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Inflammation-initiating illnesses, inflammation-related proteins, and cognitive impairment in extremely preterm infants

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Abstract

Neonatal inflammation is associated with perinatal brain damage. We evaluated to what extent elevated blood levels of inflammation-related proteins supplement information about the risk of impaired early cognitive function provided by inflammation-related illnesses. From 800 infants born before the 28th week of gestation, we collected blood spots on days 1, 7 and 14, for analysis of 25 inflammation-related proteins, and data about culture-positive bacteremia, necrotizing enterocolitis (Bell stage IIIb), and isolated perforation of the intestine, during the first two weeks, and whether they were ventilated on postnatal day 14. We considered a protein to be persistently or recurrently elevated if its concentration was in the top quartile (for gestational age and day blood was collected) on two separate days one week apart. We assessed the children at 2 years of age with the Bayley Mental Development Index (MDI). The combinations of NEC and ventilation on day 14, and of bacteremia and ventilation on day 14 consistently provided information about elevated risk of MDI <55, regardless of whether or not a variable for an elevated protein concentration was included in the model. A variable for a persistently or recurrently elevated

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concentration of each of the following proteins provided additional information about an increased risk of MDI <55: CRP, SAA, IL-6, TNF-alpha, IL-8, MIP-1beta, ICAM-1, E-SEL, and IGFBP-1. We conclude that elevated blood concentrations of inflammation-related proteins provide information about the risk of impaired cognitive function at age 2 years that supplements information provided by inflammation-associated illnesses.

Keywords

cognitive impairment; necrotizing enterocolitis; extreme prematurity; systemic inflammatory response; neonatal chronic lung disease; neonatal sepsis

1. INTRODUCTION

Extremely preterm infants (born before 28 weeks of gestation) are at a ten-fold increased risk of cognitive impairment compared to full term infants (Johnson et al., 2009). The underlying structural brain pathology is probably multifocal, but one component consists of periventricular white matter abnormalities that can be identified during the first postnatal weeks using ultrasonography or magnetic resonance imaging (MRI). The periventricular echolucent lesions correspond to histologically defined cerebral white matter necrosis (Paneth et al., 1990). These lesions, as well as MRI evidence of thinning of the corpus callosum, loss of periventricular white matter volume (Woodward et al., 2006), low hippocampal volume (Thompson et al., 2008), and increased apparent diffusion coefficient in the white matter (Krishnan et al., 2007), predict cognitive impairment evident years later.

Epidemiologic studies repeatedly link perinatal infections and inflammation with later childhood brain dysfunctions, including cerebral palsy and cognitive impairment (Dammann and O'Shea, 2008; Malaeb and Dammann, 2009; Shatrov et al., 2010; O'Shea et al., 2012); while experimental models that initiate systemic inflammation with lipopolysaccharide document later brain structure alterations and brain dysfunctions (Wang et al., 2006). Based on such models, molecular mechanisms that lead to perinatally acquired periventricular white matter damage include aberrant activation of developmentally regulated apoptotic pathways and microglial activation (Kaindl et al., 2009). Acutely, these lead to axonal damage and depletion of oligodendrocyte precursors, and subsequently to damaged subplate neurons, disordered white matter tracts, and reduced brain "connectivity."

Preterm newborns are capable of mounting a vigorous inflammatory response to antenatal intra-uterine bacteria and other inflammatory stimuli that lead to preterm birth and placenta inflammation (Fichorova et al., 2011; Hecht JL et al., 2010; McElrath et al., 2011; Leviton et al., 2011b). Inflammatory stimuli evident after delivery, such as bacteremia, necrotizing enterocolitis, and those associated with processes leading to bronchopulmonary dysplasia (*i.e.*, ventilator-associated barotrauma and oxidative stress), also appear capable of inducing a postnatal inflammatory response (Ng et al., 2010; Bose et al., 2011; Leviton et al., 2011a; Edelson et al., 1999). Systemic inflammation during the first weeks of life, as reflected in elevations of specific inflammation proteins, is predictive of neonatal cerebral white matter injury (Procianoy and Silveira, 2012; Leviton A et al., 2011b) as well as microcephaly (Leviton A et al. 2011a) and cognitive impairment at two years of age (O'Shea et al., 2012).

