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Systemic inflammation and cerebral palsy risk in extremely preterm infants

KCK Kuban, MD[1], **TM O'Shea, MD**[2], **EN Allred, MS**[3,4,5], **N Paneth, MD**[6], **D Hirtz, MD**[7], **RN Fichorova, MD, PhD**[3,8], and **A Leviton, MD**[3,4] **for the ELGAN Study Investigators**

[1]Department of Pediatrics, Boston Medical Center, Boston, MA, USA

[2]Wake Forest University School of Medicine, Winston-Salem, NC, USA

[3]Harvard Medical School, Boston, MA, USA

[4]Boston Children's Hospital, Boston, MA, USA

[5]Harvard School of Public Health, Boston, MA, USA

[6]Department of Epidemiology and Biostatistics, Michigan State University, East Lansing, MI, USA

[7]National Institute of Neurological Disorders and Stroke, Bethesda, MD, USA

[8]Department of Obstetrics Gynecology & Reproductive Biology, Brigham and Women's Hospital, Boston, MA, USA

Abstract

We hypothesized that among extremely preterm infants, elevated concentrations of inflammation-related proteins in neonatal blood are associated with cerebral palsy (CP) at 24 months.

Methods—In 939 infants born before 28 weeks gestation, we measured blood concentrations of 25 proteins on postnatal days 1, 7, and 14 and evaluated associations between elevated protein concentrations and CP diagnosis.

Results—Protein elevations within three days of birth were not associated with CP. Elevations of TNF-α, TNF-R1, IL-8, ICAM-1, on at least two days were associated with diparesis. Recurrent-persistent elevations of IL-6, E-SEL, or IGFBP-1 were associated with hemiparesis. Diparesis and hemiparesis were more likely among infants who had at least four of nine proteins elevations that previously have been associated with cognitive impairment and microcephaly.

Interpretation—Repeated elevations of inflammation-related proteins during the first two postnatal weeks are associated with increased risk of CP.

Address for Correspondence: Karl C. K. Kuban, Department of Pediatrics, Boston University School of Medicine-Boston Medical Center, 771 Albany Street, Boston, MA 02118, karl.kuban@bmc.org; phone number 617-414-4548; fax number: 617-414-4502.

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The authors declare no conflicts of interest.

Introduction

Among infants born preterm, elevated concentrations of inflammation-related proteins in umbilical cord and neonatal blood are associated with neonatal cerebral white matter damage [1-3], which, in turn, is associated with subsequent diagnosis of cerebral palsy (CP) [4,5]. Studies of these associations have been limited by small samples, selection of children based on birth weight rather than gestational age, measurement of a small number of proteins at a single time, non-standard methods for diagnosing CP, and by aggregation of heterogeneous forms of CP into a single outcome.

In our prospective study of infants born before the 28th week of gestation, we measured concentrations of 25 inflammation-related proteins in blood samples from nearly a thousand newborns within three days of birth, and approximately one week and two weeks later. Elevated concentrations of nine of these proteins (CRP, SAA, IL-1β, IL-6, IL-8, TNF-α, MIP-1β, ICAM-1, E-SEL, and IGFBP-1) on 2 days a week or more apart were associated with an increased risk of severe early cognitive impairment (MDI < 55 on the Bayley Scales of Infant Development II) [6] and microcephaly [7], but not when the elevated concentration occurred on a single day [6,7].

In this report we describe the relationships between elevated concentrations of inflammation-associated proteins and CP form -- hemiparetic, diparetic, or quadriparetic.

Methods

The ELGAN Study

The ELGAN study was designed to identify characteristics and exposures that increase the risk of structural and functional neurologic disorders in ELGANs (the acronym for Extremely Low Gestational Age Newborns). During the years 2002-2004, women delivering before 28 weeks gestation at one of 14 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.

