the chorionic plate or causing fetal vasculitis should be regarded as a fetal inflammatory response. This information may be useful in stratifying ELGAN for studies of intervention.

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Table 1

Percent of children classified by gestational age who also had the characteristics listed on the left. These are column percents.

		Gestational age (weeks)		
Characteristic		**23–24**	**25–26**	**27**
Birth weight (grams)	500–749	88	32	13
Birth weight Z-score	<−2	2	8	4
Number of fetuses	One (singleton)	68	70	60
Antenatal steroids	Full course	61	63	71
Indication for delivery	Labor	51	44	43
	Membrane rupture	19	22	22
	Cervical insufficiency	5	12	18
	Placenta abruption	15	13	9
	Preeclampsia	9	5	3
	Fetal indication	1	4	6
Placenta bacteriology	Any organism	68	46	40
Inflammation of chorionic plate	Stage 1	10	11	10
	Stage 2	12	13	8
	Stage 3	42	28	20
Inflammation of umbilical cord	Grade 1–2	10	14	12
	Grade 3	12	9	6
	Grade 4–5	6	12	7
Inflammation of fetal stem vessels	Present	31	29	20
Infarct	Present	13	17	20
Syncytial knots	Increased	15	17	28
Column N		169	367	262

Table 2

Odds ratios (99% confidence intervals) of a concentration in the top quartile (for gestational age) of the protein listed on the left among children whose placenta had the stage of chorionic plate inflammation that heads each column compared to the infants whose placenta had no chorionic plate inflammation. Adjustment has been made for gestational age category (*i.e.*, 23–24, 25–26, and 27 weeks).

	Acute inflammation of the chorionic plate		
Protein	**Stage 1**	**Stage 2**	**Stage 3**
Cytokines and their receptors			
IL-1β	1.4 (0.7, 2.9)	2.2 (1.1, 4.3)*	4.4 (2.7, 7.1)*
IL-6	2.2 (1.1, 4.5)*	3.1 (1.6, 6.1)*	2.8 (1.7, 4.6)*
IL-6R	1.1 (0.5, 2.4)	1.5 (0.7, 3.1)	3.4 (2.1, 5.7)*
TNF-α	1.4 (0.7, 2.9)	1.5 (0.7, 3.0)	3.6 (2.2, 5.9)*
TNF-R1	1.3 (0.6, 2.8)	1.5 (0.7, 3.1)	3.6 (2.2, 6.0)*
TNF-R2	1.3 (0.6, 3.0)	2.4 (1.2, 5.0)*	5.9 (3.5, 9.9)*
Chemokines			
IL-8 (CXCL8)	1.2 (0.6, 2.6)	1.8 (0.9, 3.6)	3.1 (1.9, 5.1)*
MCP-1 (CCL2)	0.9 (0.4, 1.9)	0.9 (0.4, 1.8)	0.8 (0.5, 1.4)
MCP-4 (CCL13)	1.0 (0.5, 1.9)	0.7 (0.4, 1.5)	0.9 (0.5, 1.4)
MIP-1 β (CCL4)	1.5 (0.7, 3.2)	2.3 (1.2, 4.6)*	3.8 (2.3, 6.3)*
RANTES (CCL5)	3.4 (1.7, 6.6)*	2.5 (1.1, 4.5)*	2.3 (1.4, 3.9)*
I-TAC (CXCL11)	1.9 (0.9, 4.0)	2.1 (1.04, 4.4)*	5.7 (3.4, 9.5)*
Adhesion molecules			
ICAM-1 (CD54)	1.4 (0.6, 3.1)	3.0 (1.5, 6.1)*	4.9 (2.9, 8.2)*
ICAM-3 (CD50)	2.5 (1.2, 5.2)*	2.7 (1.3, 5.6)*	6.2 (3.7, 11)*
VCAM-1 (CD106)	1.4 (0.7, 2.8)	1.3 (0.6, 2.6)	1.8 (1.1, 2.9)*
E-selectin (CD62E)	2.7 (1.2, 5.9)*	4.2 (2.1, 8.7)*	10 (5.8, 18)*
Matrix metalloproteinases (MMPs)			
MMP-1	0.8 (0.4, 1.8)	2.0 (1.03, 3.9)*	2.2 (1.3, 3.5)*
MMP-9	2.4 (1.1, 5.2)*	2.5 (1.2, 5.3)*	6.8 (4.0, 12)*
Other indicators of inflammation			
CRP	1.8 (0.8, 3.9)	2.5 (1.2, 5.1)*	4.9 (2.9, 8.3)*
SAA	2.2 (1.00, 4.7)	4.2 (2.1, 8.4)*	5.4 (3.1, 9.2)*
MPO	1.4 (0.6, 3.0)	2.9 (1.5, 5.7)*	4.7 (2.8, 7.8)*
Growth factors, their receptors, and binding proteins			
VEGF	1.9 (0.9, 4.0)	2.9 (1.5, 5.9)*	6.2 (3.8, 10)*
VEGF-R1 (Flt-1)	0.6 (0.3, 1.2)	0.5 (0.2, 1.1)	0.5 (0.3, 0.9)**
VEGF-R2 (KDR)	2.0 (0.98, 3.9)	1.9 (0.95, 3.7)	2.8 (1.7, 4.5)*
IGFBP-1	0.7 (0.4, 1.5)	0.5 (0.3, 1.1)	0.4 (0.2, 0.6)**