Possible relationships among perinatal inflammation stimuli (e.g., bacteremia, necrotizing enterocolitis, ventilator-induced lung injury), inflammation, as reflected by elevations of inflammation-related proteins in the blood, and brain injury are shown in Figure 1. As illustrated, inflammation stimuli could result in brain injury via mechanisms mediated by inflammation proteins (Model 1) or via mechanisms that do not involve these proteins

(Model 2), or both of these mechanisms might be operative (Model 3). Further, different inflammation stimuli could cause injury via different pathways (Model 4).

In Model 1, a stimulus leads to an inflammatory response, which in turn, damages the brain. Support for the first part of this model comes from observations that inflammatory stimuli, such as necrotizing enterocolitis (Edelson et al., 1999)(Martin CR et al., 2012), sepsis (Ng et al., 2003)(Leviton et al., 2012b), and mechanical ventilation (Bose et al., 2012), are associated with increased blood levels of inflammatory cytokines in preterm newborns. Systemic inflammation, in turn, has been associated with such indicators of brain damage as ventricular enlargement seen on early ultrasound scans of the brain(Leviton A et al., 2012) and microcephaly at age 2 years (Leviton A et al., 2011a), as well as such indicators of brain dysfunction as low mental and motor indices of the Bayley Scales of Infant Development (O'Shea et al., 2012).

In Model 2, a stimulus capable of inducing inflammation, damages the brain by means other than inflammation. Such stimuli can contribute to damage by: 1) sensitizing the brain to later adverse exposures (Eklind et al., 2005), 2) introducing other brain damaging exposures such as neurotoxic antibiotics (Floersheim GL and Logara-Kalantzis A, 1972), and 3) promoting coagulation, which might lead to blood vessel occlusion and resulting brain damage(Leviton and Dammann, 2004). Recurrent/prolonged acidemia, which has been identified as an antecedent of systemic inflammation(Leviton et al., 2011a), can also contribute to, or be a marker of, other processes leading to brain damage (Wajner and Goodman, 2011; Lee et al., 2008; Leviton et al., 2010).

Model 3 combines elements of Models 1 and 2, as does Model 4. Model 3 differs from Model 4 by invoking two separate stimuli.

To further our understanding of the relationship of perinatal inflammation stimuli, neonatal systemic inflammation, and brain injury, we evaluated the hypothesis that information about both inflammation-related proteins as well as neonatal clinical illnesses predict subsequent cognitive impairment. If Model 1 explains the observed links between elevations of inflammation-related proteins and brain injury, and between neonatal illnesses that induce inflammation (e.g., bacteremia, necrotizing enterocolitis, ventilator-induced lung injury) and brain injury, then statistical adjustment for elevations of blood levels of inflammation-related proteins should completely mask associations between the neonatal illnesses and brain injury. To evaluate this possibility, we used multivariable methods to analyze data from the Extremely Low Gestational Age Newborn (ELGAN) Study (O'Shea et al., 2008).

The ELGAN Study is an observational and longitudinal cohort study of infants born before the 28th week of gestation. The over-arching objective is to test the hypothesis that perinatal infection/inflammation is a risk factor for later brain dysfunctions, such as cerebral palsy, cognitive impairment, autism spectrum disorder, epilepsy, and behavioral abnormalities (O'Shea et al., 2009). To assess potential initiators of inflammation, placentas were cultured for microorganisms and examined histologically, and the concentrations of 25 inflammation-related proteins were measured in neonatal blood specimens collected on postnatal days 1, 7, and 14. Clinical assessments completed close to age two years include a standardized neurological examination and assessment of gross motor function, Bayley Scales of Infant Development, and screening for autism spectrum disorders. Ongoing clinical assessments close to age 10 years include neuropsychological assessments for intelligence and executive functioning, gold-standard assessments for autism spectrum disorders, an evaluation for epilepsy, assessments of language and communication abilities, and a behavioral assessment.