Mothers were approached for consent either upon antenatal admission or shortly after delivery, depending on clinical circumstance and institutional preference. 1249 mothers of 1506 infants consented. 939 children from whom blood specimens were collected during the first two postnatal weeks for biomarker analyses were examined at approximately 24 months post-term equivalent.

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 intra-uterine 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%), last menstrual period (7%), and gestational age recorded in the log of the neonatal intensive care unit (1%).

Each infant was assigned a birth weight Z-score, which represents the number of standard deviations the infant's birth weight was above or below the median weight of infants at the same gestational age in a standard data set.

Cerebral palsy

Eleven percent of children evaluated at 24 months were diagnosed with a diagnosis of quadriparetic, diparetic, or hemiparetic cerebral palsy[8]. Risk factors and correlated clinical outcomes differ for these three forms of CP [8-10].

Blood Protein measurements

Drops of whole blood were collected on (Schleicher & Schuell 903) filter paper 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). Twenty-five proteins were measured in the Laboratory of Genital Tract Biology, Brigham and Women's Hospital, using the Meso Scale Discovery multiplex platform and Sector Imager 2400 (Meso Scale Discovery, Gaithersburg, MD), which has been validated against ELISA. Details about the procedure for processing the blood spots and for measuring protein concentrations and absolute value ranges for proteins are explained elsewhere[6, 11].

Data analysis

Since 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 three postnatal days of blood collection. Because the concentrations of most proteins did not follow a normal distribution, we dichotomized the concentration distribution of each protein into the highest quartile and the lower three quartiles for each of the 9 gestational age-postnatal day groups.

We evaluated the following three null hypotheses. First, infants with an elevated concentration (i.e., in the top quartile for gestational age) of an inflammation-related protein on a specific day (days 1, 7, or 14) were not at higher risk of any form of cerebral palsy than infants whose concentration of that protein was in the lower three quartiles on that day. Second, infants with elevated concentrations of a single inflammation-related protein on 2 or more days (which we refer to here as a "recurrent-persistent protein elevation") were not at higher risk for cerebral palsy than infants without a recurrent-persistent protein elevation. Third, the risk of cerebral palsy did not vary with the number of proteins whose concentrations were in the highest quartile on 2 or more days. To test this hypothesis we compared children who had 1, 2-3, or 4+ proteins with concentrations in the top quartile on 2 days to children who did not have any protein with concentrations in the top quartile on 2 days. We limited our analyses to the nine proteins (IL-6, TNF-α, TNF-R1, IL-8, ICAM-1, E-SEL, CRP, SAA and IGFBP-1) whose recurrently elevated concentrations were associated with increased risk of severe early cognitive impairment[7], microcephaly[7], and CP.

The strength of association between each CP diagnosis and a protein concentration in the highest quartile is presented as a risk ratio and its 99% confidence interval. We selected this

confidence interval, rather than the conventional 95% interval, to account for multiple comparisons (25 proteins measured at 3 times), while not appreciably increasing the risk of a type 2 (false negative) error. Because our outcomes are mutually exclusive and each is appropriately compared to the same referent group (i.e., children who did not have a CP diagnosis), we created multinomial (polytomous or polychotomous) logistic regression models (Stata 13.0, StataCorp, College Station, TX) .

Results

Sample description

In this cohort, we classified 105 children as having cerebral palsy. Fifty two percent were classified as having quadriparesis (N=55), 30% as having diparesis (N=32), and 17% as having hemiparesis (N=18). All forms of CP were associated with low PDI and MDI scores on the Bayley Scales of Infant Development II and with microcephaly. Children with diparesis were less likely than children with quadriparesis or hemiparesis to have other clinical indicators of brain damage or dysfunction.

Single-day elevated protein concentrations

None of the 25 proteins we evaluated had a day-1 elevated concentration associated with any CP diagnosis On day 7, IL-6R was the only protein whose elevated concentration was associated with reduced risk of quadriparesis, while MCP-1 was the only protein whose elevated concentration was associated with increased risk of quadriparesis No protein elevation on day-7 was associated with increased or reduced risk of diparesis or hemiparesis.

