RESEARCH ARTICLE

Maternal Microbe-Specific Modulation of Inflammatory Response in Extremely Low-Gestational-Age Newborns

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ABSTRACT The fetal response to intrauterine inflammatory stimuli appears to contribute to the onset of preterm labor as well as fetal injury, especially affecting newborns of extremely low gestational age. To investigate the role of placental colonization by specific groups of microorganisms in the development of inflammatory responses present at birth, we analyzed 25 protein biomarkers in dry blood spots obtained from 527 newborns delivered by Caesarean section in the 23rd to 27th gestation weeks. Bacteria were detected in placentas and characterized by culture techniques. Odds ratios for having protein concentrations in the top quartile for gestation age for individual and groups of microorganisms were calculated. Mixed bacterial vaginosis (BV) organisms were associated with a proinflammatory pattern similar to those of infectious facultative anaerobes. *Prevotella* and *Gardnerella* species, anaerobic streptococci, peptostreptococci, and genital mycoplasmas each appeared to be associated with a different pattern of elevated blood levels of inflammation-related proteins. *Lactobacillus* was associated with low odds of an inflammatory response. This study provides evidence that microorganisms colonizing the placenta provoke distinctive newborn inflammatory responses and that *Lactobacillus* may suppress these responses.

IMPORTANCE Despite improved intensive care, preterm and especially extremely low-gestation-age neonates continue to be at a considerably increased risk of morbidity, mortality, and developmental problems. The fetal inflammatory response appears to contribute to the onset of preterm labor, fetal injury, and complications, underlying lifetime health challenges facing these children. This study provides evidence that bacterial colonization of the very preterm placenta is associated with distinct microorganism-specific inflammatory protein profiles in the newborn blood specimens. We also provide evidence that *Lactobacillus* reduces inflammatory responses in newborns. Our data support the concept that targeting of placental colonization by specific drugs or probiotics during early pregnancy holds promise for preventing not only preterm birth but also subsequent and long-lasting, inflammation-provoked late sequelae.

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Preterm birth, a medical, social, and economic problem, especially in developed countries, occurs in nearly a half million pregnancies each year in the United States alone (1, 2). Despite improved intensive care, preterm and especially extremely low-gestational-age newborns (ELGANs) continue to be at a significantly higher risk of morbidity, mortality, and developmental problems (1, 2). The systemic fetal inflammatory response to intrauterine exposures, especially intrauterine infections, is regarded as an important contributor to the onset of preterm labor, fetal injury, and the many and sometimes lifelong sequelae of early organ damage (1–3). In addition to clinically established infections, noninvasive vaginal microorganisms that ascend into the uterus also appear to contribute to preterm birth, especially when criteria are met for the syndrome of disturbed vaginal microflora known as bacterial vaginosis (BV) (3–6). Microbiologically, BV is defined by decreased vaginal concentrations of *Lactobacillus* species and increased numbers of anaerobic Gram-negative rods such as *Prevotella*, *Gardnerella* species, and genital mycoplasma species, e.g., *Ureaplasma urealyticum* and *Mycoplasma hominis* (7). Although the importance of BV as a risk factor of preterm birth is well established, routine BV treatment does not appear to reduce the risk of preterm birth (8–11).

Approximately half of all placentas delivered before the second trimester and 41% of those delivered by Caesarean section harbor organisms in their chorions, detectable by culture techniques (12). The high colonization rate decreases with increasing gestational age. Some of these microorganisms are typically part of the vaginal microflora that might have evolved mechanisms to colonize the placenta during early pregnancy. We are not aware of studies that evaluated to what extent these organisms induce systemic inflammatory responses in the fetus and newborn. In an effort to explore the relationships among specific groups of microorganisms recovered from the placenta and blood protein indicators of a fetal inflammatory response, we analyzed 25 protein biomarkers in dry

blood spots obtained from 527 newborns born by Caesarean section in the 23rd to 27th gestation weeks. The effects of other variables, e.g., placental histology, pregnancy complications, and postnatal exposures, on the inflammatory responses in the same study population have been addressed elsewhere (13, 14; T. F. McElrath, R. N. Fichorova, E. N. Allred, J. L. Hecht, M. A. Ismail, H. Yuan, and A. Leviton, submitted for publication).

RESULTS

The risks of systemic inflammation measured by distinct profiles of inflammatory proteins in newborn blood associated with placental colonization by specific groups of bacteria are presented as odds ratios in Tables 1 and 2. The number of samples that tested positive for each organism or group of organisms is stated in the bottom row of each column. The odd ratios indicate the relationship of culture-positive cases with those whose placentas did not harbor any culturable microorganism rather than with all cases studied.

Of the 527 placentas studied, 313 were culture negative, and 214 were culture positive for one or more microorganisms of the groups shown in Table 1. Lactobacilli (detected alone or mixed with other microorganisms in 21 cases or in 9.8% of all culture-positive placentas) were associated with a prominently low risk of systemic inflammatory reaction in the preterm newborn in contrast to the groups of pathogenic microorganisms and organisms characteristic of bacterial vaginosis (BV), which were all associated with increased risks of specific sets of elevated proteins compared to placentas that did not harbor any microorganisms (Table 1). The group of BV organisms was most frequently detected, with *Gardnerella vaginalis*, *Prevotella bivia*, anaerobic *Streptococcus*, and *Peptostreptococcus* present alone or in mixed culture in 47% or 22% of all culture-positive placentas, respectively. The BV organisms were associated with a heightened proinflammatory pattern similar to those of the infectious facultative anaerobes *Escherichia coli* and alpha *Streptococcus* (each detectable in 28% or 13% of the culture-positive placentas, respectively) and distinct from those of the various genital mycoplasma species (collectively detected in 38% or 18% of the culture-positive placentas, respectively). The mixed BV microorganisms and the infectious facultative anaerobes shared an identical pattern of highest odds of elevated levels of interleukin-1β (IL-1β), E-selectin, and serum amyloid A (SAA). In addition, the organisms within these two groups variably shared odds for elevated levels of IL-6, tumor necrosis factor alpha (TNF-α), TNF receptors, IL-8, intercellular adhesion molecule 1 (ICAM-1), ICAM-3, C-reactive protein (CRP), myeloperoxidase (MPO), vascular endothelial growth factor (VEGF), and VEGF receptor 2 (VEGF-R2). In total, the proinflammatory protein profile of the cases of mixed BV culture included 9 of the 25 proteins studied and was not duplicated by any single BV microorganism, suggesting that the proinflammatory effect should be attributable to the cumulative effects of the BV community rather than that of an individual microbial species.