2. METHODS AND MATERIALS

2.1. The ELGAN Study

During the years 2002–2004, women delivering before 28 weeks gestation at one of 14 participating institutions in 11 cities in 5 states were asked to enroll in the study. The enrollment and consent processes were approved by the individual institutional review boards.

At the time of delivery, biopsies of the placenta were obtained using sterile technique and flash frozen for shipping to a central laboratory for culture of Mycoplasmas, anaerobic bacteria, and aerobic bacteria (Onderdonk et al., 2008). Placentas were examined histologically using standardized definitions for vascular abnormalities and inflammation (Hecht et al., 2008a; Hecht et al., 2008b). Mothers were interviewed about their pre-pregnancy and reproductive health and about medications taken during pregnancy. The mother's medical record was reviewed to obtain information about complications and treatments during pregnancy, labor, and delivery. We reviewed neonatal medical records to obtain anthropometric data, data for calculation of an illness severity measure (Richardson et al., 2001), treatments given to the neonate, and neonatal illnesses. In preparation for collecting data specifically for this study, pathologists, radiologists, and ophthalmologists worked with their colleagues to create a manual with definitions and procedures all could agree on. They also conducted exercises to minimize observer variability. All ultrasound scans were read by two independent readers who were not provided clinical information (Kuban K et al., 2007).

Approximately two years from each infant's expected date of delivery, we interviewed her/his mother to obtain information about the infant's health and obtained the mother's responses to an autism screening questionnaire, and we evaluated each infant with anthropometric measurements, a standardized neurological examination (Kuban K et al., 2008; Kuban et al., 2005), and a standardized assessment of infant cognitive and motor functioning, and behavioral competencies (the Bayley Scales of Infant Development-Second Edition) (Bayley N, 1993). Examiners who performed neurological and developmental assessments were not aware of infants' neonatal complications, brain ultrasound abnormalities, or blood levels of inflammation-related proteins.

The sample for this report consists of the 800 newborns for whom we had information about protein measurements on at least two of the three protocol days (days 1, 7, and 14), data about whether they had Bell stage IIIb NEC or isolated bowel perforation (IP) ¹³, culture-positive bacteremia (Patel et al., 2011), or were mechanically ventilated on postnatal day 14 (Bose et al., 2011), and who had a developmental assessment at age 2 years post-term equivalent [Table 1].

2.2. Newborn variables

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

Information was collected about blood cultures for each week, but not for each day (Patel et al., 2011). The recovery of an organism from blood was reported, but details about the organism were not. An infection was identified as documented when the cultured organism was considered a potential pathogen and not a contaminant.

Necrotizing enterocolitis was classified according to modified Bell criteria (Walsh and Kliegman, 1986). For the analyses reported here, a child was considered to have NEC if the stage was IIIb (surgical NEC). Infants were classified as having isolated intestinal perforation if they had no clinical features of NEC, but had a gastrointestinal perforation documented on radiograph and an isolated intestinal perforation confirmed at the time of exploratory laparotomy or strongly suspected clinically (if managed with Penrose drain and exploratory laparotomy was not done).

Ventilation status was recorded routinely on postnatal day 14 (Laughon et al., 2009a). Here we classify a child as ventilated if s/he received conventional mechanical or high frequency ventilation that day.

2.3. 24-month developmental assessment

Developmental assessments at 24-months corrected age included the Bayley Scales of Infant Development-Second Edition (BSID-II) (Bayley N, 1993), and an assessment of gross motor function using the Gross Motor Function Classification System (Palisano et al., 1997). The overall follow up rate was 85% (1018/1200) and blood protein data were available for 92% (n=939) of the infants who were evaluated at 24 months.

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 one-day workshop where 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 the child's medical history.

When a child's visual or neurological impairments precluded assessment with the BSID-II, or more than 2 items were omitted or judged to be 'unscorable,' the child was classified as not testable on that scale. The Adaptive Behavioral Composite of the Vineland Adaptive Behavior Scales (Sparrow et al., 1984), obtained for 26 of 33 children who were considered not testable with the BSID-II Mental Scale (i.e., Mental Development Index), was used to approximate the Mental Scale score. Among infants not testable with the BSID-II Motor Scale (i.e., Psychomotor Development Index), 32 were assessed with the Vineland Adaptive Behavior Scales, and the Vineland Adaptive Behavior Scales Motor Skills Domain score was used to approximate the Motor Scale score.