On day 14, IL-6R continued to be the only protein whose elevated concentration was associated with reduced risk of quadriparesis. ICAM-1 was the only protein whose elevated day-14 concentration was associated with increased risk of diparesis, while elevated concentrations of IL-6, E-SEL, and IGFBP-1 on day 14 were associated with increased risk of hemiparesis.

Multiple-day elevated individual protein concentrations (Table 1)

Elevated concentrations on two occasions of two proteins, IL-8 and MCP-1, were associated with increased risk of quadriparesis and repeated IL-6R elevation was associated with reduced risk. Elevated concentrations of 4 proteins, TNF-α, TNF-R1, IL-8, and ICAM-1, were associated with increased risk of diparesis, while a different set of 4 protein elevations, IL-6 IL-8, E-SEL, and IGFBP-1, was associated with increased risk of hemiparesis. These elevated concentrations were much more likely to be seen on days 7 and 14 than on the first postnatal day (data not shown).

Multiple-day elevations of multiple proteins (Table 2)

The risks of diparesis and of hemiparesis were significantly increased when at least four of the nine proteins associated with severe early cognitive impairment and microcephaly were in the highest quartile.

Discussion

Elevated blood concentrations of inflammation-related proteins during the first two postnatal weeks were associated, to varying degrees, with the diagnosis of each form of cerebral palsy. No single-day elevation of any inflammation-related protein observed within 3 days of birth was associated with a diagnosis of quadriparesis, diparesis, or hemiparesis in our study.

Subacute/chronic inflammation

Elevated protein concentrations on more than one day were associated with increased risk of CP, but transient elevations were not. These findings suggest that systemic inflammation persisting or recurring over at least two early postnatal weeks is more important than transient elevations of inflammatory proteins. This pattern of stronger associations with persisting or recurring inflammation, rather than with transient inflammation, has been found also for severe early cognitive impairment [6] and microcephaly [7]. Although the most likely inference is that recurrent or persistent inflammation is needed to injure the brain, it is also possible that systemic inflammation is a consequence of ongoing brain damage [12Malaeb, 2009 #6612;].

We do not know how much of the persistence of elevated concentrations reflects a long half-life, and how much reflects continued synthesis in response to new or persistent inflammatory stimuli, such as prolonged ventilation [13] or bacteremia [14]. Although the half-life of circulating inflammation-associated cytokines is short in adult rabbits and rodents [15], the half-life in preterm human newborns remains unknown [16]. In the ELGAN cohort, persistently/recurrently elevated protein concentrations tended to occur on days 7 and 14. This observation is consistent with the notion that postnatal events contribute to risk of CP.

Is inflammation a cause or consequence of CP-related brain damage?

The presence of high concentrations of inflammation-associated proteins in neonates who later develop adverse neurological outcomes might reflect primarily the process of clearing away damaged brain cells [17]. Nonetheless, such inflammation-resolution processes can promote feedback-loops between brain damage and the immune system [18], further enhancing the degree of neurological damage [19], which, in turn, can heighten inflammation and perpetuate brain-damaging processes. In this model, perinatal brain damage is an ongoing process and what we measured might actually contribute to damage and the risk of such adverse outcomes as CP.[12]. Consequently, we take the view that the elevated concentrations of inflammation-related proteins might convey information about both the processes leading to brain damage and the risk of later dysfunctions. Whether or not systemic inflammation actually contributes to brain damage in human newborns remains to be documented although evidence suggests it does in adult humans and rodents [20].

White matter abnormalities found on perinatal cranial ultrasound studies are highly associated with both elevated pro-inflammatory peptides[21] and CP[22]. We do not know when the white matter abnormalities developed in the ELGAN cohort, so we cannot know the

relative contribution, if any, of white matter abnormalities and systemic inflammation in the pathogenesis of CP in this cohort.

