Plasma and Cerebrospinal Fluid Candidate Biomarkers of Neonatal Encephalopathy Severity and Neurodevelopmental Outcomes

Barbara Dietrick, MSc[1], Eleanor Molloy, MB, PhD[2], An N. Massaro, MD[3], Tammy Strickland, MSc[2], Jie Zhu, BS[1,4], Marie Slevin, MA[5], Veronica Donoghue, MB[5], Deirdre Sweetman, MB[5], Lynne Kelly, MB[2], Mary O'Dea, MB[2], Meaghan McGowan, BA[3], Gilbert Vezina, MD[3], Penny Glass, PhD[3], Dhananjay Vaidya, MBBS, MPH, PhD[1], Sandra Brooks, MD[6], Frances Northington, MD[1,4], and Allen D. Everett, MD[1,4]

Objectives To identify candidate biomarkers in both plasma and cerebrospinal fluid (CSF) that are associated with neonatal encephalopathy severity measured by encephalopathy grade, seizures, brain injury by magnetic resonance imaging (MRI), and neurodevelopmental outcomes at 15-30 months.

Study design A retrospective cohort study of plasma (N = 155, day of life 0-1) and CSF (n = 30, day of life 0-7) from neonates with neonatal encephalopathy and healthy neonates born at term (N = 30, ≥36 weeks of gestation) was conducted. We measured central nervous system necrosis (glial fibrillary acidic protein [GFAP], neurogranin [NRGN], tau), inflammatory (interleukin [IL]-6, IL-8, IL-10), and trophic (brain-derived neurotrophic factor [BDNF], vascular endothelial growth factor) proteins. Clinical outcomes were Sarnat scores of encephalopathy, seizures, MRI scores, and Bayley Scales of Infant and Toddler Development III at 15-30 months.

Results Plasma NRGN, tau, IL-6, IL-8, and IL-10 were greater, whereas BDNF and vascular endothelial growth factor were lower in patients with neonatal encephalopathy vs controls. In plasma, tau, GFAP, and NRGN were directly and BDNF inversely associated with encephalopathy grade. IL-6 was inversely related to seizures. Tau was directly related to MRI abnormalities. Tau was inversely associated with Bayley Scales of Infant and Toddler Development III cognitive and motor outcomes. In CSF, NRGN was inversely associated with cognitive, motor, and language measures. GFAP, IL-6, and IL-10 were inversely related to cognitive and motor outcomes. IL-8 was inversely related to motor outcomes. CSF candidate biomarkers showed no significant relationships with encephalopathy grade, seizures, or MRI abnormalities.

Conclusions Plasma candidate biomarkers predicted encephalopathy severity, seizures, MRI abnormalities, and neurodevelopmental outcomes at 15-30 months. *(J Pediatr 2020;226:71-9)*.

Neonatal encephalopathy is a syndrome defined by clinical features of neurologic dysfunction during the first few days of life, including difficulty initiating or maintaining respiration, altered consciousness, and seizures.[1,2] Neonatal encephalopathy occurs in as many as 3 per 1000 live births.[3] Common causes include hypoxic–ischemic insult leading to hypoxic–ischemic encephalopathy, perinatal infections, maternal conditions including placental abnormalities, and neonatal conditions including metabolic disorders, coagulopathies, and neonatal stroke.[1,4] The only validated treatment for this syndrome remains therapeutic hypothermia with intensive supportive care.[5] Despite the successful implementation of therapeutic hypothermia, approximately 29% of neonates with neonatal encephalopathy still have unfavorable outcomes of neurologic disability and death.[6] The modest efficacy of therapeutic hypothermia may depend on accurate assessment of injury severity for targeted intervention.[7,8] Presently, current clinical methods are insufficient to identify risk, stratify severity, and

Bayley-III	Bayley Scales of Infant and Toddler Development III	GFAP	Glial fibrillary acidic protein
BDNF	Brain-derived neurotrophic factor	IL-6	Interleukin-6
CNS	Central nervous system	IL-8	Interleukin-8
CSF	Cerebrospinal fluid	IL-10	Interleukin-10
DOL	Day of life	MRI	Magnetic resonance imaging
ELISA	Enzyme-linked immunosorbent assay	MSD	Meso scale discovery
		NRGN	Neurogranin
		VEGF	Vascular endothelial growth factor

From the [1]Johns Hopkins University School of Medicine, Baltimore, MD; [2]Paediatrics, Trinity College, the University of Dublin & Coombe Women and Infants University Hospital, Dublin, Ireland; [3]Children's National Health Systems, Washington, DC; [4]Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, MD; [5]National Maternity Hospital, Dublin, Ireland; and [6]Johns Hopkins All Children's Hospital, St Petersburg, FL

Supported by National Institutes of Health *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (R01HD086058 [to A.E. and F.N.]); Health Research Board, Ireland; Trinity College Dublin; National Children's Research Centre; Clinical and Translational Science Institute at Children's National (UL1TR000075, 1KL2RR031987-01 [to A.M.]); and the Intellectual and Developmental Disabilities Research Consortium (NIH P30HD040677 [to A.M.]). Under a license agreement between ImmunArray Ltd and the Johns Hopkins University, the University and A.E. are entitled to royalties on an invention described in this study and discussed in this publication. This arrangement has been reviewed and approved by the Johns Hopkins University in accordance with its conflict of interest policies. The other authors declare no conflicts of interest.

Portions of this study were presented at the Pediatric Academic Societies annual meeting, April 24-May 1, 2019, Baltimore, Maryland.

https://doi.org/10.1016/j.jpeds.2020.06.078

monitor therapeutic efficacy, which warrants further research into multidimensional approaches to assess and monitor evolving brain injury in neonates with neonatal encephalopathy.[9,10]

As a potential component of a multidimensional diagnostic/prognostic approach, biological fluid markers can provide objective, serial, and noninvasive information to identify pathologic processes reflected by distinct peripheral concentrations. Biomarker assays have been studied extensively in adults to predict neurologic pathologies, including traumatic brain injury and neurodegenerative diseases.[11-13] However, there is limited research and clinical application of pediatric biomarkers due to the need for large sample validation studies, especially in neonatal brain injury.[14]

Several candidate biomarkers, including central nervous system (CNS) necrosis, inflammatory, and trophic proteins, were identified in adult studies on brain injury and some have been investigated in neonatal populations. Glial fibrillary acidic protein (GFAP), a CNS-specific astrocyte cytoskeletal intermediate filament protein, was associated with abnormal magnetic resonance imaging (MRI) and neurodevelopmental outcomes.[15-18] Neurogranin (NRGN), a brain-specific protein kinase C substrate, has not yet been studied in neonatal populations but has associations with adult traumatic brain injury and neurodegeneration.[11,19-22] Tau, another CNS necrosis factor, is associated with asphyxia and neonatal encephalopathy.[23-25] Inflammation plays an important role in brain injury, and increased cytokines, especially interleukins 6, 8, and 10 (IL-6, IL-8, IL-10), have been associated with infants with brain injury.[17,24,26-29] Brain-derived neurotrophic factor (BDNF), a trophic protein, was associated with severity of injury and worse neurodevelopmental outcomes.[24,30,31] Another trophic factor, vascular endothelial growth factor (VEGF), was also associated with worse neonatal encephalopathy severity, abnormal imaging, and mortality.[17,32,33] Research in brain injury biomarkers suggests that a combination of biomarkers is the most effective for providing evidence of injury.[9,34,35] However, review of the current literature reports a need for validation studies before these biomarkers can be introduced for routine clinical care.[9,10]

We investigated a candidate multibiomarker panel to identify molecules in both plasma and cerebrospinal fluid (CSF) that are associated with neonatal encephalopathy severity measured by encephalopathy grade, seizures, brain injury by MRI, and neurodevelopmental outcomes at 15-30 months.

Methods

In this multicenter retrospective cohort study of neonatal encephalopathy, we identified and analyzed the concentrations of CNS necrosis markers (GFAP, NRGN, tau), inflammation markers (IL-6, IL-8, IL-10), and trophic-factor markers (BDNF, VEGF) from a cohort of neonates with neonatal encephalopathy and a cohort of healthy neonatal controls. The study received institutional review board approval at all hospitals, and signed informed consent was obtained from the parent of each participant. Johns Hopkins University institutional review board approved the use of all cohorts in this study.

Control Patient Cohort

Healthy neonates born at term (≥36 weeks of gestation) had plasma samples collected from National Maternity Hospital Dublin and Coombe Women and Infants University Hospital (Trinity College) from 2016 to 2018 from day of life (DOL) 0-7 with a median collection of DOL 2. The samples were stored in the Trinity Translational Medicine Institute Biobank in Dublin, Ireland. Deidentified plasma samples (n = 30) from healthy neonates born at term were analyzed in collaboration with the Neonatal Inflammation and Multiorgan Dysfunction and Brain Injury Research group (NIMBUS) at Trinity College Dublin, Ireland (JHU MTA A33285).