The BSID-II manual defines a significant delay as a Mental or Motor Scale below 70, i.e., 2 standard deviations below the mean for the standardization sample. However, in very preterm infants, the predictive ability of a Mental Scale below 55 is higher than that of a score below 70 (Roberts et al., 2010). In addition, neonatal illness is more strongly associated with a score below 55 than with a score below 70 (Laughon et al., 2009b). We excluded infants with significantly impaired gross motor function, defined as an inability to walk independently (a Gross Motor Function Classification System level 1) because motor impairment can preclude accurate assessment of cognitive ability in preschool children.

2.4. Blood spot collection

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

2.5. Protein measurement

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

Proteins were measured in duplicate with an electrochemiluminescence detection system (MSD multiplex platform, Sector Imager 2400, Discovery Workbench Software) that has been validated by comparisons with traditional ELISA (Fichorova et al., 2006; Fichorova et al., 2008).

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 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 analyte normalized to mg total protein. Each protein was measured in duplicate.

We measured the following 25 proteins: C-Reactive Protein (CRP), Serum Amyloid A (SAA), Myeloperoxidase (MPO), Interleukin-1 β (IL-1 β), 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-1 β (MIP-1 β ; 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), 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).

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, 27 weeks) and the day of blood collection. Because we were interested in the contribution of high concentrations, and the concentrations of most proteins did not follow a normal distribution, we dichotomized the distribution of each protein's concentration into the highest quartile among children in each of the 9 gestational age and postnatal day groups and the lower three quartiles.

2.6. Data analysis

We evaluated the hypothesis that once variables for inflammatory stimuli (bacteremia in week one or two, NEC stage IIIb/IP, or mechanical ventilation on day 14) are in multivariable models of the risk of an MDI < 55, the concentrations of inflammation-related proteins do not provide additional information. In previous analyses that did not consider bacteremia, protein elevations in the top quartile on two separate days a week apart provided considerably more discriminating risk information than did elevations on just one day (Leviton A et al., 2011b; Leviton A et al., 2011a; O'Shea et al., 2012). Thus, we focus here on protein elevations that persisted or recurred.

We express the strength of association between exposures (risk factors) and the outcome we studied (cognitive impairment) using odds ratios. In the current context, odds ratios indicate the odds of developing cognitive impairment for an infant with the risk factor, divided by the odds of developing cognitive impairment for an infant without the risk factor. Thus an odds ratio of 2 indicates that infants with the risk factor were at twice the risk as compared to those without the risk factor, a 100% increase in the odds.

To calculate odds ratios and 95% confidence intervals, we created a number of logistic regression models of the risk of an MDI < 55. The first model did not include any protein. Rather this model evaluated how well combinations of the three prototype inflammatory stimuli assessed in this analysis (NEC stage IIIB/IP, bacteremia in week one or two, and ventilation on day 14) provide risk information in the absence of information about protein concentrations (Table 2). Variables for gestational age categories (23–24, 25–26, and 27 weeks) were included as well.

We then took the model displayed in Table 2 (and Table 3, top line) and added a variable for concentrations of one protein in the top quartile (for gestational age and day specimen was obtained) on two days at least one week apart. The result is 25 separate models, one for each protein.

3. RESULTS

3.1. Combinations of inflammation-provoking stimuli (Table 2)

We evaluated associations between an MDI < 55 and combinations of the three prototype clinically evident inflammation-provoking stimuli (NEC stage IIIB/IP, bacteremia in week 1 or 2, or mechanical ventilation on day 14). Individually, none of these stimuli was associated with MDI < 55 (see 4th, 6th, and 7th rows in Table 2). In contrast, infants with more than one of these stimuli were more than three times as likely to have an MDI < 55, as infants with none of these stimuli (see 1st, 3rd, and 5th rows). The tripling of risk among the 13 infants who were exposed to all three inflammation-stimuli was not statistically significant.