When BV-associated microorganisms were evaluated separately, each had its own profile of elevated concentrations of proteins in the newborn blood samples (Table 1). This analysis included all cases with a specific microorganism recovered (including monocultures and mixed cultures) versus those that did not have any microorganism recovered. While the presence of *Prevotella* was associated with a relatively low risk of inflammatory response, *Gardnerella* was marked by an increased risk of TNF-α, IL-8, ICAM-1, and VEGF-R2, anaerobic *Streptococcus* was associated with a pattern of elevated IL-1β, IL-6, TNF receptor type 1 (TNF-R1), TNF-R2, and E-selectin, and *Peptostreptococcus* was characterized by a high risk of elevated macrophage inflammatory protein 1β (MIP-1β), ICAM-3, matrix metalloproteinase 9 (MMP-9), MPO, and VEGF and undetectable insulin growth factor binding protein 1 (IGF-BP-1).

Cases with cultured genital mycoplasma species (including *Ureaplasma urealyticum* and other *Mycoplasma* spp.) showed a reproducible inflammatory protein pattern that closely resembled only that of *Peptostreptococcus* and was dominated by increased odds of elevated MIP-1β, I-TAC (interferon-inducible T cell alpha-chemoattractant), ICAM-3, MMP-9, MPO, and VEGF and decreased odds of elevated VEGF-R1 and IGF-BP-1. Interestingly, in contrast to the BV-mixed group and the facultative anaerobes *E. coli* and alpha *Streptococcus*, they were not associated with elevated proinflammatory cytokines or the hepatic markers of systemic inflammation CRP and SAA.

Skin-associated organisms (*Staphylococcus* and *Propionibacterium* species), detectable in 13 to 17% of the culture-positive placentas, were not associated with elevated or reduced concentrations of inflammatory proteins in the newborn blood samples.

The levels of IL-6 receptor (IL-6R), monocyte chemotactic protein 1 (MCP-1), MCP-4, RANTES (regulated upon activation, normal T cell expressed and [presumably] secreted), and MMP-1 in the newborns' blood samples did not appear to be significantly affected by the presence of any type of cultural bacteria in their placentas (Table 1).

Because of the distinct low inflammatory profile of the *Lactobacillus*-positive cases (Table 1) and because most of them (62%) represented mixed cultures, we broke down the group into monoculture and mixed-culture *Lactobacillus*-positive subgroups, and we further contrasted those two subgroups with cases where BV organisms were found alone or in combination with non-BV organisms (Table 2). Again, odds ratios of having top-quartile protein concentrations were calculated by comparison to those of cases with no microorganisms recovered.

Although the number of placentas colonized by *Lactobacillus* alone was relatively small ($n = 8$), resulting in lack of statistical power, it was striking that most inflammatory proteins, including those associated with individual or mixed BV microorganisms (IL-1β, IL-6, TNF-R1, TNF-R2, IL-8, MIP-1β, ICAM-1, ICAM-3, E-selectin, MMP-9, CRP, SAA, and VEGF), were completely undetectable in placentas that harbored *Lactobacillus* alone (Table 2). In addition, even when mixed with others ($n = 13$), lactobacilli continued to be associated with undetectable ICAM-1 and VEGF, and despite the wide confidence intervals due to the overall low prevalence of *Lactobacillus* in the ELGAN placentas, the mixed *Lactobacillus* cultures remained at low odds for all studied proteins.

The opposite was observed in the group positive for BV-associated species. The cases that were exclusively BV organism positive ($n = 18$) represented 38% of the group. When these cases were separately analyzed, they showed an even higher risk of elevated protein concentrations, including IL-1β, IL-6, TNF-α, IL-8, ICAM-1, ICAM-3, vascular cell adhesion molecule 1 (VCAM-1), VEGF-R2, and both hepatic markers of systemic inflammation, CRP and SAA (Table 2). In contrast, the group harboring BV mixed with other microorganisms ($n = 29$), of which a significant part (24%) was mixed with *Lactobacillus*, showed lower odds for top-quartile concentrations of all cytokines, chemokines, and hepatic markers of inflammation (Table 2, last column), possibly due to the modulatory effect of *Lactobacillus* in the mixed cultures.

TABLE 1 Risk of neonate systemic inflammatory response associated with placental colonization by specific types of microorganisms