	Acute inflammation of the chorionic plate		
Protein	**Stage 1**	**Stage 2**	**Stage 3**
N	82	88	223

* Significantly increased risk, p<0.01

** Significantly reduced risk, p<0.01

Table 3

Odds ratios (99% confidence intervals) of a concentration in the top quartile (for gestational age) of the protein listed on the left among children whose placenta had the grade of umbilical cord inflammation that heads each column compared to the infants whose placenta had no umbilical cord inflammation. Adjustment has been made for gestational age category.

	Acute inflammation of the umbilical cord		
Protein	**Grade 1–2**	**Grade 3**	**Grade 4–5**
Cytokines and their receptors			
IL-1β	2.5 (1.4, 4.6)*	2.7 (1.3, 5.3)*	5.4 (2.7, 11)*
IL-6	1.9 (1.1, 3.6)*	1.7 (0.8, 3.6)	2.7 (1.4, 5.3)*
IL-6R	2.9 (1.6, 5.2)*	2.9 (1.4, 5.8)*	2.3 (1.2, 4.7)*
TNF-α	2.2 (1.2, 4.0)*	3.2 (1.6, 6.5)*	4.7 (2.4, 9.2)*
TNF-R1	2.3 (1.2, 4.2)*	2.1 (1.01, 4.4)*	4.0 (2.0, 7.9)*
TNF-R2	3.1 (1.7, 5.7)*	4.4 (2.2, 8.9)*	5.1 (2.5, 10)*
Chemokines			
IL-8 (CXCL8)	1.8 (0.97, 3.5)	3.5 (1.7, 7.1)*	3.9 (1.9, 7.6)*
MCP-1 (CCL2)	0.9 (0.5, 1.8)	0.7 (0.3, 1.7)	0.7 (0.3, 1.6)
MCP-4 (CCL13)	0.9 (0.5, 1.8)	1.1 (0.5, 2.2)	1.0 (0.5, 2.0)
MIP-1 β (CCL4)	3.0 (1.6, 5.4)*	2.8 (1.4, 5.8)*	3.2 (1.6, 6.3)*
RANTES (CCL5)	1.5 (0.8, 2.8)	2.0 (0.99, 4.1)	1.6 (0.8, 3.2)
I-TAC (CXCL11)	3.7 (2.0, 6.7)*	3.1 (1.5, 6.4)*	6.2 (3.1, 12)*
Adhesion molecules			
ICAM-1 (CD54)	2.7 (1.5, 5.0)*	3.6 (1.8, 7.4)*	5.1 (2.5, 10)*
ICAM-3 (CD50)	3.6 (2.0, 6.6)*	4.3 (2.1, 8.7)*	4.2 (2.1, 8.4)*
VCAM-1 (CD106)	1.1 (0.6, 2.0)	1.2 (0.6, 2.6)	1.9 (0.98, 3.8)
E-selectin (CD62E)	5.2 (2.8, 9.5)*	8.2 (4.0, 17)*	6.6 (3.3, 13)*
Matrix metalloproteinases (MMPs)			
MMP-1	1.6 (0.9, 3.0)	1.8 (0.9, 3.8)	1.5 (0.7, 3.0)
MMP-9	3.4 (1.9, 6.3)*	5.4 (2.6, 11)*	3.9 (1.9, 7.7)*
Other indicators of inflammation			
CRP	3.6 (1.9, 6.2)*	3.4 (1.6, 7.0)*	5.7 (2.9, 11)*
SAA	4.1 (2.2, 7.6)*	4.2 (2.0, 8.5)*	4.9 (2.4, 9.7)*
MPO	3.2 (1.7, 5.7)*	3.9 (1.9, 7.9)*	3.9 (2.0, 7.8)*
Growth factors, their receptors, and binding proteins			
VEGF	3.5 (1.9, 6.4)*	4.5 (2.2, 9.1)*	4.9 (2.5, 9.8)*
VEGF-R1 (Flt-1)	0.8 (0.4, 1.4)	0.4 (0.1, 0.99)**	0.7 (0.3, 1.5)
VEGF-R2 (KDR)	2.1 (1.1, 3.9)*	3.0 (1.5, 6.0)*	2.8 (1.4, 5.6)*
IGFBP-1	0.3 (0.2, 0.8)**	0.4 (0.1, 0.9)**	0.7 (0.3, 1.4)

	Acute inflammation of the umbilical cord		
Protein	**Grade 1–2**	**Grade 3**	**Grade 4–5**
N	103	68	74

* Significantly increased risk, p<0.01

** Significantly reduced risk, p<0.01

Table 4

Odds ratios (99% confidence intervals) of a concentration in the top quartile (for gestational age) of the protein listed on the left among children whose placenta had infarct, increased syncytial knots or decidual/ hemorrhage/fibrin deposition compared to children whose placenta did not have that histologic characteristic. Adjustment has been made for gestational age category.

