Elevated Concentrations of Inflammation-Related Proteins in Postnatal Blood Predict Severe Developmental Delay at 2 Years of Age in Extremely Preterm Infants

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Objective To evaluate the hypothesis that elevated levels of inflammation-related proteins in early postnatal blood predict impaired mental and motor development in extremely preterm infants.

Study design We measured concentrations of 25 inflammation-related proteins in blood collected on postnatal days 1, 7, and 14 from 939 infants born before 28 weeks gestation. An elevated level was defined as a concentration in the highest quartile for gestational age and day of blood collection. We identified impaired development at age 24 months using the *Bayley Scales of Infant Development, Second Edition*. The primary outcomes were scores on the Mental Scale or the Motor Scale of <55 (more than 3 SDs below the mean).

Results For 17 of the 25 inflammation-related proteins, 1 or more statistically significant associations ($P < .01$) was found between an elevated blood level of the protein and a developmental impairment. Elevations on multiple days were more often associated with developmental impairment than were elevations present for only 1 day. The highest number of predictive elevations was found in day-14 blood.

Conclusion In extremely preterm infants, elevated levels of inflammation-related proteins in blood collected on postnatal days 7 and 14, especially when sustained, are associated with impaired mental and motor development at age 2 years. *(J Pediatr 2012;160:395-401)*.

Preterm newborns are at increased risk for long-term impairments, especially cognitive impairments.[1] Factors likely contributing to the underlying brain damage include infection and inflammation.[2] Experimental inflammation-induced brain damage is mediated in part by inflammatory cytokines and other inflammation-related proteins.[3] Elevated levels of such proteins are present in preterm infants who sustained brain damage identified with neuroimaging,[4] but we are aware of only 1 study describing associations between such proteins and clinical dysfunctions at age 2 years. In that study of 67 infants born before 32 weeks gestation, levels of proinflammatory and modulatory cytokines in blood obtained during the first 72 postnatal hours were associated with motor impairment, but not cognitive impairment.[5]

We evaluated the hypothesis that elevated levels of inflammation-related proteins in blood collected on day 1, 7, and 14 after extremely preterm birth can predict impaired mental and motor development at age 24 months.

Methods

The Extremely Low Gestational Age Newborn (ELGAN) Study was designed to identify characteristics and exposures that increase the risk of structural and functional neurologic disorders in children born before 28 weeks gestation.[6] During the years 2002-2004, we invited the participation of women who delivered before 28 weeks gestation at any of 14 participating institutions. The study was approved by the Institutional Review Board of each institution.

Gestational Age

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

BSID-II	*Bayley Scales of Infant Development, Second Edition*
ELGAN	Extremely Low Gestational Age Newborn
GMFCS	Gross Motor Function Classification System

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Supported by the National Institute of Neurological Disorders and Stroke (Grant NS 40069). This study was completed as a cooperative agreement with the National Institute of Neurological Disorders and Stroke. The study sponsor participated in the study design, but not in the data collection or data analysis. The authors declare no conflicts of interest.

 10.1016/j.jpeds.2011.08.069

or intrauterine insemination, or fetal ultrasound before week 14 (62%). When these data were not available, we used (in order of preference) fetal ultrasound at 14 or more weeks (29%), last menstrual period without fetal ultrasound (7%), and gestational age recorded in the log of the neonatal intensive care unit (1%).

Blood Protein Measurements

Blood Spot Collection. Drops of blood were collected on filter paper on postnatal day 1 (range, day 1-3), day 7 (range, day 5-8), and day 14 (range, day 12-15). Dried blood spots were stored at −70°C in sealed bags with desiccant until processing.

Elution of Proteins from Blood Spots. For protein elution, 12-mm punched biopsy specimens from frozen blood spots were submerged in 300 μL of phosphate-buffered saline containing 0.1% Triton X100 and 0.03% Tween-20 (Fisher, 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 and centrifuged at 2000 × *g*, followed by collection of the filtered eluted blood. An additional wash of the punch was performed in 100 μL, for a final elution volume of 400 μL.