	Odds ratio (95% confidence intervals)[c]												
		BV-associated spp.					Facultative anaerobe		Genital mycoplasma			Skin organism	
Protein	*Lactobacillus*[d]	*P. bivia*	*G. vaginalis*	Anaerobic *Streptococcus*	*Peptostreptococcus*	Any BV-associated species	*E. coli*	Alpha *Streptococcus*	*U. urealyticum*	*Mycoplasma* spp.	Any mycoplasma species	*Staphylococcus*[a]	*Propionibacterium*
Cytokines and their receptors													
IL-1β	0.2 (0, 1.6)	1.3 (0.4, 4.1)	2.6 (0.9, 8.3)	3.0 (1.1, 8.1)[b]	2.1 (0.4, 12)	2.2 (1.1, 4.2)[b]	3.2 (1.4, 7.0)[b]	3.2 (1.4, 7.0)[b]	1.8 (0.7, 4.7)	0.6 (0.1, 2.7)	1.4 (0.6, 3.0)	0.5 (0.2, 1.8)	1.6 (0.7, 3.5)
IL-6	0.4 (0.1, 1.9)	2.2 (0.8, 6.2)	2.5 (0.8, 8.0)	4.6 (1.7, 12)[b]	2.0 (0.4, 11)	2.5 (1.3, 4.8)[b]	2.2 (0.98, 5.1)	4.0 (1.8, 8.9)[b]	1.8 (0.7, 4.5)	1.3 (0.4, 4.3)	1.7 (0.8, 3.7)	0.3 (0.1, 1.4)	1.6 (0.7, 3.4)
IL-6R	1.6 (0.6, 4.3)	1.7 (0.6, 4.9)	2.5 (0.9, 7.8)	1.2 (0.4, 3.9)	4.0 (0.8, 20)	1.9 (0.9, 3.6)	0.7 (0.2, 2.0)	1.3 (0.5, 3.2)	1.1 (0.4, 3.1)	0.9 (0.3, 3.3)	1.1 (0.5, 2.5)	1.1 (0.4, 2.9)	0.6 (0.2, 1.7)
TNF-α	0.7 (0.2, 2.3)	0.5 (0.1, 2.4)	8.9 (2.7, 30)[b]	2.2 (0.8, 6.1)	2.0 (0.4, 11)	2.2 (1.2, 4.3)[b]	2.6 (1.1, 5.8)[b]	1.9 (0.8, 4.6)	1.4 (0.5, 3.7)	1.8 (0.6, 5.4)	1.5 (0.7, 3.2)	0.3 (0.1, 1.4)	1.5 (0.7, 3.3)
TNF-R1	0.6 (0.2, 2.1)	1.1 (0.4, 3.6)	2.3 (0.7, 7.2)	3.3 (1.2, 8.8)[b]	1.8 (0.3, 10)	1.9 (0.98, 3.7)	0.8 (0.3, 2.3)	1.2 (0.5, 3.0)	1.3 (0.5, 3.4)	0.8 (0.2, 3.1)	1.2 (0.5, 2.6)	1.3 (0.5, 3.2)	1.0 (0.5, 2.4)
TNF-R2	0.4 (0.1, 2.0)	1.3 (0.4, 4.1)	2.6 (0.8, 8.3)	3.7 (1.4, 10)[b]	0.8 (0.1, 7.4)	2.2 (1.1, 4.2)[b]	2.3 (1.03, 5.3)[b]	1.7 (0.7, 4.0)	1.5 (0.6, 3.9)	0.6 (0.1, 2.7)	1.2 (0.5, 2.7)	0.5 (0.2, 1.8)	1.0 (0.4, 2.4)
Chemokines													
IL-8 (CXCL8)	0.2 (0, 1.4)	0.8 (0.2, 2.8)	4.2 (1.4, 13)[b]	2.0 (0.7, 5.5)	0.7 (0.1, 6.3)	1.9 (0.96, 3.6)	2.0 (0.9, 4.5)	2.3 (1.04, 5.2)[b]	2.3 (0.96, 5.6)	1.2 (0.4, 3.8)	1.7 (0.8, 3.6)	0.3 (0.1, 1.2)	0.9 (0.4, 2.1)
MCP-1 (CCL2)	1.7 (0.7, 4.3)	1.2 (0.4, 3.4)	0.5 (0.1, 2.4)	2.5 (0.9, 6.7)	2.8 (0.6, 14)	1.3 (0.7, 2.6)	1.9 (0.9, 4.3)	1.1 (0.5, 2.7)	0.6 (0.2, 1.8)	0.4 (0.1, 1.8)	0.5 (0.2, 1.4)	0.5 (0.2, 1.5)	1.8 (0.9, 3.7)
MCP-4 (CCL13)	0.9 (0.3, 2.4)	1.1 (0.4, 3.3)	0.5 (0.1, 2.3)	1.5 (0.5, 4.1)	1.4 (0.2, 7.6)	1.0 (0.5, 2.1)	0.7 (0.3, 1.9)	0.7 (0.3, 1.9)	0.6 (0.2, 1.7)	0.6 (0.2, 2.2)	0.6 (0.3, 1.5)	0.6 (0.2, 1.7)	1.7 (0.8, 3.5)
MIP-1β (CCL4)	1.4 (0.5, 3.9)	1.8 (0.6, 5.4)	1.3 (0.4, 4.9)	0.9 (0.3, 3.4)	8.8 (1.6, 49)[b]	1.9 (0.9, 3.7)	2.1 (0.9, 4.8)	1.8 (0.7, 4.2)	5.7 (2.4, 14)[b]	1.0 (0.3, 3.7)	3.3 (1.6, 6.8)[b]	0.8 (0.3, 2.3)	1.5 (0.7, 3.3)
RANTES (CCL5)	1.0 (0.4, 2.9)	0.7 (0.2, 2.5)	2.8 (0.9, 8.6)	0.4 (0.1, 2.0)	1.6 (0.3, 9.2)	1.3 (0.6, 2.5)	1.3 (0.6, 3.1)	0.5 (0.2, 1.6)	1.2 (0.4, 3.1)	1.5 (0.5, 4.4)	1.2 (0.6, 2.6)	1.2 (0.5, 2.8)	0.5 (0.2, 1.4)
I-TAC (CXCL11)	0.7 (0.2, 2.6)	1.8 (0.6, 5.4)	1.3 (0.4, 4.9)	1.8 (0.6, 5.4)	2.2 (0.4, 12)	1.5 (0.7, 3.1)	1.8 (0.7, 4.2)	2.4 (1.1, 5.6)[b]	3.4 (1.4, 8.1)[b]	1.5 (0.5, 4.7)	2.7 (1.3, 5.5)[b]	0.5 (0.2, 1.9)	1.1 (0.4, 2.5)