Patients with Neonatal Encephalopathy Cohort

Neonatal encephalopathy was defined as requiring resuscitation at birth and having an abnormal neurologic examination. Inclusion criteria were as follows: all infants with neonatal encephalopathy Sarnat score 2 or 3[36] requiring therapeutic hypothermia, neonatal encephalopathy in the first 48 hours of life without therapeutic hypothermia, or postnatally diagnosed with brain injury on cranial ultrasound.[33,37] Exclusion criteria consisted of maternal substance abuse and major congenital abnormalities. The neonatal encephalopathy cohort was drawn from National Maternity Hospital Dublin and Children's National Health System, Washington, DC. The National Maternity Hospital (Trinity College) neonatal encephalopathy neonates had plasma and CSF samples collected as previously described. At Children's National, neonates with neonatal encephalopathy had plasma samples collected from 2012 to 2016 as part of a prospective study evaluating candidate biomarkers of brain injury in neonatal encephalopathy. From both studies, a single deidentified plasma sample at enrollment from DOL 0-1 and clinical data (n = 155) were analyzed. From the Trinity College neonatal encephalopathy cohort only, deidentified CSF samples from DOL 0-7, with a median of DOL 3, and clinical data (n = 30) were analyzed.

Clinical outcomes of neurologic injury severity were evaluated for significant relationships with candidate biomarker concentrations. To measure clinical severity, degree of encephalopathy was determined by the Sarnat classification and stratified as mild (Sarnat score 0-1) or moderate-to-severe (Sarnat score 2-3).[36] Clinical evidence of brain injury was defined as the presence of seizures or severity of injury by MRI on DOL 5-15 according to the Barkovich scale, evaluating the basal ganglia area, watershed area, and the combined basal ganglia/watershed area.[38,39] The Barkovich scale was categorized into two groups: score of 0 and scores 1-5.

Neurodevelopmental outcomes were evaluated using cognitive, motor, and language scores of the Bayley Scales

of Infant and Toddler Development (Bayley-III) at 15-30 months.[40] We evaluated the Bayley-III as both a continuous and binary variable. For our binary analysis, we categorized the scores into two groups normal, scores ≥85 and abnormal, scores <85 or death.[41] The patients who died before neurodevelopmental follow-up were assigned a score of 39 for the continuous Bayley-III analysis.[42]

Laboratory Methods

All primary plasma samples were stored up to 24 hours at 4°C until aliquoted and stored at −80°C. All sample aliquots were exposed to 1-2 freeze/thaws before assaying. All assays were performed from 2018-2019 in the same laboratory (Everett Laboratory) at the Johns Hopkins University School of Medicine in Baltimore, Maryland.

A custom multiplex enzyme-linked immunosorbent assay (ELISA) was developed to measure BDNF, IL-6, IL-8, IL-10, and VEGF simultaneously using robotically spotted capture antibodies on the 96-well plate format (Meso Scale Discovery [MSD], Rockville, Maryland). Capture antibody-spotted plates were washed with 1 × phosphate-buffered saline supplemented with 0.05% TWEEN. Calibrators for BDNF, VEGF, and IL-6, IL-8, and IL-10 (MSD) were produced using commercially provided diluent (product number R50AG-2, MSD). The detection antibody cocktail was prepared in commercial diluent (product number R51BA-5, MSD). Plasma and CSF samples were diluted 5-fold. The lower limits of quantification for the BDNF, IL-6, IL-8, IL-10, and VEGF assays were 48.47 pg/mL, 0.47 pg/mL, 0.56 pg/mL, 1.26 pg/mL, and 1.59 pg/mL, respectively, with interassay coefficients of variation of 9.7%, 4.7%, 1.8%, 1.7%, and 2.7%, respectively.

A custom duplex ELISA was developed to measure GFAP and NRGN simultaneously using robotically spotted capture antibodies on the 96-well plate format (MSD). The development of the capture antibodies, detection antibodies, and calibrators of NRGN and GFAP have been previously described.[21,43] Plasma and CSF samples were diluted 2-fold. The lower limits of quantification for the GFAP and NRGN assays were 0.014 and 0.016 ng/mL, respectively, with interassay coefficients of variation of 2.6% and 2.8%, respectively.

Tau was measured using a commercial ELISA (product number N451LAA-1; MSD). Plasma and CSF samples were diluted 4-fold and assayed according to manufacturer instructions. The lower limits of quantification for Tau was 82.03 pg/mL, with an interassay coefficient of variation of 5.1%.

Statistical Analyses

Demographic and functional data are presented as median and IQR or percentages, as appropriate. As demographic, candidate biomarker, and clinical outcome data were not normally distributed for the neonatal encephalopathy cohort, the Mann–Whitney *U* test was used for categorical variables, and Spearman correlation was used for continuous variables. For the binary demographic data, the Fisher exact test was used for comparison. Adjusted analysis was performed by logistic and linear regression using log-transformed candidate biomarker concentrations and adjusted for gestational age and sex. For all statistical analyses, a P value of ≤.05 was considered significant. Statistical analysis was performed using GraphPad Prism (Version 8.0.0 [131]; 2018; GraphPad Software, San Diego, California) and Stata (Version 15, StataCorp LLC, College Station, Texas).

Results

Subject Demographics

There were 30 healthy control neonates born at term and 155 neonates with neonatal encephalopathy available for analysis. From the neonatal encephalopathy cohort, 155 plasma samples and 30 CSF samples were available for analysis (**Table I**).

Clinical outcomes of the neonatal encephalopathy cohort are summarized in **Table II**. The neonatal encephalopathy cohort were predominately (92%) moderate-to-severe neonatal encephalopathy (Sarnat score of 2-3). In total, 54% of the neonatal encephalopathy cohort had seizures. For MRI-derived brain injury, the majority had low Barkovich scores of 0 for basal ganglia (76%), watershed (74%), and combined basal ganglia/watershed area (67%). At 15-30 months, the majority of the neonatal encephalopathy cohort had normal (Bayley-III score ≥85) Bayley-III cognitive (75%), motor (72%), and language (70%) composite scores. Clinical outcomes were not available for the control cohort.

Plasma Candidate Biomarkers in Patients with Neonatal Encephalopathy and Healthy Neonates Born at Term

Plasma candidate biomarker concentrations significantly differed in patients with neonatal encephalopathy compared with controls (**Table III**; available at www.jpeds.com). CNS necrosis markers NRGN ($P = .03$) and tau ($P < .001$) and inflammation markers IL-6 ($P < .001$), IL-8 ($P < .001$), and IL-10 ($P < .001$) were greater, whereas trophic-factors BDNF ($P < .001$) and VEGF ($P < .001$) were lower in patients with neonatal encephalopathy compared with controls.

Candidate Biomarkers and Clinical Outcomes of Brain Injury

In univariate analysis of plasma candidate biomarkers, greater tau ($P < .001$), GFAP ($P = .05$), and NRGN ($P = .03$) and lower BDNF ($P = .002$) and VEGF ($P = .05$) were associated with moderate-to-severe encephalopathy (**Table IV**; available at www.jpeds.com). IL-6, IL-8, and IL-10 were all greater but not significantly. When adjusted for gestational age and sex, tau ($P = .002$), GFAP ($P = .03$), and NRGN ($P = .05$) were directly related and BDNF ($P = .04$) was inversely related to severity of encephalopathy (**Table V**). There were no significant associations of CSF candidate biomarkers with moderate-to-severe encephalopathy in univariate or adjusted analysis (**Table VI**; available at www.jpeds.com).

Table I. Demographic data of healthy neonates born at term and neonates with neonatal encephalopathy

Demographic measures	Control cohort (n = 30)	Total neonatal encephalopathy cohort (n = 155)	Trinity college cohort (n = 57)	Children's National cohort (n = 98)	*P* value
Median (IQR)					
Gestational age, wk	39.1 (37.9, 40.0)	39.6 (38, 40.7)	40.7 (40.0, 41.6)	39.0 (38.0, 40.0)	.22*
Birth weight, kg	3.3 (3.1, 3.6)	3.3 (2.9, 3.8)	3.6 (3.2, 4.0)	3.1 (2.8, 3.6)	.73*
First blood gas pH	**7.3 (7.2, 7.4)**	**7.0 (6.9, 7.1)**	7.0 (6.9, 7.2)	7.0 (6.8, 7.1)	**<.001***
5-min Apgar score	**10 (10, 10)**	**4 (2, 6)**	5 (3, 7)	3.5 (2, 5)	**<.001***
Sex, n (%)					
Male	12 (40)	88 (57)	38 (68)	50 (51)	.11†
Female	18 (60)	66 (43)	18 (32)	48 (49)	
Mode of delivery, n (%)					
Cesarean	**9 (30)**	**66 (52)**	20 (36)	46 (64)	**.04†**
Vaginal	**21 (70)**	**61 (48)**	35 (64)	26 (36)	
Therapeutic hypothermia, n (%)					
Yes	**0 (0)**	**135 (88)**	37 (66)	98 (100)	**<.001†**
No	**30 (100)**	**19 (12)**	19 (34)	0 (0)	

*Mann–Whitney *U* test was used for comparison of the control cohort and the total neonatal encephalopathy cohort.
†Fisher exact test was used for comparison of control cohort and the total neonatal encephalopathy cohort.
Bold values indicate statistical significance.