Why are selected multiple proteins associated with CP risk?

The proteins we have identified also have been associated previously with increased risks of neurological impairments, including stroke, CP, and cognitive impairments [4,23-29]. When we evaluated the role of these proteins in predicting CP, we found that risk was related to the number of protein elevations, which became significant when at least four of the circulating proteins were elevated. This observation suggests that the breadth of the inflammatory response, represented by the number of elevated inflammation-related protein concentrations among those we evaluated, is associated with increased risk of brain damage and consequent adverse neurological outcomes, including CP.

Inflammation-related proteins tend to be highly interrelated, with an inflammatory stimulus increasing the expression of hundreds of proteins [30]. Indeed, in our sample, the concentrations of most of the 25 proteins we assessed tended to vary with one another [31].

Although a portion of the organ damage that accompanies sepsis is attributed to a “cytokine storm” [32], for the most part our subjects did not have life-threatening sepsis, nor did most children have damage to multiple organs [33]. Consequently, explosive, potentially fatal inflammation is probably not a suitable model for what we see. Rather, a slower sub-acute or chronic set of inflammatory processes may be more consistent with our findings. The small number of proteins whose elevated concentrations were most clearly associated with a CP form might reflect the concept that a small subset of inflammation-related proteins is especially relevant to brain damage in extremely preterm newborns [11].

It is also possible that a larger number of proteins would have been found elevated in the ELGAN children if blood sampling was extended beyond two weeks after birth. The two cytokines, IL-6 and TNF-α, are often the primary initiators of inflammatory cascades via NF-κB, in response to environmental cues. Also, IL-8, ICAM-1 and E-selectin are among the earliest secondary responders to inflammatory stimulation e.g. via IL-6 and TNF-α. Elevations of soluble IL-6 and TNF-α receptors show contrasting effects. While solubleTNF-R1 may extend the half-life of TNF-α thus contributing to inflammation, the role of soluble IL-6R in inflammation is more controversial and complex [34.]

Postnatal, not prenatal inflammation

In our sample, day-1 elevated concentrations were associated with spontaneous indications for delivery [35], histologic inflammation of the placenta [36], and recovery of organisms from placenta parenchyma [37], suggesting that day-1 elevations reflect prenatal influences. Our finding that early protein elevations appear to be less predictive of CP risk in ELGANs than protein elevations at one and two postnatal weeks suggests that postnatal events contribute appreciably to risk for long-term neurological impairments, while antenatal phenomena contribute considerably less.

Heterogeneity of CP

The stronger association of elevated protein concentrations with hemiparetic and diparetic forms of CP than with quadriparetic CP suggests that the pathophysiology differs among CP forms. This possibility prompted us to distinguish different forms of CP in our analyses [8].

Exposure to higher concentrations of inflammation-associated proteins in ventricular or transependymal CSF might account for the predilection for damage to the periventricular white matter fibers subserving lower extremities seen in diparetics [38]. Other mechanisms, involving genetic or epigenetic-associated risks [39,40,] might have a stronger role in contributing to the risk in certain forms of CP, such as quadriparesis. The relatively small number of children with hemiparesis limits the inferences that might be drawn about contributory risks.

Our findings imply that the risk of brain damage in extremely preterm infants might be influenced by both the occurrence of inflammation-related illnesses and the severity of the systemic inflammation that accompanies such illnesses [41]. For this reason, and because the effects of inflammation on synaptic integrity [42,43], plasticity [43-46], oligodendroglia cell survival [47], and dysregulated apoptosis[48] might extend over months to years, the therapeutic window for intervention might also be longer than the immediate postnatal period [49].