3.2. Models with protein variables (Table 3)

To evaluate whether protein elevations provide risk information that is not redundant with that provided by the three clinical events that we studied, and whether clinical events provided risk information that is non-redundant, we created 26 multivariable logistic regression models that included the protein elevation in the top quartile on two days a week apart), a combination of clinically evident stimuli (NEC stage IIIB/IP and mechanical ventilation on day 14 or bacteremia in week one or two and mechanical ventilation on day 14), and gestational age. When a protein has a statistically significantly elevated odds ratio, it provides information about the risk of severe cognitive limitation above and beyond the risk information provided by the combination of inflammation-promoting clinical exposures (*i.e.*, either the combination of necrotizing enterocolitis and prolonged ventilation assistance, or the combination of bacteremia and prolonged ventilation assistance) Regardless of what protein was added to the model, the combinations of mechanical ventilation on day 14 with NEC stage IIIB/IP, and mechanical ventilation on day 14 with bacteremia in week one or two, conveyed statistically significant information about the risk of MDI < 55. For nine proteins (CRP, SAA, IL-6, TNF- α , IL-8, MIP-1 β , ICAM-1, E-SEL, and IGFBP-1), the odds ratios associated with the proteins were significantly greater than 1, suggesting that elevated concentrations provide risk information that supplements information provided by the combinations of clinically evident stimuli.

4. DISCUSSION

Our main finding is that quantitatively assessed indicators of neonatal systemic inflammation such as the proteins measured in this study supplement the information about the risk of impaired development at age 2 years provided by NEC IIIb/IP, bacteremia and ventilation. Very preterm newborns who had NEC IIIb/IP, bacteremia, or were exposed to prolonged mechanical ventilation are at increased risk of low MDI (Stoll et al., 2004; Walsh et al., 2005; Hintz et al., 2005; Shah et al., 2008; Martin et al., 2010). So are children who had systemic inflammation (O'Shea et al., 2012). Our study appears to be the first to evaluate the contribution of the neonate's systemic inflammatory response in light of information about inflammation-provoking exposures.

Although we report that quantitative indicators of systemic inflammation provide supplemental information, we are not certain why. One explanation for the supplemental information invokes the quality of information about the inflammation-provoking disorders and exposures. The dichotomous nature of our clinical variables (e.g. NEC IIIb/IP, bacteremia and ventilation), does not convey information about the intensity of the inflammation associated with these clinical disorders. For example, we did not collect identifying information about the organisms recovered from the blood, nor did we quantify the bacterial load. Consequently, bacteremia with *Staphylococcus epidermidis* was treated the same as bacteremia with a more virulent organism.

Another explanation invokes infant characteristics. The magnitude/severity of the inflammatory response (Patel et al., 2011; Stoll et al., 2002; Bartels et al., 2007) and the vulnerability to inflammation (Brichtova and Kozak, 2008) are influenced by infant characteristics. Among preterm newborns exposed to placenta inflammation, the lower the gestational age, the higher the blood concentrations of inflammation-related proteins (Leviton et al., 2011b). Therefore, the same stimulus might produce a more vigorous or more damaging response in some newborns than in their peers. This explanation assumes that the response contributes to the brain damage.

A third possibility is that some of the inflammatory response might have been stimulated by secondary processes involved in clearing away damaged brain cells that may continue after a stimulus that has caused damage has been removed. Necrotic cells release "damage-associated molecular patterns," (Kono and Rock, 2008) which are identified by inflammatory cells that can then initiate an inflammatory response to clear away the debris. (Rock and Kono, 2008) Part of this inflammatory response includes creating signals to recruit more inflammatory cells. (McDonald et al., 2010) In this explanation, the systemic inflammatory response is secondary to brain damage or in other words, it is an indicator that brain damage had already begun. At the same time, this process might be part of the immunologic "danger signal," (Matzinger, 2002) promoting feedback-loops between innate and adaptive immune responses, (Leviton et al., 2005) which might, in turn, promote feedback loops between brain damage and immune responses. If true, this scenario would further support the notion that perinatal brain damage is an ongoing or reverberating process, not a single insult (Dammann, 2007; Malaeb and Dammann, 2009; Fleiss B and Gressens P, 2012).