In univariate analysis of plasma candidate biomarkers and seizure occurrence, lower IL-6 ($P = .02$) was associated with seizures (**Table VII**; available at www.jpeds.com). In adjusted analysis, IL-6 showed an inverse relationship ($P = .02$) with seizure occurrence (**Table V**). There were no significant associations of CSF candidate biomarkers with seizures in univariate or adjusted analysis (**Table VIII**; available at www.jpeds.com).

Candidate Biomarkers and Brain Injury by MRI

In adjusted analysis, only plasma tau was directly related to Barkovich basal ganglia ($P = .002$), watershed ($P = .03$), and basal ganglia/watershed ($P = .006$) scores (**Table V**). CSF candidate biomarkers did not have any significant relationships with MRI abnormalities (**Table IX**; available at www.jpeds.com).

Candidate Biomarkers and Neurodevelopmental Outcomes

In univariate analysis of plasma candidate biomarkers, tau and IL-6 negatively correlated with Bayley-III cognitive ($P = .02$, $P = .05$) and motor ($P = .02$, $P = .03$) composite scores (**Table X**; available at www.jpeds.com). IL-8 negatively correlated with cognitive ($P = .005$), motor ($P < .001$), and language ($P = .02$) composite scores. IL-10 negatively correlated with motor composite scores only ($P = .03$). VEGF positively correlated with motor composite scores ($P = .03$). In univariate analysis of CSF candidate biomarkers, NRGN and IL-6 negatively correlated with Bayley-III cognitive ($P = .04$, $P = .05$) and motor ($P = .03$, $P = .02$) scores, respectively (**Table XI**; available at www.jpeds.com). IL-8 negatively correlated with motor scores only ($P = .03$).

In adjusted analysis of plasma candidate biomarkers, only tau was associated with decreased cognitive ($P = .03$) and motor ($P = .03$) outcomes (**Table XII**). In adjusted analysis of CSF candidate biomarkers, NRGN was inversely related to all 3 outcomes of cognitive ($P = .001$), motor ($P < .001$), and language ($P = .007$) measures (**Table XII**). GFAP, IL-6, and IL-10 were inversely related to cognitive ($P = .02$, $P = .02$, $P = .02$) and motor ($P = .04$, $P = .009$, $P = .008$) outcomes, respectively. IL-8 was inversely related to motor outcomes only ($P = .03$).

In univariate analysis of plasma candidate biomarkers and binary neurodevelopmental outcomes (abnormal [Bayley-III <85 or death] or normal [≥85]), greater tau and IL-8 were associated with abnormal cognitive ($P = .03$, $P = .05$), motor ($P = .01$, $P = .009$), and language ($P = .04$, $P = .005$) outcomes, respectively (**Table XIII**; available at www.jpeds.com). In adjusted analysis, greater than 1 SD of increased tau between individuals presented a 1.76 times increased likelihood for abnormal cognitive outcomes ($P = .04$), 1.82 times increased likelihood for abnormal motor outcomes ($P = .03$), and 1.71 times increased likelihood for abnormal language outcomes ($P = .05$).

Discussion

In this multicenter retrospective cohort study of neonatal encephalopathy, we demonstrated a candidate multibiomarker panel of CNS necrosis, inflammatory, and trophic-factor proteins differentiated neonates with neonatal encephalopathy from healthy neonates born at term within the first 24 hours of life. Moreover, this candidate biomarker panel, with the best performance from tau, differentiated severity of neonatal encephalopathy measured by clinical encephalopathy, seizures, brain injury by MRI, and neurodevelopmental outcomes at 15-30 months. Although previous research focused on candidate biomarkers such as cytokines, tau, and GFAP, our study contributes novel investigation of less-studied candidate biomarkers, NRGN, VEGF, and BDNF, providing important performance comparisons.

We found that CNS necrosis markers (NRGN and tau) and inflammation markers (IL-6, IL-8, and IL-10) were greater,

Table II. Clinical data of neonates with neonatal encephalopathy

Clinical measures	Total neonatal encephalopathy cohort (n = 155)	Trinity college cohort (n = 57)	Children's National cohort (n = 98)
Degree of encephalopathy, n (%)			
Sarnat 0	3 (2)	3 (5)	0 (0)
Sarnat 1	9 (6)	9 (16)	0 (0)
Sarnat 2	116 (75)	36 (63)	80 (82)
Sarnat 3	27 (17)	9 (16)	18 (18)
Seizures, n (%)			
Yes	74 (54)	33 (67)	41 (47)
No	63 (46)	16 (33)	47 (53)
Brain injury by MRI: Barkovich score, n (%)			
Barkovich basal ganglia (n = 117)			
Barkovich 0	89 (76)	26 (84)	63 (73)
Barkovich 1	5 (4)	0 (0)	5 (6)
Barkovich 2	6 (5)	1 (3)	5 (6)
Barkovich 3	9 (8)	0 (0)	9 (10)
Barkovich 4	8 (7)	4 (13)	4 (5)
Barkovich watershed (n = 117)			
Barkovich 0	86 (74)	22 (71)	64 (74)
Barkovich 1	7 (6)	3 (10)	4 (5)
Barkovich 2	7 (6)	2 (6)	5 (6)
Barkovich 3	1 (1)	0 (0)	1 (1)
Barkovich 4	11 (9)	2 (6)	9 (10)
Barkovich 5	5 (4)	2 (6)	3 (4)
Barkovich basal ganglia/watershed (n = 117)			
Barkovich 0	78 (67)	21 (68)	57 (66)
Barkovich 1	7 (6)	1 (3)	6 (7)
Barkovich 2	12 (10)	5 (16)	7 (8)
Barkovich 3	16 (14)	3 (10)	13 (15)
Barkovich 4	4 (3)	1 (3)	3 (4)
Neurodevelopmental outcomes: Bayley-III, median (IQR)			
Cognitive score (n = 99)	99.5 (85, 105)	105 (95, 110)	95 (39, 100)
Motor score (n = 99)	97 (76, 107)	103 (97, 118)	91.5 (39, 100)
Language score (n = 96)	94 (74, 106)	100 (86, 112)	91 (39, 106)
Neurodevelopmental outcomes: Bayley-III, n (%)			
Cognitive score (n = 99)			
Normal (≥85)	74 (75)	34 (87)	40 (67)
Abnormal (<85)	5 (5)	1 (3)	4 (7)
Dead	20 (20)	4 (10)	16 (27)
Motor score (n = 99)			
Normal (≥85)	71 (72)	34 (87)	37 (62)
Abnormal (<85)	8 (8)	1 (3)	7 (12)
Dead	20 (20)	4 (10)	16 (27)
Language score (n = 96)			
Normal (≥85)	67 (70)	32 (82)	35 (61)
Abnormal (<85)	9 (9)	3 (8)	6 (11)
Dead	20 (21)	4 (10)	16 (28)

Bayley-III indicates Bayley Scales of Infant and Toddler Development III at 15-30 months.
Patients with missing data: Sarnat n = 0, seizures n = 18, Barkovich scores n = 38, Bayley-III Cognitive n = 56, Motor n = 56, Language n = 59.

whereas trophic factors (BDNF and VEGF) were lower in patients with neonatal encephalopathy compared with controls. Greater IL-6[27–29] and tau,[23] and lower VEGF[44] in neonates with neonatal encephalopathy compared with controls is consistent with previous smaller, single center studies. The findings of greater NRGN[21,22] and lower BDNF[45-47] in patients with brain injury compared with healthy controls has also been reported in adults with traumatic brain injury and delirium. Conversely, others showed greater BDNF in neonates with asphyxia compared with controls using cord blood samples.[30,31] Olin et al found that cord blood is not representative of neonatal blood, and therefore our use of plasma samples from the first 24 hours of life is a strength of our study.[48] In addition, timing variation of sample collection and therapeutic hypothermia also may have contributed to discrepancies, as cooling may affect candidate biomarker concentrations.[32,44] However, because the standard of care is to begin therapeutic hypothermia within 6 hours of birth, and the plasma samples in our study span DOL 0-1, therapeutic hypothermia was considered a mediator, not a confounder, and was not included in the adjusted analysis. The median plasma concentrations of GFAP and tau are greater in the control cohort than the infants with mild encephalopathy in the neonatal encephalopathy cohort. This difference was driven by 3 control outliers not excluded from analysis that each had elevated GFAP, tau, and NRGN. In addition, although the control cohort had no overt clinical brain injury at birth, they were neonates admitted to the neonatal intensive care unit and could have had subclinical brain injury to account for the elevated

Table V. Association of plasma candidate biomarker concentrations of neonates with neonatal encephalopathy and clinical encephalopathy, seizure occurrence, and brain injury by MRI*