Strengths of this study

Our study has several strengths. First, we included a large number of infants. Second, to minimize confounding due to factors related to fetal growth restriction, we recruited infants based on gestational age and not birth weight. Third, we collected our data prospectively. Fourth, attrition in the first two years was modest, with neurological outcomes established in almost 90% of surviving infants. Fifth, examiners at two years were not aware of the medical histories of the children they examined, thereby minimizing “diagnostic suspicion bias”. Sixth, we minimized observer variability in assessments of motor function. Seventh, we used a structured objective algorithm to classify CP subtypes [8]. Eighth**,** our protein data are of high quality, with high content validity [11,35,37].

Limitations of this study

As with all observational studies, we are unable to distinguish between causation and association as explanations for what we found. Second, the small numbers of children with each of the three CP forms, limit the power of our analyses. Third, although we sampled a wide range of inflammation-associated proteins, including specific proteins known to be associated with neurological damage, we did not evaluate all known inflammation-associated proteins. The proteins measured were selected on the basis of their likely involvement in the fetal/neonatal inflammatory response and the accuracy with which they could be measured within linearity ranges using the Meso Scale Discovery multiplex platform.

Conclusion

Extremely preterm newborns who have repeatedly elevated concentrations of a number of inflammation-related proteins in their blood during the first two postnatal weeks are at increased risk of a cerebral palsy diagnosis two years later. This conclusion carries implications for clinical practice, including extending clinical interventions beyond the conventional postnatal period.

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Abbreviations

ELGAN	extremely low gestational age newborn
BSID	Bayley Scales of Infant Development
CP	cerebral palsy
IL-1β	interleukin-1β
IL-6	interleukin-6
IL-6R	interleukin-6 receptor
TNF-α	tumor necrosis factor-α
TNF-R1	tumor necrosis factor-α-receptor-1
TNF-R2	tumor necrosis factor-α-receptor-2
IL-8	interleukin-8 (CXCL8)
MCP-1	monocyte chemotactic protein-1 (CCL2)
MCP-4	monocyte chemoattractant protein-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	intercellular adhesion molecule-1(CD54)
ICAM-3	intercellular adhesion molecule-3 (CD50)
VCAM-1	vascular cell adhesion molecule-1 (CD106)

E-SEL	E-selectin (CD62E)
MMP-1	matrix metalloproteinase-1
MMP-9	matrix metalloproteinase-9
CRP	C-reactive protein
SAA	serum amyloid A
MPO	myeloperoxidase
VEGF	vascular endothelial growth factor
VEGF-R1	vascular endothelial growth factor-receptor-1
VEGF-R2	vascular endothelial growth factor-receptor-2
IGFBP-1	insulin-lIke growth factor binding protein-1

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Table 1

Risk of Cerebral Palsy Forms Associated with High Protein Concentrations On More Than One Day *