Our observations suggest that systemic inflammation might contribute to cognitive impairment

A fourth possibility is that none of the inflammatory stimuli on which we focused is the stimulus for the elevated protein levels we identified. Inflammatory stimuli that we did not evaluate include viral infections, urinary tract infections, and tracheitis.

Regardless of which of these possibilities most closely reflects the biology that underlies our observations, the major inference prompted by our findings is that the newborn's systemic inflammation response provides important information about the risk of brain damage indicators, even when adjusting for presumed stimuli for the systemic inflammation.

Inflammation is a complex process with hundreds of genes either up- or down-regulated (Zak and Aderem, 2009). Consequently, individual proteins should be seen as only a small part of a much larger and complex process with highly inter-related components (Srisakandam and Altmann, 2008; Leviton et al., 2012a). Why some proteins provided more risk information than others is open to many interpretations. The proteins we identified here are among those most closely linked to perinatal brain damage in the very preterm newborns (Dammann and O'Shea, 2008; Malaeb and Dammann, 2009). The elevated blood concentrations of inflammation-associated proteins could be regarded as snap-shot biomarkers of inflammation. Perhaps a continuously available biomarker of inflammation, such as decreased heart rate variability might provide better risk information than our snap-shots (Fairchild and O'Shea, 2010; Addison et al., 2009). Continuously available biomarkers might also be useful for characterizing baseline risk for infants enrolled in trials of post-NICU interventions to improve developmental outcome.

The association between perinatal inflammation and brain damage merely identifies where more basic work is needed if perinatal brain damage is to be prevented. More information is needed about the timing of injury and repair. For example, what is the role of antenatal inflammation? Does it sensitize the brain making it more vulnerable to subsequent insults, (Eklind et al., 2005) or is preconditioning more likely, making the brain less vulnerable? (Mallard and Hagberg, 2007) How long do inflammation and acute damage continue? Accumulating evidence suggests that the therapeutic window of opportunity might extend weeks or even months beyond delivery of an extremely preterm infant. (Fleiss B and Gressens P, 2012) However, is an anti-inflammatory intervention sufficient once inflammatory damage has been initiated?

Could interventions to inhibit inflammation, in fact, be detrimental? We ask this question because neuroinflammation induces not only a broad spectrum of anti-inflammatory but also immunomodulatory responses, and other processes involved in inflammation resolution (Serhan, 2009) and brain injury repair (Malaeb and Dammann, 2009).

Interventions to prevent inflammation-related perinatal brain injury are likely to be derived from interdisciplinary collaborations involving clinical and laboratory-based scientists. This is exemplified by studies of melatonin in animals (Gressens et al., 2008; Welin et al., 2007) that have led to initiation of a randomized trial in humans (ClinicalTrials.gov Identifier: NCT00649961).

Techniques for measurement of inflammation-related proteins in minute quantities of neonatal blood, (Fichorova et al., 2008) as used in our study, provide an opportunity to assess the effect of therapies to diminish/eliminate systemic inflammation when these agents are tested in randomized trials. Also, these techniques allow investigation of the anti-inflammatory effects of potentially neuroprotective interventions. (Schmidt et al., 2012; Brocklehurst et al., 2011; Loron et al., 2011)

Our study has several strengths. First, we evaluated a large number of infants thereby providing power to perceive a doubling of risk of most indicators of perinatal brain damage. Second, to minimize confounding due to factors related to fetal growth restriction, we recruited infants based on gestational age and not birth weight. (Arnold CC et al., 1991) Third, we collected all of our data prospectively. Fourth, attrition in the first two years was modest, with information about MDI scores for almost 90% of surviving infants. Fifth,

examiners were not aware of the medical histories of the children they examined, thereby minimizing “diagnostic suspicion bias.” (Sackett DL, 1979) Sixth, our protein data are of high quality, with high content validity.(Leviton et al., 2011b; Hecht JL et al., 2010; Fichorova et al., 2011; McElrath et al., 2011)

4.1 Limitations

Our major limitation is not having collected information about the organisms recovered from the blood of our subjects. Consequently, what we classify as documented bacteremia includes such highly virulent organisms as *Staphylococcus aureus* along with much less virulent organisms, such as *Staphylococcus epidermidis*. The other major limitation of our study is that we do not have precise information about the timing of the inflammatory stimuli we identified, limiting the confidence with which we can attribute protein elevations to inflammatory disorders or inflammatory disorders to protein elevations.