	Clinical encephalopathy		Seizure occurrence		MRI Barkovich score					
					Basal ganglia		Watershed		Basal ganglia/Watershed	
Biomarkers	Coefficient (95% CI)	*P* value	Coefficient (95% CI)	*P* value	Coefficient (95% CI)	*P* value	Coefficient (95% CI)	*P* value	Coefficient (95% CI)	*P* value
GFAP	**0.38 (0.04 to 0.71)**	**.03**	−0.007 (−0.15 to 0.14)	.93	0.11 (−0.07 to 0.30)	.22	0.08 (−0.10 to 0.26)	.39	0.003 (−0.16 to 0.17)	.98
NRGN	**0.48 (−0.001 to 0.95)**	**.05**	−0.06 (−0.22 to 0.11)	.50	0.14 (−0.06 to 0.34)	.16	0.06 (−0.13 to 0.25)	.54	0.04 (−0.14 to 0.22)	.70
BDNF	**−0.61 (−1.18 to −0.03)**	**.04**	−0.09 (−0.29 to 0.11)	.37	−0.23 (−0.47 to 0.01)	.06	−0.20 (−0.45 to 0.04)	.10	−0.17 (−0.40 to 0.06)	.14
IL-6	0.04 (−0.24 to 0.31)	.80	**−0.19 (−0.36 to −0.03)**	**.02**	0.03 (−0.18 to 0.23)	.81	0.03 (−0.18 to 0.24)	.76	−0.004 (−0.19 to 0.18)	.97
IL-8	0.12 (−0.18 to 0.43)	.43	−0.002 (−0.19 to 0.19)	.98	0.09 (−0.18 to 0.35)	.51	0.27 (−0.04 to 0.58)	.09	0.11 (−0.13 to 0.36)	.37
IL-10	0.16 (−0.16 to 0.49)	.32	−0.008 (−0.18 to 0.16)	.93	−0.05 (−0.27 to 0.17)	.69	0.11 (−0.12 to 0.33)	.36	−0.06 (−0.26 to 0.14)	.56
VEGF	−0.23 (−0.53 to 0.07)	.14	−0.02 (−0.16 to 0.13)	.84	−0.06 (−0.23 to 0.11)	.50	−0.09 (−0.26 to 0.08)	.30	−0.04 (−0.20 to 0.12)	.65
Tau	**1.46 (0.53 to 2.38)**	**.002**	0.08 (−0.23 to 0.39)	.62	**0.74 (0.26 to 1.22)**	**.002**	**0.47 (0.05 to 0.88)**	**.03**	**0.60 (0.18 to 1.03)**	**.006**

*Adjusted for gestational age and sex, the natural log of candidate biomarker concentrations was used in adjusted analyses. Clinical encephalopathy: duplex (GFAP and NRGN): n = 141; multiplex (BDNF, IL-6, IL-8, IL-10, VEGF): n = 141; tau: n = 123. Seizure occurrence: duplex (GFAP and NRGN): n = 125; multiplex (BDNF, IL-6, IL-8, IL-10, VEGF): n = 125; tau: n = 109. MRI: duplex (GFAP and NRGN): n = 107; multiplex (BDNF, IL-6, IL-8, IL-10, VEGF): n = 107; tau: n = 93.
Bold values indicate statistical significance.

GFAP and tau. Finally, there are multiple factors as previously discussed, including the influence of therapeutic hypothermia and timing variation of sample collection that may contribute to this observation.

The identification of early candidate biomarkers to discriminate neonatal encephalopathy severity is becoming increasingly important with potential adjuvant therapies to therapeutic hypothermia, such as xenon, erythropoietin, and stem cells.[24,49-52] In our study, using adjusted analysis, plasma tau, GFAP, NRGN, and BDNF differentiated between mild and moderate-to-severe encephalopathy. The greater tau, GFAP, and NRGN concentrations in moderate-to-severe encephalopathy supported the hypothesis that these CNS necrosis markers are indicators of acute and possibly ongoing neuronal injury.[11,17,23,35] Meanwhile, BDNF was lower in moderate-to-severe encephalopathy. BDNF is a neuronal survival factor, suggesting an inadequate concentration to protect the brain in more severe injuries.[53-55] CSF candidate biomarkers did not differentiate encephalopathy severity in our study, although others reported significant results for CSF GFAP,[56,57] CSF IL-6,[29] and CSF VEGF.[32] This could possibly be due to availability of CSF samples at a single time point compared with 0-7 days of life.

Seizures are a common sequelae of neonatal encephalopathy, and infants with seizures are associated with worse neurodevelopmental outcomes or death.[7,8,36,58] Seizures were common in our cohort, and neonates with seizures had predominately moderate-to-severe neonatal encephalopathy (99% Sarnat score 2-3). We found lower plasma IL-6 concentrations could differentiate and were predictive of seizures in neonates with neonatal encephalopathy. Conversely, Numis et al showed greater IL-6 was associated with epilepsy in newborns with neonatal encephalopathy.[59] This discrepancy could reflect variation in the dynamic inflammatory process and possible dysregulation in severely injured neonates. Furthermore, our study did not control for infectious etiologies, due to limited sample size and availability of clinical data. Further evaluation of inflammatory cytokines considering infectious etiologies is warranted in larger cohorts. Candidate biomarkers in CSF were not able to differentiate seizure occurrence.

Evidence of structural injury, especially the basal ganglia, in neonatal encephalopathy is well described.[38] We found with adjusted analysis that plasma tau directly related to Barkovich score for basal ganglia, watershed, and combined basal ganglia/watershed area injury. Previous research supports our findings that plasma tau is associated with worse brain injury severity on MRI.[24] Tau is an axonal protein and particularly enriched in the brain white matter.[60,61] The presence of elevated tau suggests additional white matter injury in neonatal encephalopathy, which has been suspected based on clinical outcomes and MRI.[62] However, MRI is not diagnostic until 24 hours after the injury and for neonates treated with therapeutic hypothermia, the therapeutic hypothermia devices are not MRI-compatible, which delays MRI use.[10,63,64] Our data suggest that plasma tau, collected on DOL 0-1, could be an effective predictor of brain injury

Table XII. Association of plasma and CSF candidate biomarker concentrations of neonates with neonatal encephalopathy and Bayley-III scores at 15-30 months*

	Plasma						CSF					
	Cognitive		Motor		Language		Cognitive		Motor		Language	
Biomarkers	Coefficient (95% CI)	*P* value	Coefficient (95% CI)	*P* value	Coefficient (95% CI)	*P* value	Coefficient (95% CI)	*P* value	Coefficient (95% CI)	*P* value	Coefficient (95% CI)	*P* value
GFAP	−0.03 (−2.8 to 2.7)	.98	−0.4 (−3.2 to 2.5)	.80	0.3 (−2.8 to 3.3)	.87	**−9.2 (−17 to −1.9)**	**.02**	**−8.7 (-17 to −0.5)**	**.04**	−7.0 (−15 to 1.1)	.09
NRGN	−0.07 (−3.4 to 3.3)	.97	−0.6 (−4.1 to 2.9)	.73	0.2 (−3.5 to 3.9)	.90	**−22.4 (−34 to −11.3)**	**.001**	**−25.3 (−37 to −13.8)**	**<.001**	**−19.1 (−32 to −6.0)**	**.007**
BDNF	1.4 (−2.3 to 5.2)	.45	1.6 (−2.3 to 5.4)	.43	0.9 (−3.3 to 5.0)	.68	4.2 (−9.5 to 18.0)	.52	5.0 (−9.8 to 19.6)	.49	1.9 (−12.4 to 16.2)	.78
IL-6	−2.2 (−5.2 to 0.8)	.16	−2.2 (−5.3 to 0.9)	.17	−2.5 (−5.8 to 0.8)	.14	**−4.4 (−8.0 to −1.0)**	**.02**	**−5.1 (−8.7 to −1.5)**	**.009**	−3.6 (−7.5 to 0.3)	.07
IL-8	−2.6 (−6.2 to 1.0)	.16	−3.0 (−6.7 to 0.9)	.13	−3.2 (−7.2 to 0.7)	.11	−2.9 (−7.1 to 1.3)	.16	**−4.8 (−8.9 to −0.6)**	**.03**	−2.5 (−7.0 to 1.9)	.25
IL-10	−0.3 (−4.0 to 3.5)	.89	−0.6 (−4.5 to 3.3)	.76	−1.6 (−5.6 to 2.5)	.45	**−34.6 (−63 to −5.9)**	**.02**	**−42.0 (−71 to −12.7)**	**.008**	−20.2 (−54 to 13.0)	.22
VEGF	0.5 (−2.5 to 3.4)	.75	0.9 (−2.2 to 3.9)	.57	1.0 (−2.3 to 4.2)	.56	−0.4 (−9.6 to 8.8)	.93	0.08 (−9.8 to 10.0)	.99	−1.5 (−11.0 to 8.1)	.75
Tau	**−5.6 (−10.6 to −0.5)**	**.03**	**−6.1 (−11.5 to −0.7)**	**.03**	−5.4 (−10.9 to 0.2)	.06	−2.1 (−6.8 to 2.7)	.37	−2.6 (−7.5 to 2.3)	.27	−0.8 (−5.8 to 4.1)	.73

*Adjusted for gestational age and sex, the natural log of candidate biomarker concentrations was used in adjusted analyses. Plasma: duplex (GFAP and NRGN): n = 93; multiplex (BDNF, IL-6, IL-8, IL-10, VEGF): n = 92; tau: n = 81. CSF: duplex (GFAP and NRGN): n = 20; multiplex (BDNF, IL-6, IL-8, IL-10, VEGF): n = 20; tau: n = 17.
Bold values indicate statistical significance.

and help to fill this crucial time gap for intervention. CSF candidate biomarkers were not associated with brain injury measured on MRI.