Adhesion molecules													
ICAM-1 (CD54)	—	1.4 (0.5, 4.6)	4.0 (1.3, 12)[b]	2.0 (0.7, 5.8)	2.3 (0.4, 14)	2.4 (1.2, 4.7)[b]	3.5 (1.6, 7.9)[b]	2.2 (0.9, 5.2)	1.0 (0.3, 3.0)	1.6 (0.5, 5.0)	1.3 (0.6, 3.0)	0.6 (0.2, 2.0)	1.1 (0.5, 2.7)
ICAM-3 (CD50)	1.4 (0.5, 4.0)	2.4 (0.9, 6.9)	2.8 (0.9, 8.9)	1.9 (0.6, 5.5)	22 (2.4, 196)[b]	3.0 (1.6, 5.8)[b]	1.6 (0.6, 3.9)	1.5 (0.6, 3.7)	2.4 (0.97, 5.9)	4.5 (1.6, 12)[b]	3.1 (1.5, 6.3)[b]	1.0 (0.4, 2.8)	1.7 (0.8, 3.8)
VCAM-1 (CD106)	1.3 (0.5, 3.5)	1.4 (0.5, 4.0)	2.8 (0.9, 8.6)	1.8 (0.6, 5.0)	1.6 (0.3, 9.2)	1.9 (0.97, 3.6)	1.2 (0.5, 2.8)	0.9 (0.4, 2.3)	0.9 (0.3, 2.5)	0.5 (0.1, 2.1)	0.8 (0.3, 1.8)	0.7 (0.3, 2.0)	0.9 (0.4, 2.2)
E-selectin (CD62E)	0.3 (0, 1.9)	1.1 (0.3, 3.9)	1.5 (0.4, 5.7)	4.5 (1.6, 12)[b]	2.5 (0.4, 14)	2.1 (1.1, 4.3)[b]	4.7 (2.1, 10)[b]	2.4 (1.02, 5.5)[b]	2.2 (0.9, 5.6)	2.3 (0.8, 6.8)	2.1 (0.98, 4.6)	0.2 (0, 1.5)	1.0 (0.4, 2.5)
MMPs													
MMP-1	0.8 (0.3, 2.6)	0.8 (0.2, 2.8)	1.1 (0.3, 4.0)	0.2 (0, 1.7)	3.6 (0.7, 18)	1.0 (0.5, 2.1)	1.0 (0.4, 2.7)	0.8 (0.3, 2.1)	1.3 (0.5, 3.4)	1.6 (0.6, 4.9)	1.5 (0.7, 3.2)	0.6 (0.2, 1.9)	0.9 (0.4, 2.1)
MMP-9	0.8 (0.2, 2.7)	1.9 (0.6, 5.6)	0.4 (0, 3.0)	1.4 (0.4, 4.5)	9.2 (1.6, 51)[b]	1.9 (0.98, 3.9)	1.3 (0.5, 3.2)	1.8 (0.8, 4.4)	2.4 (0.99, 6.1)	2.8 (0.96, 7.9)	2.8 (1.4, 5.8)[b]	0.4 (0.1, 1.6)	1.3 (0.6, 3.0)
Other inflammation indicators													
CRP	0.2 (0, 1.7)	1.9 (0.6, 5.6)	2.9 (0.9, 9.1)	1.9 (0.6, 5.6)	0.9 (0.1, 8.0)	2.2 (1.1, 4.2)[b]	3.0 (1.3, 6.7)[b]	2.2 (0.9, 5.1)	1.0 (0.3, 3.0)	2.1 (0.7, 6.2)	1.5 (0.7, 3.3)	0.4 (0.1, 1.6)	0.7 (0.3, 2.0)
SAA	0.3 (0, 1.9)	2.7 (0.97, 7.7)	1.5 (0.4, 5.7)	2.7 (0.97, 7.7)	1.0 (0.1, 8.8)	2.1 (1.1, 4.3)[b]	5.0 (2.3, 11)[b]	5.0 (2.3, 11)[b]	1.4 (0.5, 3.9)	1.2 (0.3, 4.2)	1.4 (0.6, 3.2)	0.6 (0.2, 2.2)	1.0 (0.4, 2.5)
MPO	1.4 (0.5, 4.0)	1.9 (0.6, 5.5)	2.0 (0.6, 6.7)	1.0 (0.3, 3.5)	9.0 (1.6, 50)[b]	2.3 (1.2, 4.5)[b]	1.8 (0.8, 4.3)	2.9 (1.3, 6.5)[b]	4.1 (1.7, 9.8)[b]	3.5 (1.2, 9.8)[b]	3.8 (1.9, 7.7)[b]	0.8 (0.3, 2.3)	1.1 (0.5, 2.6)
Growth factors, their receptors, and binding proteins													
VEGF	—	1.6 (0.5, 5.2)	1.0 (0.2, 4.4)	1.1 (0.3, 4.1)	11 (1.9, 59)[b]	1.8 (0.9, 3.7)	2.5 (1.1, 5.8)[b]	2.1 (0.9, 5.0)	2.3 (0.9, 5.9)	3.2 (1.1, 9.1)[b]	2.5 (1.2, 5.4)[b]	0.9 (0.3, 2.8)	1.1 (0.4, 2.7)
VEGF-R1 (Flt1)	1.0 (0.4, 2.5)	0.3 (0.1, 1.2)	0.4 (0.1, 1.6)	1.4 (0.5, 3.7)	1.0 (0.2, 5.4)	0.7 (0.3, 1.3)	0.8 (0.3, 1.9)	0.4 (0.2, 1.1)	0.3 (0.1, 1.01)	0.1 (0, 1.00)	0.2 (0.1, 0.7)[b]	0.6 (0.2, 1.4)	1.1 (0.5, 2.3)
VEGF-R2 (KDR)	0.4 (0, 1.7)	1.2 (0.4, 3.6)	3.2 (1.04, 9.9)[b]	0.8 (0.2, 2.9)	0.7 (0.1, 6.5)	1.6 (0.8, 3.1)	2.4 (1.1, 5.4)[b]	0.8 (0.3, 2.2)	0.8 (0.3, 2.4)	0.9 (0.2, 3.1)	0.2 (0.4, 2.1)	0.7 (0.2, 1.9)	0.6 (0.2, 1.6)
IGF-BP-1	0.9 (0.4, 2.5)	0.3 (0.1, 1.4)	0.4 (0.1, 1.9)	0.7 (0.2, 2.3)	—	0.4 (0.2, 0.9)[b]	0.8 (0.3, 2.0)	0.4 (0.1, 1.1)	0.2 (0.1, 0.97)[b]	0.5 (0.1, 1.9)	0.4 (0.1, 0.97)	0.5 (0.2, 1.4)	1.0 (0.5, 2.2)
No. of positive placentas	21	17	13	17	6	47	28	28	23	16	37	27	36