Protein	Infarct	Increased syncytial knots	Decidual hem/ fibrin deposition
Cytokines and their receptors			
IL-1β	0.8 (0.5, 1.4)	0.6 (0.4, 1.1)	0.8 (0.4, 1.4)
IL-6	0.6 (0.3, 1.05)	0.5 (0.3, 0.96)**	0.7 (0.4, 1.3)
IL-6R	0.7 (0.4, 1.2)	0.6 (0.4, 1.1)	1.1 (0.7, 2.0)
TNF-α	0.7 (0.4, 1.2)	0.7 (0.4, 1.2)	0.9 (0.6, 1.6)
TNF-R1	0.5 (0.3, 1.00)	0.7 (0.4, 1.2)	1.1 (0.6, 1.9)
TNF-R2	0.7 (0.4, 1.2)	0.7 (0.4, 1.2)	1.0 (0.6, 1.8)
Chemokines			
IL-8 (CXCL8)	0.6 (0.3, 1.2)	0.7 (0.4, 1.3)	1.0 (0.6, 1.7)
MCP-1 (CCL2)	1.1 (0.6, 1.9)	0.9 (0.5, 1.5)	0.7 (0.4, 1.3)
MCP-4 (CCL13)	0.9 (0.5, 1.5)	0.9 (0.5, 1.5)	1.1 (0.7, 1.9)
MIP-1 β (CCL4)	0.4 (0.2, 0.9)	0.7 (0.4, 1.2)	1.0 (0.6, 1.7)
RANTES (CCL5)	0.4 (0.2, 0.7)**	0.5 (0.3, 0.9)**	1.1 (0.6, 1.8)
I-TAC (CXCL11)	0.5 (0.3, 1.01)	0.7 (0.4, 1.2)	1.0 (0.6, 1.7)
Adhesion molecules			
ICAM-1 (CD54)	0.5 (0.3, 0.99)**	0.9 (0.5, 1.5)	0.8 (0.5, 1.5)
ICAM-3 (CD50)	0.2 (0.1, 0.5)**	0.3 (0.2, 0.6)**	0.8 (0.4, 1.4)
VCAM-1 (CD106)	0.5 (0.3, 0.9)**	0.7 (0.4, 1.2)	1.2 (0.7, 2.1)
E-selectin (CD62E)	0.5 (0.2, 0.9)**	0.5 (0.3, 0.96)**	1.1 (0.6, 1.9)
Matrix metalloproteinases (MMPs)			
MMP-1	0.3 (0.2, 0.7)**	0.6 (0.4, 1.1)	1.0 (0.6, 1.7)
MMP-9	0.5 (0.3, 0.99)**	0.4 (0.2, 0.7)**	0.8 (0.5, 1.5)
Other indicators of inflammation			
CRP	0.7 (0.4, 1.2)	0.9 (0.5, 1.5)	0.6 (0.3, 1.1)
SAA	0.6 (0.3, 1.04)	0.7 (0.4, 1.3)	0.7 (0.4, 1.3)
MPO	0.5 (0.3, 0.95)**	0.4 (0.2, 0.8)**	0.8 (0.4, 1.4)
Growth factors, their receptors, and binding proteins			
VEGF	0.6 (0.3, 1.1)	0.5 (0.3, 0.9)**	1.3 (0.8, 2.2)
VEGF-R1 (Flt-1)	1.3 (0.7, 2.1)	2.3 (1.4, 3.8)*	0.9 (0.5, 1.6)
VEGF-R2 (KDR)	0.5 (0.3, 0.97)**	0.8 (0.5, 1.3)	0.9 (0.5, 1.6)
IGFBP-1	2.0 (1.2, 3.3)*	2.2 (1.3, 3.5)*	1.2 (0.7, 2.1)
N	135	159	137

*Significantly increased risk, p<0.01

** Significantly reduced risk, p<0.01

Table 5

Odds ratios (99% confidence intervals) of a concentration in the top quartile (for gestational age) of the protein listed on the left among children whose placenta had the combination of histologic lesions listed at the top relative to the risk among those whose placenta had no inflammatory lesion. Adjustment has been made for gestational age category.

Inflammation of	Odds ratios (and 95% confidence intervals)			
plate or membranes	Yes	Yes	No	No
umbilical cord	Yes	No	Yes	No
CRP	5.3 (3.0, 9.6)*	3.1 (1.8, 5.5)*	2.5 (0.4, 15)	1.0
SAA	5.6 (3.1, 10)*	4.1 (2.4, 7.1)*	2.7 (0.4, 17)	1.0
MPO	4.3 (2.5, 7.6)*	2.5 (1.4, 4.2)*	2.8 (0.5, 15)	1.0
N	124	164	10	446

* Significantly increased risk, $p<0.01$