Protein [a]	Quadriparesis (55)	Diparesis (32)	Hemiparesis (18)
CRP	1.2 (0.6, 2.5)	1.7 (0.7, 4.1)	1.9 (0.5, 6.7)
SAA	1.0 (0.4, 2.3)	2.2 (0.9, 5.4)	2.9 (0.8, 10)
MPO	1.4 (0.7, 2.7)	0.8 (0.3, 2.1)	1.0 (0.3, 3.5)
IL-1β	1.5 (0.7, 3.1)	2.0 (0.8, 5.4)	1.5 (0.6, 4.5)
IL-6	1.4 (0.6, 3.2)	1.3 (0.5, 3.4)	**7.1 (1.8, 27)**
IL-6R	***0.2 (0.1, 0.6)***	0.5 (0.2, 1.5)	0.3 (0.1, 1.3)
TNF-α	1.6 (0.8, 3.1)	**3.5 (1.4, 8.6)**	2.5 (0.8, 7.5)
TNF-R1	1.4 (0.7, 3.0)	**2.4 (1.04, 5.5)**	1.8 (0.6, 5.5)
TNF-R2	0.8 (0.3, 1.7)	2.2 (0.9, 5.3)	1.5 (0.4, 5.2)
IL-8 (CXCL8)	**2.1 (1.03, 4.2)**	**3.1 (1.2, 8.1)**	**3.1 (1.0, 9.9)**
MCP-1 (CCL2)	**2.5 (1.2, 5.1)**	0.8 (0.3, 2.1)	0.8 (0.2, 3.1)
MCP-4 (CCL13)	0.9 (0.4, 2.1)	1.0 (0.4, 2.4)	1.3 (0.4, 4.0)
MIP-1β (CCL4)	1.0 (0.4, 2.1)	2.1 (0.8, 5.3)	1.4 (0.5, 4.3)
RANTES (CCL5)	0.4 (0.1, 0.98)	1.4 (0.6, 3.4)	0.2 (0, 1.3)
I-TAC (CXCL11)	0.7 (0.3, 1.5)	0.9 (0.3, 2.3)	2.2 (0.7, 6.6)
ICAM-1 (CD54)	2.0 (0.98, 3.9)	**3.0 (1.2, 7.4)**	1.6 (0.5, 5.7)
ICAM-3 (CD50)	1.2 (0.6, 2.4)	0.7 (0.3, 1.8)	1.7 (0.6, 5.0)
VCAM-1 (CD106)	0.8 (0.4, 1.6)	1.1 (0.5, 2.6)	0.7 (0.2, 2.7)
E-SEL (CD62E)	1.0 (0.5, 2.0)	2.0 (0.8, 4.7)	**3.2 (1.1, 9.5)**
MMP-1	0.4 (0.1, 1.01)	1.2 (0.5, 2.9)	0.9 (0.3, 2.8)
MMP-9	0.6 (0.2, 1.4)	1.4 (0.5, 3.6)	1.7 (0.5, 6.3)
VEGF	0.8 (0.3, 1.3)	1.2 (0.5, 3.1)	1.3 (0.4, 3.6)
VEGF-R1	2.0 (0.9, 4.3)	1.1 (0.5, 2.8)	1.9 (0.6, 5.4)
VEGF-R2	0.6 (0.3, 1.5)	1.9 (0.8, 4.2)	0.7 (0.2, 2.5)
IGFBP-1	1.1 (0.5, 2.5)	1.5 (0.5, 4.1)	**3.6 (1.1, 12)**

* Risk ratios (and 99% confidence interval) of the form of CP associated with a protein concentration in the top quartile (for GA and day specimen was obtained) of the protein listed on the left **on two separate days** at least a week apart relative to that of children who did not. The sample for this table consists of all children with blood measurements on ≥ 2 days. These were multinomial analyses with children who did not have CP as the referent group and are adjusted for gestational age. **Bold** items are significantly elevated at p <.01 and ***italicized bold*** items are significantly low at p <.01.

[a] The legend for proteins listed in the table are found at the beginning of this publication.

Table 2

Risk of Cerebral Palsy Forms Associated with Multiple Elevated Proteins *

Cerebral Palsy forms	Number of proteins elevated on 2+ days			
	4+	2-3	1	0
Quadriparesis	1.8 (0.8, 4.0)	1.1 (0.5, 2.7)	2.0 (0.95, 4.0)	1.0
Diparesis	**3.0 (1.3, 7.1)**	1.3 (0.5, 3.6)	0.8 (0.3, 2.6)	1.0
Hemiparesis	**4.2 (1.3, 14)**	1.0 (0.2, 5.0)	1.3 (0.3, 5.6)	1.0

* Risk ratios (**95**% confidence intervals) for each form of cerebral palsy associated with elevated concentrations of the number of proteins identified at the top of each column on two separate days a week apart. The nine proteins are IL-6, TNF-α, TNF-R1, IL-8, ICAM-1, E-SEL, CRP, SAA and IGFBP-1. The referent category (identified with an odds ratio of 1.0) consists of all children who did not have any protein with a concentration in the top quartile on any two days. **Bold** items are significantly elevated at $p < .05$.