[a] All were coagulase negative.
[b] Significantodds ratio; $P < 0.05$.
[c] Odds ratios (95% confidence intervals) of blood protein concentrations in the top quartile comparing ELGANs whose placentas harbored a specific type of bacteria (numbers stated in the bottom row of each column) with those whose placentas did not harbor any culturable microorganism ($n = 313$). Values in red indicate statistically significant elevations and values in blue indicate significantly reduced odds ratios. 0 cells, so odds ratio cannot be calculated.
[d] Common in vaginal health.

TABLE 2 Risk of neonate systemic inflammatory response associated with placental colonization by *Lactobacillus* species alone or mixed with others in contrast to that by BV-associated species

Protein	Odds ratio (95% confidence intervals)[b]					
	Lactobacillus			BV-associated spp.[c]		
	All (alone and mixed)	Alone (monoculture)	Mixed	All (alone and mixed)	Alone	Mixed with any other microorganism
Cytokines and their receptors						
IL-1β	0.2 (0, 1.6)	—	0.4 (0, 2.8)	2.2 (1.1, 4.2)[a]	3.4 (1.3, 8.9)[a]	1.6 (0.7, 3.8)
IL-6	0.4 (0.1, 1.9)	—	0.7 (0.2, 3.4)	2.5 (1.3, 4.8)[a]	3.2 (1.2, 8.5)[a]	2.1 (0.9, 4.8)
IL-6R	1.6 (0.6, 4.3)	0.6 (0.1, 4.7)	2.5 (0.9, 7.8)	1.9 (0.9, 3.6)	2.0 (0.7, 5.5)	1.8 (0.8, 4.1)
TNF-α	0.7 (0.2, 2.3)	0.6 (0.1, 4.7)	0.7 (0.2, 3.3)	2.2 (1.2, 4.3)[a]	5.0 (1.9, 13)[a]	1.3 (0.5, 3.1)
TNF-R1	0.6 (0.2, 2.1)	—	1.1 (0.3, 4.1)	1.9 (0.98, 3.7)	2.3 (0.9, 6.3)	1.7 (0.7, 3.8)
TNF-R2	0.4 (0.1, 2.0)	—	0.8 (0.2, 3.6)	2.2 (1.1, 4.2)[a]	2.1 (0.8, 5.8)	2.2 (0.98, 5.0)
Chemokines						
IL-8 (CXCL8)	0.2 (0, 1.4)	—	0.3 (0, 2.4)	1.9 (0.96, 3.6)	2.9 (1.1, 7.6)[a]	1.4 (0.6, 3.2)
MCP-1 (CCL2)	1.7 (0.7, 4.3)	2.8 (0.7, 12)	1.3 (0.4, 4.2)	1.3 (0.7, 2.6)	1.1 (0.4, 3.1)	1.5 (0.7, 3.3)
MCP-4 (CCL13)	0.9 (0.3, 2.4)	—	0.5 (0.1, 2.3)	1.0 (0.5, 2.1)	1.7 (0.7, 4.6)	0.7 (0.3, 1.8)
MIP-1β (CCL4)	1.4 (0.5, 3.9)	—	2.7 (0.9, 8.7)	1.9 (0.9, 3.7)	1.7 (0.6, 4.9)	2.0 (0.9, 4.6)
RANTES (CCL5)	1.0 (0.4, 2.9)	—	2.1 (0.7, 6.5)	1.3 (0.6, 2.5)	1.3 (0.4, 3.7)	1.3 (0.5, 2.9)
I-TAC (CXCL11)	0.7 (0.2, 2.6)	0.6 (0.1, 5.2)	0.8 (0.2, 3.7)	1.5 (0.7, 3.1)	1.7 (0.6, 4.9)	1.4 (0.6, 3.4)
Adhesion molecules						
ICAM-1 (CD54)	—	—	—	2.4 (1.2, 4.7)[a]	4.7 (1.8, 12)[a]	1.5 (0.6, 3.7)
ICAM-3 (CD50)	1.4 (0.5, 4.0)	—	2.8 (0.9, 8.9)	3.0 (1.6, 5.8)[a]	3.6 (1.4, 9.5)[a]	2.7 (1.2, 6.1)[a]
VCAM-1 (CD106)	1.3 (0.5, 3.5)	0.5 (0.1, 3.9)	2.1 (0.7, 6.5)	1.9 (0.97, 3.6)	2.6 (1.00, 6.9)[a]	1.5 (0.6, 3.4)
E-selectin (CD62E)	0.3 (0, 1.9)	—	0.4 (0.1, 3.3)	2.1 (1.1, 4.3)[a]	2.5 (0.9, 7.0)	1.9 (0.8, 4.6)
MMPs						
MMP-1	0.8 (0.3, 2.6)	2.2 (0.5, 9.3)	0.3 (0, 2.4)	1.0 (0.5, 2.1)	1.0 (0.3, 3.2)	0.9 (0.4, 2.4)
MMP-9	0.8 (0.2, 2.7)	—	1.4 (0.4, 5.2)	1.9 (0.98, 3.9)	1.8 (0.6, 5.2)	2.1 (0.9, 4.8)
Other inflammation indicators						
CRP	0.2 (0, 1.7)	—	0.4 (0, 3.0)	2.2 (1.1, 4.2)[a]	4.6 (1.7, 12)[a]	1.2 (0.5, 3.1)
SAA	0.3 (0, 1.9)	—	0.4 (0.1, 3.3)	2.1 (1.1, 4.3)[a]	3.2 (1.2, 8.6)[a]	1.6 (0.6, 3.9)
MPO	1.4 (0.5, 4.0)	0.6 (0.1, 5.3)	2.0 (0.6, 6.7)	2.3 (1.2, 4.5)[a]	2.2 (0.8, 6.2)	2.4 (1.04, 5.4)[a]
Growth factors, their receptors, and binding proteins						
VEGF	—	—	—	1.8 (0.9, 3.7)	2.0 (0.7, 5.9)	1.7 (0.7, 4.1)
VEGF-R1 (Flt1)	1.0 (0.4, 2.5)	1.2 (0.3, 5.0)	0.9 (0.3, 2.9)	0.7 (0.3, 1.3)	0.8 (0.3, 2.3)	0.6 (0.3, 1.5)
VEGF-R2 (KDR)	0.4 (0, 1.7)	—	0.7 (0.1, 3.1)	1.6 (0.8, 3.1)	3.0 (1.1, 7.9)[a]	1.0 (0.4, 2.5)
IGF-BP-1	0.9 (0.4, 2.5)	2.3 (0.6, 9.5)	0.4 (0.1, 1.9)	0.4 (0.2, 0.9)[a]	0.7 (0.2, 2.1)	0.3 (0.1, 0.9)[a]
No. of positive placentas	21	8	13	47	18	29

[a] Significant odds ratio; $P < 0.05$.
[b] Odds ratios (95% confidence intervals) of blood protein concentrations in the top quartile comparing ELGANs whose placentas harbored a specific type of bacteria (numbers stated in the bottom row of each column) with those whose placentas did not harbor any culturable microorganism ($n = 313$). Values in red indicate statistically significant elevations and values in blue indicate significantly reduced odds ratios. 0 cells, odds ratio cannot be calculated.
[c] Including *G. vaginalis*, *P. bivia*, anaerobic *Streptococcus*, and *Peptostreptococcus*.