5. SUMMARY

Among ELGANs, recurrently or persistently elevated blood concentrations of inflammation-related proteins during the first two postnatal weeks appear to supplement information about the risk of an MDI < 55 provided by NEC/IP, bacteremia, and prolonged ventilation. Thus, the intensity of the systemic inflammatory response adds risk information beyond that provided by NEC/IP, bacteremia, and “prolonged” ventilation.

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Highlight

Elevated blood inflammation-related proteins predict impaired early cognitive functioning, supplementing information provided by inflammation-associated illnesses.

Model 1 **Model 2** **Model 3** **Model 4** **Figure 1.**

Four models of the interrelationships among an inflammation stimulus (one or more inflammation-related illnesses), and inflammation response, and an indicator of brain damage

Table 1

Sample description.

	Yes	No
Enrolled	1506	
Survived to 24 months	1200	306
GMFCS <1	962	238
Proteins measured in blood spots on 2 days	805	157
Necrotizing enterocolitis [*] /isolated bowel perforation	50	750
Bacteremia during the first 2 weeks	134	666
Mechanical ventilation on day 14	458	342
Mental Development Index (MDI) <55	89	711

Abbreviations: GMFCS – Gross Motor Function Classification System; MDI – Mental Development Index

^{*} Bell stage IIIb

Table 2

Odds ratio (and 95% CI) for Mental Development Index < 55* in logistic regression models that include necrotizing enterocolitis (Bell stage IIIb), bacteremia, and mechanical ventilation on postnatal day 14 either as isolated exposures or in combination with one another. All models adjust for gestational age. The referent group consists of infants with none of the three exposures. Bold text indicates odds ratios that are significance at $p < 0.05$.

NEC IIIb/IP	Bacteremia in week 1 or 2	Ventilated on day14	OR (95% CI)	Row N
+	+	+	3.1 (0.8, 12)	13
+	+	-	----	0
+	-	+	4.1 (1.4,11)	28
+	-	-	1.4 (0.2, 12)	9
-	+	+	3.5 (1.7, 7.1)	80
-	+	-	2.0 (0.8, 5.4)	41
-	-	+	1.0 (0.5, 1.9)	337
-	-	-	1.0	292

Abbreviations: NEC – necrotizing enterocolitis

* among children with a GMFCS < 1

Table 3

Odds ratio (and 95% CI) for Mental Development Index < 55* associated with necrotizing enterocolitis (Bell stage IIIb or IP) and ventilation on day-14, and with bacteremia in week 1 or 2 and ventilation on day-14 when protein information is not included (top line), and when information is included about elevated blood concentrations (defined as in the top quartile on two days at least one week apart) of the protein listed on the left. All models adjust for gestational age. Bold text indicates odds ratios that are significance at $p < 0.05$.