When evaluating neurodevelopmental outcomes related to brain injury severity, we found with adjusted analysis, that plasma tau and CSF GFAP, NRGN, IL-6, IL-8, and IL-10 were negatively associated with Bayley-III scores at 15-30 months. Specifically, greater plasma tau collected on DOL 0-1 predicted abnormal cognitive and motor outcomes at 15-30 months. Adding multiple candidate biomarkers to the regression model did not significantly improve the association of outcomes beyond that seen with tau. Other studies using plasma and CSF also demonstrated greater levels of GFAP, IL-6, IL-8, and tau were associated with abnormal neurodevelopmental outcomes at 15-30 months.[14,15,17,24,25,29,65,66] Previous research also supports that lower VEGF is associated with abnormal neurodevelopmental outcomes.[32] Our study suggests that greater levels of CNS necrosis, especially tau, and inflammatory markers could predict poor neurodevelopmental performance in the future.

Furthermore, we analyzed the baseline characteristics of infants without reported neurodevelopmental assessments to evaluate the impact of candidate biomarker association with abnormal outcomes because the data from neonates without MRI Barkovich scores (n = 38) and without Bayley-III scores at 15-30 months (n = 56) were not included in analysis. Those without reported MRI Barkovich scores appeared to be healthier infants with greater gestational age, higher proportion of vaginal deliveries, lower rates of therapeutic hypothermia, and lower proportion of moderate-to-severe Sarnat scores. Those without reported Bayley-III scores importantly did not differ in degree of encephalopathy and therefore should not affect our conclusions about abnormal neurodevelopmental outcomes. Future studies should include analyses of factors that can impact follow-up rates and neurodevelopmental outcomes at follow-up, including socioeconomic status.

Limitations of this study include small sample size, heterogeneity of neonatal encephalopathy cases, a single time point for some samples, sample variation over 24 hours, and unavailability of clinical data, which limited the ability to evaluate some outcomes. The neonatal encephalopathy criteria for this study included infants with neonatal encephalopathy identified <6 hours with therapeutic hypothermia, <48 hours without therapeutic hypothermia, and postnatally with brain injury on cranial ultrasound. This reflects a heterogeneity of cases; however, all infants had perinatal asphyxia. Due to the limited availability of samples and clinical data, the absence of CSF samples from healthy neonates born at term precluded any comparison of CSF candidate biomarkers in the neonatal encephalopathy cohort compared with controls. In addition, CSF candidate biomarker analysis of neurodevelopmental outcomes was limited by a lack of CSF samples from neonates with abnormal (<85) Bayley-III scores (n = 1). In addition, for the CSF analysis, there was a small number of neonates with mild encephalopathy (n = 4) compared with moderate-to-severe encephalopathy (n = 26). Lastly, the inclusion of CSF

samples spanning DOL 0-7 (median DOL 3) may influence the associations with clinical outcomes.

In conclusion, the ideal biomarker for identifying, stratifying, and monitoring neonatal encephalopathy would need to be stable, measurable at a high sensitivity in an easy-to-access biofluid, have peak concentrations early in life, and have the ability to discriminate neonatal encephalopathy severity and predict neurodevelopmental outcomes. Our study provided novel insight into a selection of candidate biomarkers that fit these optimal criteria for neonatal encephalopathy, with tau as the best performer in multiple measures of brain injury and outcomes. Furthermore, plasma candidate biomarkers were able to identify neonates with neonatal encephalopathy, discriminate clinical severity, and predict seizures, brain injury by MRI, and neurodevelopmental outcomes at 15-30 months. Our study also identified potential adjunctive therapies for neonatal encephalopathy. Larger validation studies are needed to further investigate this candidate biomarker panel and its implementations into clinical settings. ■

We thank the patients and their families for their participation and contributions to this study. We thank the Everett research group at Johns Hopkins University School of Medicine for their support and contributions. We thank the Johns Hopkins University School of Medicine Scholarly Concentration mentor Dr Meredith Atkinson and the Johns Hopkins University School of Medicine Dean's Funding for their support and contributions.

Submitted for publication Mar 24, 2020; last revision received May 25, 2020; accepted Jun 25, 2020.

References

1. Molloy EJ, Bearer C. Neonatal encephalopathy versus hypoxic-ischemic encephalopathy. Pediatr Res 2018;84:574.
2. Volpe JJ. Neonatal encephalopathy: an inadequate term for hypoxic-ischemic encephalopathy. Ann Neurol 2012;72:156-66.
3. Kurinczuk JJ, White-Koning M, Badawi N. Epidemiology of neonatal encephalopathy and hypoxic–ischaemic encephalopathy. Early Hum Dev 2010;86:329-38.
4. Nelson K, Bingham P, Edwards E, Horbar J, Kenny J, Inder T, et al. Antecedents of neonatal encephalopathy in the Vermont Oxford Network Encephalopathy Registry. Pediatrics 2012;130:878-86.
5. Jacobs S, Berg M, Hunt R, Tarnow-Mordi W, Inder T, Davis P. Cooling for newborns with hypoxic ischaemic encephalopathy. Cochrane Database Syst Rev 2013:CD003311.
6. Shankaran S, Laptook AR, Pappas A, McDonald SA, Das A, Tyson JE, et al. Effect of depth and duration of cooling on death or disability at age 18 months among neonates with hypoxic-ischemic encephalopathy a randomized clinical trial. JAMA 2017;318:57-67.
7. Gluckman PD, Wyatt JS, Azzopardi D, Ballard R, Edwards AD, Ferriero DM, et al. Selective head cooling with mild systemic hypothermia after neonatal encephalopathy: multicentre randomised trial. Lancet 2005;365:663-70.
8. Wyatt JS, Gluckman PD, Liu PY, Azzopardi D, Ballard R, Edwards AD, et al. Determinants of outcomes after head cooling for neonatal encephalopathy. Pediatrics 2007;119:912-21.
9. Graham EM, Everett AD, Delpech JC, Northington FJ. Blood biomarkers for evaluation of perinatal encephalopathy: state of the art. Curr Opin Pediatr 2018;30:199-203.
10. American College of Obstetricians and Gynecologists' Task Force on Neonatal Encephalopathy. Neonatal encephalopathy and neurologic outcome, second edition. Obstet Gynecol 2014;123:896-901.
11. Blennow K. A review of fluid biomarkers for Alzheimer's disease: moving from CSF to Blood. Neurol Ther 2017;6:15-24.
12. Pelinka LE, Kroepfl A, Leixnering M, Buchinger W, Raabe A, Redl H. GFAP versus S100B in serum after traumatic brain injury: relationship to brain damage and outcome. J Neurotrauma 2004;21:1553-61.
13. Missler U, Wiesmann M, Wittmann G, Magerkurth O, Hagenström H. Measurement of glial fibrillary acidic protein in human blood: analytical method and preliminary clinical results. Clin Chem 1999;44:138-41.
14. Ramaswamy V, Horton J, Vandermeer B, Buscemi N, Miller S, Yager J. Systematic review of biomarkers of brain injury in term neonatal encephalopathy. Pediatr Neurol 2009;40:215-26.
15. Ennen CS, Huisman TAGM, Savage WJ, Northington FJ, Jennings JM, Everett AD, et al. Glial fibrillary acidic protein as a biomarker for neonatal hypoxic–ischemic encephalopathy treated with whole-body cooling. Am J Obstet Gynecol 2011;205:251.e1-7.
16. Massaro AN, Jeromin A, Kadom N, Vezina G, Hayes RL, Wang KKW, et al. Serum biomarkers of MRI brain injury in neonatal hypoxic ischemic encephalopathy treated with whole-body hypothermia: a pilot study. Pediatr Crit Care Med 2013;14:310-7.
17. Chalak LF, Sánchez PJ, Adams-Huet B, Laptook AR, Heyne RJ, Rosenfeld CR. Biomarkers for severity of neonatal hypoxic-ischemic encephalopathy and outcomes in newborns receiving hypothermia therapy. J Pediatr 2014;164:468-74.e1.
18. Stewart A, Tekes A, Huisman TAGM, Jennings JM, Allen MC, Northington FJ, et al. Glial fibrillary acidic protein as a biomarker for periventricular white matter injury. Am J Obstet Gynecol 2013;209:27.e1-7.
19. Represa A, Deloulme JC, Sensenbrenner M, Ben-Ari Y, Baudier J, et al. Neurogranin: immunocytochemical localization of a brain-specific protein kinase C substrate. J Neurosci 1990;10:3782-92.
20. Guadaño-Ferraz A, Viñuela A, Oeding G, Bernal J, Rausell E. RC3/neurogranin is expressed in pyramidal neurons of motor and somatosensory cortex in normal and denervated monkeys. J Comp Neurol 2005;493:554-70.
21. Yang J, Korley FK, Dai M, Everett AD. Serum neurogranin measurement as a biomarker of acute traumatic brain injury. Clin Biochem 2015;48:843-8.
22. Çevik S, Özgenç MM, Güneyk A, Ş Evran, Akkaya E, Çalış F, et al. NRGN, S100B and GFAP levels are significantly increased in patients with structural lesions resulting from mild traumatic brain injuries. Clin Neurol Neurosurg 2019;183:105380.
23. Toorell H, Zetterberg H, Blennow K, Sävman K, Hagberg H. Increase of neuronal injury markers Tau and neurofilament light proteins in umbilical blood after intrapartum asphyxia. J Matern Neonatal Med 2017;31:1-5.
24. Massaro AN, Wu YW, Bammler TK, Comstock B, Mathur A, McKinstry RC, et al. Plasma biomarkers of brain injury in neonatal hypoxic-ischemic encephalopathy. J Pediatr 2018;194:67-75.
25. Takahashi K, Hasegawa S, Maeba S, Fukunaga S, Motoyama M, Hamano H, et al. Serum tau protein level serves as a predictive factor for neurological prognosis in neonatal asphyxia. Brain Dev 2014;36:670-5.
26. Lv H, Wang Q, Wu S, Yang L, Ren P, Yang Y, et al. Neonatal hypoxic ischemic encephalopathy-related biomarkers in serum and cerebrospinal fluid. Clin Chim Acta 2015;450:282-97.
27. Çelik Y, Atlcl A, Gülaşl S, Makharoblldze K, Eskandari G, Sungur MA, et al. The effects of selective head cooling versus whole-body cooling on some neural and inflammatory biomarkers: arandomized controlled pilot study. Ital J Pediatr 2015;41:1-8.
28. Chaparro-Huerta V, Flores-Soto ME, Merin Sigala ME, Barrera de León JC, Lemus-Varela M de L, Torres-Mendoza BM de G, et al. Proinflammatory cytokines, enolase and S-100 as early biochemical indicators of hypoxic-ischemic encephalopathy following perinatal asphyxia in newborns. Pediatr Neonatol 2017;58:70-6.
29. Leifsdottir K, Mehmet H, Eksborg S, Herlenius E. Fas-ligand and interleukin-6 in the cerebrospinal fluid are early predictors of hypoxic-ischemic encephalopathy and long-term outcomes after birth asphyxia in term infants. J Neuroinflammation 2018;15:1-11.

