The Relationship between Early Concentrations of 25 Blood Proteins and Cerebral White Matter Injury in Preterm Newborns: The ELGAN Study

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Objective To evaluate whether concentrations of inflammation-related proteins are elevated in the blood of preterm newborns who develop cerebral white matter damage.

Study design We measured 25 proteins in blood collected on days 1, 7, and 14 from 939 infants born before the 28th week of gestation. Brain ultrasound scans were read by at least two sonologists, who agreed on the presence or absence of lesions. A protein concentration was considered elevated if it was in the highest quartile for gestational age and the day on which the specimen was collected.

Results In time-oriented models, elevated concentrations of vascular endothelial growth factor receptor 1, serum amyloid A, and macrophage inflammatory protein 1β on day 1 and interleukin-8 on day 7 were associated with increased risk of ventriculomegaly. Elevated concentrations of macrophage inflammatory protein 1β on day 1 and intercellular adhesion molecule 1 on day 7 were associated with increased risk of an echolucent lesion. Infants with elevated concentrations of inflammation-related proteins on two separate days were at significantly increased risk for ventriculomegaly, but at only modestly increased risk for an echolucent lesion.

Conclusions Concentrations of inflammation-related proteins in the circulation in the first days after preterm birth provide information about the risk of sonographic white matter damage. The inflammatory process might begin in utero. *(J Pediatr 2011;158:897-903)*.

The evidence that a systemic infection or inflammatory process can contribute to cerebral white matter damage in newborns comes from studies of animals, human autopsy material, and measurements of inflammation-related proteins in amniotic fluid, umbilical cord blood, and early postnatal blood.[1] Previous studies that measured inflammation-related proteins in amniotic fluid and in blood were limited by the number of proteins measured at one time, small samples, selection on the basis of birth weight rather than gestational age, and the lack of attention to observer variability in reading cranial ultrasound scans. The present study avoided these limitations, allowing us to report on associations between the levels of 25 proteins measured during the first 2 postnatal weeks and the presence of ventriculomegaly and an echolucent lesion identified by two separate sonologists, who independently evaluated the scans of 728 infants born before the 28th week of gestation.

Methods

The ELGAN Study was designed to identify characteristics and exposures that increase the risk of structural and functional neurologic disorders in extremely low gestational age newborns (ELGANs).[2] During the years 2002-2004, women delivering before 28 weeks gestation at one of 14 institutions in 11 cities in 5 states were enrolled in the study. The enrollment and consent procedures were approved by the individual institutional review boards.

Mothers were approached for consent either on antenatal admission or shortly after delivery, depending on clinical circumstance and institutional preference. A

CRP	C-reactive protein	MMP	Matrix metalloproteinase
ELGAN	Extremely low gestational age newborn	MPO	Myeloperoxidase
ICAM	Intercellular adhesion molecule	RANTES	Regulated upon activation, normal T-cell expressed, and (presumably) secreted
IGFBP	Insulin growth factor–binding protein	SAA	Serum amyloid A
IL	Interleukin	TNF	Tumor necrosis factor
IL-R	Interleukin receptor	TNF-R	Tumor necrosis factor receptor
I-TAC	Interferon-inducible T-cell alpha-chemoattractant	VCAM	Vascular cell adhesion molecule
MCP	Monocyte chemotactic protein	VEGF	Vascular endothelial growth factor
MIP	Macrophage inflammatory protein	VEGF-R	Vascular endothelial growth factor receptor

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total of 1249 mothers of 1506 infants consented. We measured the concentrations of proteins in early blood specimens of 728 children examined at approximately 24 months postterm equivalent.

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 performed before the 14th week of gestation (62%). When these data were not available, reliance was placed sequentially on fetal ultrasound at 14 or more weeks (29%), date of last menstrual period without fetal ultrasound (7%), and gestational age recorded in the log of the neonatal intensive care unit (1%).