The difference between the groups of *Lactobacillus*-positive and -negative cases is illustrated in Fig. 1 by concentration distributions for selected proteins representing the cytokines (IL-6), chemokines (IL-8), and hepatic inflammatory markers (CRP and SAA). Although the analysis of raw concentration values could not have the nominal statistical value of the percentile odds ratio analysis (Tables 1 and 2) due to the overall low prevalence of *Lactobacillus* species cultured from the ELGAN placentas, the data demonstrate the contrast between the observed narrow distribution and lower levels of proteins in the *Lactobacillus*-positive cases.

DISCUSSION

Our study supports the concept that the placental colonization with vaginal microorganisms can induce a systemic inflammatory response in the fetus and newborn and that the dominating molecular features of this response can be dependent on the type of bacteria. We report molecular patterns distinguishing the groups of *Lactobacillus* species, BV-associated species, infectious facultative anaerobes, genital mycoplasmas, and skin organisms.

In agreement with a widely accepted belief that BV-associated bacteria are capable of inducing preterm labor, we found that mixed BV microorganisms are associated with elevated concentrations of primary proinflammatory cytokines (IL-1β, IL-6, and TNF-α), capable of inducing acute phase reactants (CRP and SAA), chemokines (IL-8), and adhesion molecules (E-selectin, ICAM-1, and ICAM-3). IL-1β, IL-6, and TNF-α, as innate immunity mediators, play protective roles at local sites of bacterial invasion; however, when simultaneously and significantly elevated in the systemic circulation, these primary cytokines can induce chemokines, adhesion molecules, and further leukocyte activation, promoting extravasation and tissue damage of noninfected tissues as shown in studies of the central nervous system (15). The mixed BV-associated blood protein patterns were similar to those of infectious facultative anaerobes, e.g., *E. coli* and alpha *Streptococcus*, confirming the pathogenic potential of the BV microorganisms. Like *E. coli* and alpha *Streptococcus*, placental colonization with mixed BV microorganisms was associated with high odds of CRP, SAA, and MPO.

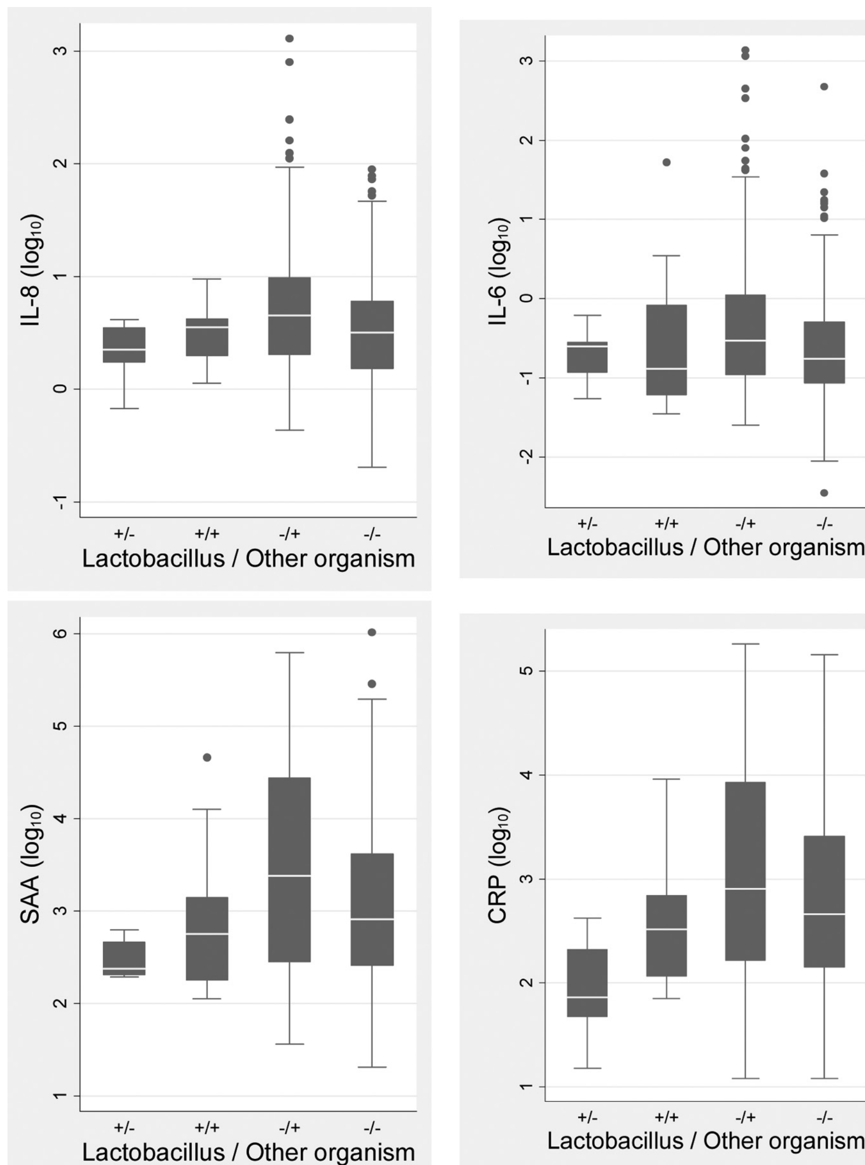

FIG 1 Comparison of risks of systemic inflammatory response measured by selected proteins in blood specimens obtained from newborns with *Lactobacillus* species versus other organisms detected in their placentas. Box plots represent logarithmically transformed interquartile ranges showing the number of pg of specific protein per mg of total protein distributed in four groups by the presence of lactobacilli and/or other microorganisms, as follows: +/− ($n = 8$), +/+ ($n = 13$), −/+ ($n = 193$), and −/− ($n = 313$). The whiskers extend to the upper and lower adjacent values. The upper adjacent value is equal to the following: the largest data point that is less than or equal to the upper quartile + 1.5 × (upper quartile − lower quartile). The lower adjacent value is equal to the following: the smallest data point that is greater than or equal to the lower quartile + 1.5 × (upper quartile − lower quartile) (51).

In contrast to BV, genital mycoplasmas did not induce primary cytokines and acute phase reactants but were associated with an invasive pattern of cytolytic activities mediated by MPO and MIP-1β, which increase nitric oxide production (16), and extracellular matrix degradation enzymes (MMP-9), which are major mediators of inflammatory tissue destruction. MMP-9 has a well-established role in parturition and is significantly increased in the amniotic fluid in preterm delivery (17). Mycoplasmas also showed increased levels of I-TAC, a chemokine for NK and T cells, which may be induced via Nod-1 activation by mycoplasma lipopeptides (18). Nod-1 activation selectively induces immune activation while avoiding upregulation of proinflammatory cytokines (18). The lack of primary cytokine upregulation may also be a result of some active suppressive properties of these microorganisms, since decreased expression of IL-1β and IL-8 characterizes mycoplasma-infected cells (19). *Ureaplasma urealyticum* and *Mycoplasma* species were analyzed separately because numerous studies, including ours, have implicated them in preterm birth (20–27). They seemed to contribute differently to the group pattern of elevated proteins, with *Ureaplasma urealyticum* being the major contributor to chemokine responses, while mixed mycoplasma colonization seemed needed for increased MMP-9 levels.