	ODDS RATIOS AND 95% CONFIDENCE INTERVALS		
Protein name	Protein alone	NEC IIIb/IP AND Ventilated on day 14	Bacteremia in week 1 or 2 AND Ventilated on day 14
none	----	4.1 (1.4, 11)	3.5 (1.7, 7.1)
CRP	1.8 (1.04, 3.0)	3.5 (1.3, 9.3)	3.2 (1.5, 6.6)
SAA	2.3 (1.4, 4.0)	3.5 (1.3, 9.5)	3.1 (1.5, 6.4)
MPO	1.2 (0.7, 2.1)	4.1 (1.5, 11)	3.5 (1.7, 7.2)
IL-1 β	1.7 (0.98, 3.0)	3.8 (1.4, 10)	3.5 (1.7, 7.3)
IL-6	2.4 (1.4, 4.0)	3.6 (1.3, 9.6)	3.1 (1.5, 6.5)
IL-6R	1.2 (0.7, 2.0)	4.2 (1.6, 11)	3.5 (1.7, 7.2)
TNF- α	1.9 (1.1, 3.2)	3.9 (1.4, 10)	3.2 (1.6, 6.7)
TNF-R1	0.9 (0.5, 1.6)	4.2 (1.6, 11)	3.4 (1.6, 7.0)
TNF-R2	1.2 (0.7, 2.2)	4.1 (1.6, 11)	3.3 (1.6, 6.9)
IL-8	2.2 (1.3, 3.8)	3.5 (1.3, 9.4)	3.0 (1.4, 6.3)
MCP-1	1.5 (0.9, 2.6)	3.9 (1.5, 10)	3.1 (1.5, 6.5)
MCP-4	0.9 (0.5, 1.6)	4.1 (1.5, 11)	3.5 (1.7, 7.1)
MIP-1 β	1.8 (1.1, 3.0)	4.7 (1.7, 13)	3.6 (1.7, 7.4)
RANTES	1.4 (0.8, 2.5)	4.5 (1.7, 12)	3.8 (1.8, 7.9)
I-TAC	0.8 (0.4, 1.5)	4.0 (1.5, 11)	3.4 (1.7, 7.1)
ICAM-1	2.1 (1.3, 3.5)	3.5 (1.3, 9.5)	2.9 (1.4, 6.1)
ICAM-3	1.4 (0.8, 2.4)	4.3 (1.6, 11)	3.4 (1.7, 7.2)
VCAM-1	1.6 (0.9, 2.7)	4.4 (1.6, 12)	3.4 (1.6, 7.0)
E-SEL	2.1 (1.3, 3.5)	4.3 (1.6, 11)	3.4 (1.6, 7.0)
MMP-1	0.8 (0.4, 1.5)	4.1 (1.5, 11)	3.2 (1.6, 6.7)
MMP-9	0.7 (0.3, 1.3)	4.0 (1.5, 11)	3.5 (1.7, 7.3)
VEGF	1.3 (0.8, 2.2)	4.3 (1.6, 12)	3.5 (1.7, 7.2)
VEGF-R1	0.6 (0.3, 1.2)	4.3 (1.6, 11)	3.4 (1.6, 7.1)
VEGF-R2	1.6 (0.97, 2.7)	4.0 (1.5, 11)	3.3 (1.6, 6.7)
IGFBP-1	2.4 (1.4, 4.1)	4.1 (1.5, 11)	3.2 (1.5, 6.6)

Abbreviations: NEC – necrotizing enterocolitis; CRP: C-Reactive Protein; SAA: serum amyloid A; MPO: myeloperoxidase; IL-1 β : Interleukin-1 β ; IL-6: Interleukin-6; IL-6R: interleukin-6 receptor; TNF- α : Tumor Necrosis Factor- α ; TNF-R1: Tumor Necrosis Factor-alpha receptor 1; TNF-R2: tumor necrosis factor-alpha-receptor-2; IL-8: Interleukin-8; MCP-1: monocyte chemotactic protein-1 (CCL2); MCP-4: monocyte chemoattractant protein-4 (CCL13); MIP-1 β : Macrophage Inflammatory Protein-1 β (CCL4); RANTES: regulated upon activation, normal T-cell expressed and (presumably) secreted; I-TAC: Interferon-inducible T cell Alpha-Chemoattractant; ICAM-1: intercellular adhesion molecule-1; ICAM-3: Intercellular adhesion molecule-3 (CD54); VCAM-1: vascular cell adhesion molecule-1 (CD106); E-SEL1: E-selectin (CD62E); MMP-1: Matrix MetalloProteinase-1; MMP-9: matrix metalloproteinase-9; VEGF: Vascular Endothelial Growth Factor; VEGF-R1: Vascular Endothelial Growth Factor-Receptor 1; VEGF-R2: vascular endothelial growth factor-receptor-2; IGFBP-1: Insulin Growth Factor Binding Protein-1

* among children with a GMFCS < 1