Ultrasound Scans

Routine scans were performed by technicians at all of the participating hospitals using digitized high-frequency transducers (7.5 and 10 MHz). All ultrasound studies included the 6 standard quasi-coronal views and 5 sagittal views, using the anterior fontanel as the sonographic window. Three sets of protocol scans were defined based on the postnatal day on which they were obtained. Protocol 1 scans were obtained between day 1 and day 4 (n = 539); protocol 2 scans, between day 5 and day 14 (n = 689); and protocol 3 scans, between day 15 and week 40 (n = 704). Efforts to minimize observer variability included conference calls to discuss aspects of images prone to different interpretations.[3] Templates of multiple levels of ventriculomegaly were part of the data collection form.[4] All ultrasound scans were read by two independent readers who were blinded to clinical information. When the two readers differed in their recognition of lesions, the films were sent to a third (tie-breaking) reader, who was unaware of the first two readers' reports. The readers agreed on the presence or absence of ventriculomegaly and of an echolucent lesion. The 60 children considered to have "late" ventriculomegaly had ventriculomegaly identified on the third scan by the first reader. A total of 67 children had an echolucent lesion identified by two readers.

Neurodevelopmental Assessment

At approximately 24 months postterm equivalent, the participating children underwent a developmental assessment that included neurologic examination, application of the Bayley Scales of Infant Development, and measurement of head circumference.[2]

Blood Spot Collection

Drops of blood were collected on filter paper on postnatal day 1 (range, days 1-3), postnatal day 7 (range, days 5-8), and postnatal day 14 (range, days 12-15). All blood for this analysis was provided from what remained of specimens obtained for clinical indications. Dried blood spots were stored at -70°C in sealed bags.

Elution of Proteins from Blood Spots

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

Protein Measurements

Proteins were measured in duplicate with an electrochemiluminescence detection system (MSD Multiplex Platform, Sector Imager 2400, Discovery Workbench Software, Meso Scale Discovery, Gaithersburg, Maryland), and measurements were validated by comparisons with those obtained with traditional enzyme-linked immunosorbent assay.[5,6] The multiplex assays were optimized to allow detection of each biomarker within the linearity range of the eluted samples. Split quality control blood pools tested on each plate showed an interassay variation of 10%-20% for each protein. The total protein concentration in each eluted sample was determined with the BCA Protein Assay Kit (ThermoFisher Scientific, Rockford, Illinois) using the VICTOR2 Multilabel Counter (PerkinElmer, Boston, Massachusetts), and the measurements of each analyte was normalized to mg of total protein. Each protein was measured in duplicate in the Laboratory of Genital Tract Biology, Brigham and Women's Hospital.

The following proteins were measured: C-reactive protein (CRP); serum amyloid A (SAA); myeloperoxidase (MPO); interleukin (IL)-1β; IL-6; IL-6 receptor (IL-6R); tumor necrosis factor (TNF)-α; TNF receptor (TNF-R) 1; TNF-R2; IL-8 (CXCL8); monocyte chemotactic protein (MCP)-1 (CCL2); 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 (ICAM)-1 (CD54); ICAM-3 (CD50); vascular cell adhesion molecule-1 (VCAM-1; CD106); E-selectin (CD62E); matrix metalloproteinase (MMP)-1; MMP-9; vascular endothelial growth factor (VEGF); VEGF receptor (VEGF-R)-1 (Flt-1), VEGF-R2 (KDR); and insulin growth factor–binding protein 1 (IGFBP-1). We chose these 25 proteins because they represent the broad groups that we consider most important, and because they can be readily, reliably, and accurately measured in the dry blood spot elution matrix with commercially available platforms.

Because protein concentrations varied with gestational age at delivery and with the postnatal day of collection, we divided our sample into 9 groups defined by gestational age category (23-24, 25-26, and 27 weeks) and by the day of blood collection. Because we were interested in the contribution of high protein concentrations, and the concentrations of most proteins did not follow a normal distribution, we