In addition to group-specific responses, this study also unveiled unique features of some individual BV-associated microorganisms. Among the BV-associated species, *Prevotella* is frequently found in preterm placentas with high-grade chorionic plate inflammation (28), and it was among the most prevalent BV-associated microorganisms colonizing the ELGAN placenta in our study. *In vitro* work has shown that some *Prevotella* strains can exhibit a strong invasive but weak cytokine-inducing capacity, and this combination of properties may lead to higher colonization rates and survival in the host (29). In agreement with these findings, our study showed relatively weak inflammatory responses to placental colonization with *Prevotella* alone. *Gardnerella*, the most common BV organism (30, 31), appeared to be the most inflammation-provoking organism and was associated with the highest odds of elevated TNF-α, IL-8, and ICAM-1. *Peptostreptococcus*, also frequently associated with high-grade chorionic plate inflammation (28) and linked to tissue destruction in the gut and oral mucosa (32, 33), showed a mycoplasma-like inflammatory protein pattern and was associated with the highest odds of tissue destructive enzymes, e.g., MMP-9 and MPO as well as MIP-1β and ICAM-3. Thus, each of the individual BV-associated microorganisms contributed uniquely to the pattern of mixed BV colonization, which demonstrated the biggest variety of elevated inflammatory proteins compared to any other group pattern. The mixed BV colonization conferred a risk of newborn inflammatory response higher than that of any of the individual BV-associated microorganisms alone, thus supporting the concept of BV as a polymicrobial pathogenic state.

This study suggests that *Lactobacillus* alone or in mixed placental colonization may suppress inflammatory responses in extremely preterm newborns. Our observations add to a growing body of experi-

mental evidence indicating that lactobacilli have anti-inflammatory properties. Lactobacilli have been found to suppress pathogen attachment and proliferation in the vaginal epithelium (9). Findings from *in vitro* studies with nonvaginal *Lactobacillus* strains have suggested that lactobacilli are active modulators of signaling pathways, leading to a strain-specific repertoire of cytokine release and immune tolerance as well as downregulation of proinflammatory signaling (34–37). Intestinal lactobacilli also inhibit epithelial inflammatory responses to *Salmonella* lipopolysaccharide (38).

In combination or independently of placental microbial colonization, a number of other prenatal or postnatal factors may influence the inflammatory protein levels in a newborn's blood. Since most of the blood specimens used for the analysis reported here were obtained on the day of birth, the blood protein concentrations in our analysis were not adjusted for postnatal phenomena and exposures (e.g., nutrition, therapeutic interventions, postnatal onset of anemia, sepsis, etc.); however, postnatal exposures and clinical outcomes have been analyzed in longitudinal samples from the same babies collected within the first two postnatal weeks and published elsewhere (13, 14; McElrath et al., submitted). The wide variance of protein concentrations in ELGANs with culture-negative placentas (illustrated for some proteins in Fig. 1) that exceed by logs those found in ELGANs with *Lactobacillus*-positive placentas and are sometimes close to those of other microorganism-positive cases raises the possibilities that part of the inflammation evident in preterm newborns may be attributable to noncultured microorganisms or noninfectious factors not addressed in this paper.

Previous publications from our group have shown that the organisms recovered from the placenta are unlikely to be contaminants (28, 39) and that recovery of these organisms provides information about the risk of preterm delivery (40) as well as structural and functional consequences of brain damage (23, 41). We have also reported that inflammation of the placental tissue, often a consequence of bacteria in the placenta, provides information about the probability of elevated concentrations of inflammation-related proteins in the newborn's blood (13, 14). Moreover, elevated concentrations of the same inflammation-related proteins provide information about the risk of sonographically defined brain damage (42). Thus, the present study provides glue to the existing construct of clinical evidence linking the organisms in the placenta to the risk of brain damage attributable to elevated systemic inflammatory proteins in the neonate.

Evidence that intrauterine inflammatory phenomena contribute to neonatal disorders (43) prompts us to consider what we found to be biologically and not just statistically significant. Although we have found some evidence for the specificity of organism-cytokine relationships, we prefer to emphasize that no inflammatory stimulus affects only a small set of proteins. Indeed, an inflammatory stimulus is capable of influencing the expression of more than a thousand genes (44). Thus, what we measured is likely only the tip of the iceberg. Our neonatal blood specimens were collected in the intensive care nursery, almost invariably within 24 h of delivery. Because many inflammatory mediators, e.g., interleukins (45, 46) and TNF-α (47), have relatively short half-lives, we are reluctant to view what we measured after birth to be limited to intrauterine phenomena. How much of what we measured should be considered evidence of neonatal rather than fetal inflammation remains to be determined. Nevertheless, our data clearly demonstrate that maternal microorganisms are associated with systemic inflammatory patterns detectable after birth.

In conclusion, we provide evidence for microbe-specific patterns of perinatal inflammatory responses. Although in addition to or independently of placental microorganisms, other prenatal as well as postnatal factors may contribute to the inflammatory condition and its perpetuation after birth. Our data suggest that placental colonization by specific groups of organisms can increase or decrease the risk of a systemic inflammatory condition independently from other factors, and moreover, colonization by specific groups of organisms can define the pattern of circulating inflammatory proteins after birth. Our findings also suggest that placenta-derived *Lactobacillus* diminishes the risk of heightened inflammatory responses in extremely low-gestation-age newborns. Although more research is needed to elucidate the molecular mechanisms and health consequences of priming the fetal and newborn systemic inflammatory responses with specific bacterial constituents of the placenta, our data suggest that the targeting of placental colonization by specific drugs or probiotics during early pregnancy may hold promise for preventing not only preterm birth but also the devastating and far-reaching inflammatory consequences in premature newborns.