30. Imam SS, Gad GI, Atef SH, Shawky MA. Cord blood brain derived neurotrophic factor: diagnostic and prognostic marker in fullterm newborns with perinatal asphyxia. Pakistan J Biol Sci 2009;12:1498-504.
31. Liu F, Yang S, Du Z, Guo Z. Dynamic changes of cerebral-specific proteins in full-term newborns with hypoxic–ischemic encephalopathy. Cell Biochem Biophys 2013;66:389-96.
32. Sweetman DU, Onwuneme C, Watson WR, Murphy JFA, Molloy EJ. Perinatal asphyxia and erythropoietin and VEGF: serial serum and cerebrospinal fluid responses. Neonatology 2017;111:253-9.
33. O'Hare FM, Watson RWG, O'Neill A, Segurado R, Sweetman D, Downey P, et al. Serial cytokine alterations and abnormal neuroimaging in newborn infants with encephalopathy. Acta Paediatr Int J Paediatr 2017;106:561-7.
34. Diaz-Arrastia R, Wang KKW, Papa L, Sorani MD, Yue JK, Puccio AM, et al. Acute biomarkers of traumatic brain injury: relationship between plasma levels of ubiquitin c-terminal hydrolase-L1 and glial fibrillary acidic protein. J Neurotrauma 2014;31:19-25.
35. Graham EM, Burd I, Everett AD, Northington FJ. Blood biomarkers for evaluation of perinatal encephalopathy. Front Pharmacol 2016;7:196.
36. Sarnat HB, Sarnat MS. Encephalopathy fetal distress a clinical and electroencephalographic study. Arch Neurol 1976;33:696-705.
37. Sweetman D, Kelly LA, Zareen Z, Nolan B, Murphy J, Boylan G, et al. Coagulation profiles are associated with early clinical outcomes in neonatal encephalopathy. Front Pediatr 2019;7:1-7.
38. Douglas-Escobar M, Weiss MD. Hypoxic-ischemic encephalopathy: a review for the clinician. JAMA Pediatr 2015;169:397-403.
39. Barkovich AJ, Hajnal BL, Vigneron D, Sola A, Partridge JC, Allen F, et al. Prediction of neuromotor outcome in perinatal asphyxia: evaluation of MR scoring systems. Am J Neuroradiol 1998;19:143-9.
40. Bayley N. Bayley scales of infant and toddler development manual. 3rd ed. San Antonio, (TX): The Pscyhological Corporation; 2006.
41. Shankaran S, Laptook AR, Ehrenkranz RA, Tyson JE, McDonald SA, Donovan EF, et al. Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy. N Engl J Med 2005;353:1574-84.
42. Tobin J. Estimation of relationships for limited dependent variables. Econometrica 1958;26:24-36.
43. Bembea MM, Savage W, Strouse JJ, Schwartz JME, Graham E, Thompson CB, et al. Glial fibrillary acidic protein as a brain injury biomarker in children undergoing extracorporeal membrane oxygenation. Pediatr Crit Care Med 2011;12:572-9.
44. Okazaki K, Kusaka T, Kondo M, Kozawa K, Yoshizumi M, Kimura H. Temporal alteration of serum G-CSF and VEGF levels in perinatal asphyxia treated with head cooling. Cytokine 2012;60:812-4.
45. Korley FK, Diaz-Arrastia R, Wu AHB, Yue JK, Manley GT, Sair HI, et al. Circulating brain-derived neurotrophic factor has diagnostic and prognostic value in traumatic brain injury. J Neurotrauma 2016;33:215-25.
46. Failla MD, Conley YP, Wagner AK. Brain-derived neurotrophic factor (BDNF) in Traumatic brain injury-related mortality: interrelationships between genetics and acute systemic and central nervous system BDNF profiles. Neurorehabil Neural Repair 2016;30:83-93.
47. Wyrobek J, Laflam A, Max L, Tian J, Neufeld KJ, Kebaish KM, et al. Association of intraoperative changes in brain-derived neurotrophic factor and postoperative delirium in older adults. Br J Anaesth 2017;119:324-32.
48. Olin A, Henckel E, Chen Y, Lakshmikanth T, Pou C, Mikes J, et al. Stereotypic immune system development in newborn children. Cell 2018;174:1277-92.e14.
49. Wu YW, Goodman AM, Chang T, Mulkey SB, Gonzalez FF, Mayock DE, et al. Placental pathology and neonatal brain MRI in a randomized trial of erythropoietin for hypoxic – ischemic encephalopathy. Pediatr Res 2020;87:879-84.
50. Dingley AJ, Tooley J. Xenon ventilation during therapeutic hypothermia in neonatal encephalopathy: a feasibility study. Pediatrics 2014;133:809-18.
51. Cotten CM, Murtha AP, Goldberg RN, Grotegut CA, Smith PB, Goldstein RF, et al. Feasibility of autologous cord blood cells for infants with hypoxic-ischemic encephalopathy. J Pediatr 2014;164:973-9.e1.
52. Azzopardi D, Robertson NJ, Bainbridge A, Cady E, Charles-edwards G, Deierl A, et al. Moderate hypothermia within 6 h of birth plus inhaled xenon versus moderate hypothermia alone after birth asphyxia (TOBY-Xe): a proof-of-concept, open-label, randomised controlled trial. Lancet Neurol 2016;15:145-53.
53. Leal G, Comprido D, Duarte CB. BDNF-induced local protein synthesis and synaptic plasticity. Neuropharmacology 2014;76:639-56.
54. Korte M, Carrolltt P, Wolf E, Brem G, Thoenent H, Bonhoeffer T. Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. Proc Natl Acad Sci U S A 1995;92:8856-60.
55. Grad S, Ertel W, Keel M, Infanger M, Vonderschmitt DJ, Maly FE. Strongly enhanced serum levels of vascular endothelial growth factor (VEGF) after polytrauma and burn. Clin Chem Lab Med 1998;36:379-83.
56. Blennow M, Savman K, Ilves P, Thoresen M, Rosengren L. Brain-specific proteins in the cerebrospinal fluid of severely asphyxiated newborn infants. Acta Paediatr 2001;90:1171-5.
57. Blennow M, Hagberg H, Rosengren L. Glial fibrillary acidic protein in the cerebrospinal fluid: a possible indicator of prognosis in full-term asphyxiated newborn infants? Pediatr Res 1995;37:260-4.
58. Pinchefsky EF, Hahn CD. Outcomes following electrographic seizures and electrographic status epilepticus in the pediatric and neonatal ICUs. Curr Opin Neurol 2017;30:156-64.
59. Numis AL, Foster-Barber A, Deng X, Rogers EE, Barkovich AJ, Ferriero DM, et al. Early changes in pro-inflammatory cytokine levels in neonates with encephalopathy are associated with remote epilepsy. Pediatr Res 2019;86:616-21.
60. Binder LI, Frankfurter A, Rebhun LI. The distribution of tau in the mammalian central nervous system. J Cell Biol 1985;101:1371-8.
61. Gorath M, Stahnke T, Mronga T, Goldbaum O, Richter-Landsberg C. Developmental changes of Tau protein and mRNA in cultured rat. Glia 2001;36:89-101.
62. Hinojosa-Rodríguez M, Harmony T, Carrillo-Prado C, Van Horn JD, Irimia A, Torgerson C, et al. Clinical neuroimaging in the preterm infant: diagnosis and prognosis. NeuroImage Clin 2017;16:355-68.
63. Barkovich AJ, Miller SP, Bartha A, Newton N, Hamrick SEG, Mukherjee P, et al. MR imaging, MR spectroscopy, and diffusion tensor imaging of sequential studies in neonates with encephalopathy. Am J Neuroradiol 2006;27:533-47.
64. McKinstry RC, Miller JH, Snyder AZ, Mathur A, Schefft GL, Almli CR, et al. A prospective, longitudinal diffusion tensor imaging study of brain injury in newborns. Neurology 2002;59:824-33.
65. Bartha AI, Foster-Barber A, Miller SP, Vigneron DB, Glidden DV, Barkovich AJ, et al. Neonatal encephalopathy: association of cytokines with MR spectroscopy and outcome. Pediatr Res 2004;56:960-6.
66. Savman K, Blennow M, Gustafson K, Tarkowski E, Hagberg H. Cytokine response in cerebrospinal fluid after birth asphyxia. Pediatr Res 1998;43:746-51.