Protein Measurements. Proteins were measured in duplicate using the Meso Scale Discovery multiplex platform and Sector Imager 2400 (MSD, Gaithersburg, Maryland). 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 total protein concentration in each eluted sample was determined by bicinchoninic acid assay, and the measurements of each analyte were normalized to milligrams of total protein. Details about the procedures used to measure inflammation-related proteins are available elsewhere.[7]

24-Month Developmental Assessment

Developmental assessments at 24 months corrected age included the *Bayley Scales of Infant Development, Second Edition* (BSID-II)[8] and an assessment of gross motor function using the Gross Motor Function Classification System (GMFCS).[9] Certified examiners administered and scored the BSID-II. All examiners were experienced users of the BSID-II and specifically for the ELGAN Study attended a 1-day workshop at which published guidelines for test administration and videotaped examinations were reviewed. Examiners were aware of the child's enrollment in the ELGAN Study and corrected age, but not of the child's medical history.

When a child's visual or neurologic impairments precluded assessment with the BSID-II, or more than 2 BSID-II items were omitted or judged "unscorable," the child was classified as not testable on that scale. In 26 of 33 children considered not testable with the BSID-II Mental Scale, the Adaptive Behavioral Composite of the Vineland Adaptive Behavior Scales[10] was used to approximate the Mental Scale score. In 32 children not testable with the BSID-II Motor Scale, the Vineland Adaptive Behavior Scales Motor Skills Domain score was used to approximate the Motor Scale score.

The BISD-II manual defines a significant delay as a Mental or Motor Scale score of <70 (ie, 2 SDs below the mean for the standardization sample). However, in very preterm infants, a Mental Scale score of <55 has a higher predictive ability than a score of <70.[11] In addition, neonatal illness is more strongly associated with a score of <55 than with a score of <70.[12] Thus, we classified the BSID-II outcomes into 3 categories: Mental or Motor Scale score of <55 (>3 SDs below the mean), 55-69 (2-3 SDs below the mean), or >69 (within 2 SDs of the mean or higher). The primary outcome was a Mental or Motor Scale score of <55 (>3 SDs below the mean).

Data Analysis

For analyses involving the BSID-II Mental Scale, we excluded infants with significantly impaired gross motor function, defined as an inability to walk independently (GMFCS level ≥1), because their failure on certain Mental Scale test items might have been attributable to impaired motor function rather than to impaired mental development.

For all analyses, we classified a protein biomarker concentration as elevated if it was in the highest gestational age–specific and postnatal day–specific quartile. To describe associations between gestational age–specific and postnatal day–specific elevations of protein concentrations and Mental or Motor Scale score of <55 or 55-69, we used multinominal logistic regression to estimate ORs. The referent group was infants with Mental or Motor Scale score of ≥70. All models were adjusted for gestational age. Given that the aim of our etiologic study was not to develop a prediction model for developmental impairment, we did not include all candidate predictors. To balance the risks of type 1 and type 2 errors, we chose to describe the precision of OR estimates with 99% CIs.

Results

Mothers (n = 1249) of 1506 infants gave their informed consent. For this analysis, we limited the sample to the 939 children from whom blood was obtained for analysis of protein levels and whose development was assessed at age 24 months. The overall follow-up rate was 85% (1018 of 1200), and blood protein data were available for 92% (n = 939) of the infants evaluated at age 24 months. Thus, 182 of the 1200 survivors were not included in this analysis because they did not return for follow-up, and 79 were not included because of a lack of blood protein data (**Figure**; available at www.jpeds.com).

On the Mental Scale, 11% of the cohort had a score of <55 and 11% had a score of 55-69. On the Motor Scale, 16% scored below 55, and 15% scored 55-69. Among children with a Mental Scale score of <55, 43% had a Motor Scale score of <55; among those with a Motor Scale of <55, 55% had a Mental Scale score of <55. Low scores were especially likely in infants born at gestational age 23-24 weeks or with a birth weight of ≤750 g, and in those born to mothers with preeclampsia (**Table I**; available at www.jpeds.com).