MATERIALS AND METHODS

ELGAN study and newborn variables. During 2002 to 2004, the ELGAN study enrolled mothers ($n = 1{,}249$) and their babies ($n = 1{,}506$) delivered before 28 weeks of gestation at 14 institutions located in 11 cities of five states. The primary goal of the study was to identify parameters and exposures increasing the risk of brain disorders in ELGANs (48). The enrollment and consent procedures were approved by the human subject research review boards of the individual institutions. This nested study was limited to 527 of the ELGANs delivered by Caesarean section for whom blood samples were available for protein measurements closest to birth (69% within 24 h and all within 72 h of birth) and whose placenta was biopsied within 1 h after delivery. The demographics of these infants and their mothers are presented in Table 3.

The gestational age was hierarchically estimated based on the quality of available information and the following sequence of reliance: (i) known dates of embryo retrieval, intrauterine insemination, or fetal ultrasound before the 14th week (62%); (ii) fetal ultrasound at $\geq$14 weeks (29%); (iii) last menstrual period with no fetal ultrasound (7%); and (iv) a record of the gestation age made in the neonatal intensive care unit log (1%).

Placentas. The placentas were biopsied under sterile conditions as soon as possible after delivery (82% within 1 h) and further microbiologically processed in an anaerobic chamber as described (39). In brief, a ~1-cm^2 tissue sample was homogenized in 1:10 (wt/vol) phosphate-buffered saline (PBS). Aliquots of each homogenate were seeded on selective and nonselective media, as follows: (i) prereduced brucella base agar containing sheep blood (5%), vitamin K_1, and hemin; (ii) tryptic soy agar with sheep blood (5%); (iii) chocolate agar; and (iv) A-7 agar. Established criteria were used to enumerate, isolate, and identify the different colony types at the Brigham and Women's Microbiology Laboratory (49). Since we have determined that constituents of the chorion parenchyma prevent the reliable detection of bacterial DNA by PCR techniques, this study assessed only placental colonization patterns obtained by culture techniques (8).

Blood spot collection and storage. In order to establish the ELGAN inflammatory response associated with placental microbiology while avoiding as much as possible the potentially independent inflammatory impact of later events related to the clinical management of the ELGANs, we analyzed the first blood sample available closest to birth (postnatal days 1 to 3). The study was limited to specimens that remained after blood samples were drawn for clinical care. A blood drop was collected on filter paper (Schleicher & Schuell 903), exposed to air at room temperature in

TABLE 3 Demographic characteristics of mothers and infants in the study of placental microbiology and systemic inflammatory response in the ELGAN study

Demographic	%
Mothers ($n = 412$)	
Age (yrs)	
<21	12
21–35	67
>35	20
No. with public medical insurance	36
Education (yrs completed)	
<12	15
12 (high school)	28
13–15	21
16 (college)	20
>16	16
Infants ($n = 527$)	
Race	
White	64
Black	25
Asian	3
Native American	1
Mixed	3
Other	6
Hispanic	10
Completed gestation wk	
23–24	18
25–26	45
27	37

dark containment until dried, and stored with desiccators in thermo-sealed plastic bags at –70°C.

Elution and measurements of proteins from dried blood spots. All dried blood specimens were eluted and analyzed in the Laboratory of Genital Tract Biology (Brigham and Women's Hospital), which is accredited by the College of American Pathologists for immunoassays and cytokine measurement. Disposable 12-mm biopsy punches were used to excise the blood spot specimens, which were then placed in 0.3 ml PBS with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) and 0.03% Tween 20 (Fisher, Hampton, NH), vortexed, and placed on a shaker for 1 h at 4°C. The buffer was transferred along with the paper to a SpinX dual-chamber filter tube (Fisher) and spun down at 2,000 × g, followed by collection of the filtered eluted blood specimens. An additional wash of the punch was performed in 0.1 ml for a final elution volume of 0.4 ml.

Protein biomarker levels in the eluted blood specimens were quantified using Meso Scale Discovery (MSD; Gaithersburg, MD) electrochemiluminescence (ECL) multiplex immunoassays and Sector Imager 2400, validated by comparison with traditional enzyme-linked immunosorbent assay (ELISA) (50). Each assay was optimized for the simultaneous detection of up to 10 proteins within the linearity range of the dry blood spot elution matrix. The ECL readouts were converted to numbers of pg/milliliters by MSD Workbench software via interpolation from calibrator curves stretching over at least 4 logs. The interassay coefficient of variation [CV = 100 × standard deviation (SD)/mean] was <10 to 20%, as assessed by quality control sample aliquots tested on each plate. The average of two measurements of each analyte was normalized to the number of mg total protein, measured by the Pierce bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL) and Victor^2 reader (PerkinElmer, Boston, MA).

The following 25 protein biomarkers were measured: IL-1β, IL-6, IL-6R, TNF-α, TNF-R1, TNF-R2, IL-8 (CXCL8), MCP-1 (CCL2), MCP-4 (CCL13), MIP-1β (macrophage inflammatory protein 1β) (CCL4), RANTES (regulated upon activation, normal T cell expressed and [presumably] secreted) (CCL5), I-TAC (interferon-inducible T cell alpha-chemoattractant) (CXCL11), ICAM-1 (CD54), ICAM-3 (CD50), VCAM-1 (CD106), E-SEL (E-selectin) (CD62E), MMP-1, MMP-9, CRP, SAA, MPO (myeloperoxidase), VEGF (vascular endothelial growth factor), VEGF-R1, VEGF-R2, and IGF-BP-1.

Data analysis. The generalized null hypothesis evaluated in this study was that the risk of a blood protein concentration in the highest quartile for newborns of a particular gestational age is not associated with the recovery of an organism from their placentas. The focus on the top-quartile concentrations was supported by the concept that the highest concentrations would be most biologically significant and by the observed nonlinear distribution of the concentrations of most proteins among the ELGAN children classified by the presence/absence of each placenta organism.

Because protein concentrations varied with gestational age at delivery, as described for the ELGAN study elsewhere (14), we divided our samples into three groups defined by the gestational age category (23 to 24, 25 to 26, and 27 weeks) and defined the highest quartile of each protein among newborns in each of these gestational age categories. Our unit of measurement is the odds ratio (and 95% confidence interval) that children whose placenta yielded an organism were more likely to have a protein measurement in the top quartile than children whose placenta did not harbor that organism (or group of organisms).

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The work was conducted in accordance with Institutional Review Board-approved procedures for protection of human subjects and in accordance with the Declaration of Helsinki. We have no conflicts of interest.

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