Table III. Comparison of plasma candidate biomarker concentrations in healthy neonates born at term and neonates with neonatal encephalopathy

Biomarkers	Control cohort median (IQR)	Neonatal encephalopathy cohort Median (IQR)	*P* value
GFAP	80 (31, 553)	221 (9.0, 1008)	.75
NRGN	**8 (8, 8)**	**34 (7, 374)**	**.03**
BDNF	**1376.9 (867.8, 2629.0)**	**407.3 (152.5, 1161.0)**	**<.001**
IL-6	**4.9 (2.6, 14.3)**	**28.4 (9.8, 119.9)**	**<.001**
IL-8	**32.2 (24.4, 47.0)**	**113.5 (52.3, 394.9)**	**<.001**
IL-10	**0.7 (0.3, 2.2)**	**8.5 (2.2, 48.8)**	**<.001**
VEGF	**276.1 (142.3, 319.9)**	**12.9 (0.9, 60.7)**	**<.001**
Tau	**111.4 (32.8, 182.3)**	**243.1 (115.1, 541.0)**	**<.001**

All analyses used the Mann–Whitney *U* test for comparisons of nonparametric data. Candidate biomarker units are pg/mL. Duplex (GFAP and NRGN): control n = 24, neonatal encephalopathy n = 141; multiplex (BDNF, IL-6, IL-8, IL-10, VEGF): control n = 29, neonatal encephalopathy n = 141; tau: control n = 27, neonatal encephalopathy n = 123.
Bold values indicate statistical significance.

Table IV. Univariate analysis of plasma candidate biomarker concentrations of neonates with neonatal encephalopathy and clinical encephalopathy

Biomarkers	Mild (Sarnat 0-1) Median (IQR)	Moderate-to-severe (Sarnat 2-3) Median (IQR)	*P* value
GFAP	**9 (9, 45)**	**251 (9, 1154)**	**.05**
NRGN	**7 (7, 7)**	**40 (7, 403)**	**.03**
BDNF	**1596.5 (1081.2, 2324.1)**	**387.2 (136.1, 1006.5)**	**.002**
IL-6	21.4 (7.2, 39.1)	29.7 (9.8, 148.5)	.57
IL-8	64.7 (46.1, 104.4)	119.6 (52.6, 404.4)	.11
IL-10	4.6 (1.3, 16.7)	9.2 (2.3, 59.5)	.23
VEGF	**87.0 (0.9, 330.2)**	**11.7 (0.9, 55.3)**	**.05**
Tau	**45.8 (45.8, 103.6)**	**276.7 (135.5, 554.4)**	**<.001**

Univariate analyses used the Mann–Whitney *U* test for comparisons of nonparametric data. Candidate biomarker units are pg/mL. Duplex (GFAP and NRGN): mild n = 11, moderate-to-severe n = 130; multiplex (BDNF, IL-6, IL-8, IL-10, VEGF): mild n = 10, moderate-to-severe n = 131; tau: mild n = 11, moderate-to-severe n = 112.
Bold values indicate statistical significance.

Table VI. Association of CSF candidate biomarker concentrations of neonates with neonatal encephalopathy and clinical encephalopathy

	Univariate analysis			Adjusted logistic regression*	
Biomarkers	Mild (Sarnat 0-1) Median (IQR)	Moderate-to-severe (Sarnat 2-3) Median (IQR)	*P* value	Coefficient (95% CI)	*P* value
GFAP	322 (220, 395)	247 (156, 415)	.54	−0.118 (−0.953 to 0.716)	.78
NRGN	7 (7, 24)	7 (7, 7)	.50	−0.783 (−2.439 to 0.873)	.35
BDNF	30.3 (30.3, 30.3)	30.3 (30.3, 30.3)	.57	–	
IL-6	4.0 (3.1, 142.6)	3.8 (0.2, 36.2)	.46	−0.135 (−0.580 to 0.311)	.55
IL-8	369.9 (160.4, 501.2)	203.5 (78.4, 606.1)	.76	−0.122 (−0.777 to 0.532)	.71
IL-10	1.0 (1.0, 1.0)	1.0 (1.0, 1.0)	.70	–	
VEGF	4.5 (2.0, 6.6)	4.9 (3.3, 9.6)	.46	0.603 (−0.784 to 1.990)	.39
Tau	3223.9 (56.6, 2.8e+04)	2626.1 (56.6, 6721.5)	.80	−0.016 (−0.446 to 0.415)	.94

Univariate analyses used the Mann–Whitney *U* test for comparisons of non-parametric data. Candidate biomarker units are pg/mL. Duplex (GFAP and NRGN): mild n = 4, moderate-to-severe n = 26; multiplex (BDNF, IL-6, IL-8, IL-10, VEGF): mild n = 4, moderate-to-severe n = 26; Tau: Mild n = 4, moderate-to-severe n = 23.
*Adjusted for gestational age and sex, the natural log of candidate biomarker concentrations were used in adjusted analyses.

Table VII. Univariate analysis of plasma candidate biomarker concentrations of neonates with neonatal encephalopathy and seizure occurrence

Biomarkers	No median (IQR)	Yes median (IQR)	*P* value
GFAP	224 (9, 878)	153 (9, 1008)	.96
NRGN	57 (7, 549)	17 (7, 313)	.64
BDNF	373.2 (149.6, 1112.5)	480.5 (183.2, 1525.5)	.52
IL-6	**43.7 (13.4, 295.5)**	**22.7 (6.5, 66.8)**	**.02**
IL-8	100.8 (53.6, 341.6)	130.7 (47.5, 439.3)	.75
IL-10	11.4 (2.9, 36.4)	7.6 (1.3, 59.5)	.39
VEGF	10.0 (0.2, 76.5)	14.3 (0.9, 52.6)	.79
Tau	251.9 (123.8, 528.2)	239.8 (119.2, 554.5)	.90

Univariate analyses used the Mann–Whitney *U* test for comparisons of nonparametric data. Candidate biomarker units are pg/mL. Duplex (GFAP and NRGN): no n = 58, yes n = 67; multiplex (BDNF, IL-6, IL-8, IL-10, VEGF): no n = 60, yes n = 65; tau: no n = 51, yes n = 58.
Bold values indicate statistical significance.

Table VIII. Association of CSF candidate biomarker concentrations of neonates with neonatal encephalopathy and seizure occurrence

	Univariate analysis			Adjusted logistic regression*	
Biomarkers	No Median (IQR)	Yes Median (IQR)	*P* value	Coefficient (95% CI)	*P* value
GFAP	258 (251, 345)	202 (156, 636)	.57	−0.048 (−0.682 to 0.587)	.88
NRGN	7 (7, 7)	7 (7, 7)	.68	0.273 (−1.157 to 1.702)	.71
BDNF	30.3 (30.3, 30.3)	30.3 (30.3, 30.3)	.48	–	
IL-6	3.1 (0.7, 196.2)	4.5 (0.2, 36.2)	.68	−0.059 (−0.387 to 0.270)	.73
IL-8	300.5 (81.3, 503.9)	203.5 (74.2, 606.1)	.78	0.036 (−0.362 to 0.433)	.86
IL-10	1.0 (1.0, 1.0)	1.0 (1.0, 1.0)	.48	–	
VEGF	5.5 (2.8, 7.3)	3.8 (2.6, 6.9)	.76	−0.008 (−0.770 to 0.754)	.98
Tau	2337.5 (56.6, 6391.4)	5290.6 (56.6, 7552.7)	.46	0.127 (−0.217 to 0.471)	.47

Univariate analyses used the Mann–Whitney *U* test for comparisons of non-parametric data. Candidate biomarker units are pg/mL. Duplex (GFAP and NRGN): no n = 9, yes n = 18; multiplex (BDNF, IL-6, IL-8, IL-10, VEGF): no n = 9, yes n = 18; tau: no n = 9, yes n = 15.
*Adjusted for gestational age and sex, the natural log of candidate biomarker concentrations was used in adjusted analyses.

Table IX. Association of CSF candidate biomarker concentrations of neonates with neonatal encephalopathy and brain injury by MRI

	Basal ganglia		Watershed		Basal ganglia/Watershed	
Biomarkers	Coefficient (95% CI)	*P* value	Coefficient (95% CI)	*P* value	Coefficient (95% CI)	*P* value
GFAP	0.53 (−0.58 t 1.63)	.35	0.10 (−0.75 to 0.96)	.82	1.46 (−0.43 to 3.346)	.13
NRGN	–		0.37 (−1.15 to 1.90)	.63	–	
BDNF	–		–		–	
IL-6	0.63 (−0.10 to 1.35)	.09	0.04 (−0.34-0.41)	.84	0.10 (−0.30 to 0.49)	.63
IL-8	0.53 (−0.28 to 1.35)	.20	−0.02 (−0.45 to 0.42)	.95	0.07 (−0.43 to 0.57)	.78
IL-10	–		–		–	
VEGF	−0.07 (−1.07 to 0.93)	.89	0.00 (−0.79 to 0.79)	.99	−0.03 (−0.82 to 0.76)	.95
Tau	0.77 (−0.53 to 2.07)	.25	0.11 (−0.34 to 0.55)	.64	0.14 (−0.33 to 0.61)	.55

All analyses used adjusted logistic regression, adjusted for gestational age and sex, the natural log of biomarker concentrations was used in adjusted analyses. Candidate biomarker units are pg/mL. Duplex (GFAP and NRGN): n = 20; multiplex (BDNF, IL-6, IL-8, IL-10, VEGF): n = 20; tau: n = 18.

Table X. Univariate analysis of plasma candidate biomarker concentrations of neonates with neonatal encephalopathy and Bayley-III scores at 15-30 months

	Cognitive		Motor		Language	
Biomarkers	**rho**	***P* value**	**rho**	***P* value**	**rho**	***P* value**
GFAP	−0.04	.72	−0.09	.38	0.01	.95
NRGN	−0.10	.36	−0.16	.12	−0.05	.67
BDNF	0.16	.14	0.13	.23	0.03	.76
IL-6	**−0.21**	**.05**	**−0.23**	**.03**	−0.15	.17
IL-8	**−0.29**	**.005**	**−0.40**	**<.001**	**−0.25**	**.02**
IL-10	−0.20	.06	**−0.23**	**.03**	−0.17	.11
VEGF	0.16	.13	**0.23**	**.03**	0.14	.20
Tau	**−0.27**	**.02**	**−0.26**	**.02**	−0.16	.17

Univariate analyses used Spearman correlation for non-parametric data. Candidate biomarker units are pg/mL. Duplex (GFAP and NRGN): Cognitive and Motor n = 93, Language n = 90; multiplex (BDNF, IL-6, IL-8, IL-10, VEGF): Cognitive and Motor n = 92, Language n = 89; tau: Cognitive and Motor n = 81, Language n = 80.
Bold values indicate statistical significance.

Table XI. Univariate analysis of CSF candidate biomarker concentrations of neonates with neonatal encephalopathy and Bayley-III scores at 15-30 months

	Cognitive		Motor		Language	
Biomarkers	**rho**	***P* value**	**rho**	***P* value**	**rho**	***P* value**
GFAP	−0.31	.19	−0.27	.25	−0.12	.62
NRGN	**−0.46**	**.04**	**−0.48**	**.03**	−0.39	.09
BDNF	0.12	.61	0.10	.69	0.03	.90
IL-6	**−0.44**	**.05**	**−0.51**	**.02**	−0.26	.26
IL-8	−0.40	.08	**−0.50**	**.03**	−0.25	.28
IL-10	−0.34	.14	−0.34	.14	−0.34	.14
VEGF	−0.06	.80	−0.06	.79	−0.07	.78
Tau	−0.23	.38	−0.25	.33	−0.07	.80

Univariate analyses used Spearman correlation for non-parametric data. Candidate biomarker units are pg/mL. Duplex (GFAP and NRGN): n = 20; multiplex (BDNF, IL-6, IL-8, IL-10, VEGF): n = 20; tau: n = 17.

Table XIII. Association of plasma candidate biomarker concentrations of neonates with neonatal encephalopathy and binary outcomes of normal and abnormal Bayley-III scores at 15-30 months

	Univariate analysis			Adjusted logistic regression*	
Bayley-III scores	Normal (≥85) Median (IQR)	Abnormal (<85 or death) Median (IQR)	*P* value	OR of SD (95% CI)	*P* value
Cognitive					
GFAP	300.4 (8.7, 1154.2)	284.6 (8.7, 821.8)	.68	.88 (0.55-1.42)	.59
NRGN	35.9 (7.1, 498.7)	49.9 (16.5, 243.2)	.68	.94 (0.58-1.53)	.81
BDNF	444.6 (134.6, 1802.3)	317.3 (77.0, 692.5)	.18	0.75 (0.46-1.22)	.25
IL-6	28.8 (11.5, 116.8)	35.1 (9.5, 2425.8)	.31	1.32 (0.80-2.16)	.28
IL-8	**115.3 (59.6, 462.6)**	**324.8 (94.5, 2458.4)**	**.05**	1.26 (0.73-2.15)	.41
IL-10	11.6 (1.7, 60.5)	13.6 (1.9, 111.8)	.76	0.92 (0.55-1.54)	.76
VEGF	11.7 (0.9, 82.2)	9.3 (0.5, 43.8)	.35	0.89 (0.54-1.49)	.66
Tau	**218.0 (102.4, 541.6)**	**454.1 (210.8, 1410.2)**	**.03**	**1.76 (1.02-3.06)**	**.04**
Motor					
GFAP	281.9 (8.7, 1154.2)	429.1 (8.7, 1039.1)	.87	0.91 (0.57-1.45)	.69
NRGN	35.1 (7.1, 498.7)	59.4 (16.5, 314.2)	.66	0.95 (0.59-1.52)	.82
BDNF	434.6 (134.6, 1720.7)	414.6 (77.0, 868.7)	.37	0.80 (0.50-1.28)	.35
IL-6	28.8 (10.9, 116.8)	33.4 (10.9, 2365.0)	.26	1.34 (0.83-2.14)	.23
IL-8	**108.9 (53.9, 398.7)**	**360.6 (106.8, 2458.4)**	**.009**	1.43 (0.83-2.48)	.20
IL-10	10.9 (1.4, 37.0)	18.8 (2.8, 134.4)	.34	1.08 (0.66-1.75)	.77
VEGF	12.6 (0.9, 83.6)	10.5 (0.5, 43.8)	.32	0.91 (0.56-1.48)	.70
Tau	**215.0 (95.4, 541.0)**	**412.8 (210.8, 1410.2)**	**.01**	**1.82 (1.06-3.12)**	**.03**
Language					
GFAP	300.4 (8.7, 1154.2)	99.1 (8.7, 902.6)	.46	0.79 (0.49-1.26)	.32
NRGN	34.3 (7.11, 498.7)	38.0 (16.5, 208.2)	.98	0.83 (0.51-1.35)	.45
BDNF	446.6 (136.1, 1808.5)	372.5 (77.0, 724.9)	.19	0.76 (0.47-1.22)	.25
IL-6	27.4 (10.8, 113.7)	35.1 (10.9, 2365.0)	.21	1.39 (0.86-2.26)	.18
IL-8	**101.3 (49.9, 390.7)**	**360.6 (114.4, 1836.6)**	**.005**	1.51 (0.87-2.63)	.15
IL-10	9.2 (1.3, 31.6)	22.1 (2.8 134.4)	.19	1.23 (0.75-2.01)	.42
VEGF	14.8 (0.9, 83.6)	7.2 (0.2, 43.8)	.18	0.83 (0.51-1.36)	.46
Tau	**218.0 (102.4, 541.0)**	**412.8 (209.1, 1410.2)**	**.04**	**1.71 (1.00-2.93)**	**.05**

Univariate analyses used the Mann–Whitney *U* test for comparisons of non-parametric data. Candidate biomarker units are pg/mL. Duplex (GFAP and NRGN): Cognitive: normal n = 69, abnormal n = 24, Motor: normal n = 66, abnormal n = 27, Language: normal n = 63, abnormal n = 27; multiplex (BDNF, IL-6, IL-8, IL-10, VEGF): Cognitive: normal n = 68, abnormal n = 24, Motor: normal n = 64, abnormal n = 28, Language: normal n = 61, abnormal n = 28; tau: Cognitive: normal n = 57, abnormal n = 19, Motor: normal n = 59, abnormal n = 22, Language: normal n = 55, abnormal n = 22.

*Adjusted for gestational age and sex, the natural log of candidate biomarker concentrations was used in adjusted analyses.

Bold values indicate statistical significance.