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Pediatric RESEARCH

CLINICAL RESEARCH ARTICLE

Serial plasma biomarkers of brain injury in infants with neonatal encephalopathy treated with therapeutic hypothermia

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BACKGROUND: Neonatal encephalopathy (NE) is a major cause of long-term neurodevelopmental disability in neonates. We evaluated the ability of serially measured biomarkers of brain injury to predict adverse neurological outcomes in this population.
METHODS: Circulating brain injury biomarkers including BDNF, IL-6, IL-8, IL-10, VEGF, Tau, GFAP, and NRGN were measured at 0, 12, 24, 48, 72, and 96 h of cooling from 103 infants with NE undergoing TH. The biomarkers' individual and combinative ability to predict death or severe brain injury and adverse neurodevelopmental outcomes beyond 1 year of age was assessed.
RESULTS: Early measurements of inflammatory cytokines IL-6, 8, and 10 within 24 HOL (AUC = 0.826) and late measurements of Tau from 72 to 96 HOL (AUC = 0.883, OR 4.37) were accurate in predicting severe brain injury seen on MRI. Late measurements of Tau were predictive of adverse neurodevelopmental outcomes (AUC = 0.81, OR 2.59).
CONCLUSIONS: Tau was consistently a predictive marker for brain injury in neonates with NE. However, in the first 24 HOL, IL-6, 8, and 10 in combination were most predictive of death or severe brain injury. The results of this study support the use of a serial biomarker panel to assess brain injury over the time course of disease in NE.

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IMPACT:

- While recent studies have evaluated candidate brain injury biomarkers, no biomarker is in current clinical use.
- This study supports the use of a serial biomarker panel for ongoing assessment of brain injury neonates with NE.
- In combination, IL6, IL8, and IL10 in the first 24 h of cooling were more predictive of brain injury by MRI than each cytokine alone.
- Individually, Tau was overall most consistently predictive of adverse neurological outcomes, particularly when measured at or after rewarming.

INTRODUCTION

Neonatal encephalopathy (NE) is a major contributor to adverse neurologic sequelae and long-term neurodevelopmental disability in affected neonates.[1–3] It is estimated that 1–3 per 1000 births result in asphyxia that can lead to NE.[4] Although there have been advancements in the identification and treatment of perinatal NE, including the introduction of therapeutic hypothermia, the rate of disability among affected neonates still remains around 45%.[3,5,6] To reduce the rate of these adverse outcomes, infants who are at an increased risk of brain injury or not responding well to treatment must be identified early for possible adjuvant neuroprotective interventions. Real-time physiological biomarkers are urgently needed to evaluate the extent of brain injury, guide treatment practices, and predict outcomes for neonates with NE.

Currently, there is no blood-based biomarker of neonatal brain injury used in clinical practice. However, several candidate circulating plasma biomarkers that can indicate the severity of hypoxic–ischemic injury to the brain have been proposed. Both pro- and anti-inflammatory immune-modulating cytokines Interleukin (IL) 6, IL-8, and IL-10 are upregulated as a part of the neuroinflammatory cascade triggered by hypoxic conditions in the brain, and have been associated with brain injury in small cohorts of babies with NE.[7] Tau protein, Neurogranin (NRGN), and Glial Acidic Fibrillary Protein (GFAP) are brain-specific proteins found concentrated within neuron axons, dendrites, and astrocytes, respectively.[8–10] Thus, the elevation of these proteins in the blood can be a sign of neuronal or astrocytic damage or apoptosis.[8,9,11,12] Growth factors vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF) are also released as a response to neuronal injury.[7,11] Thus, these markers can reflect distinct aspects of pathogenesis when assessed quantitatively in the blood of affected newborns.

Recent studies have examined the use of circulating plasma biomarkers as a real-time indicator of brain injury in neonates with NE undergoing therapeutic hypothermia (TH) and have investigated the efficacy of these markers to predict adverse CNS

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outcomes in infants with NE.[7,8,10,13,14] However, the temporality of serial measurements and combinative value of these biomarkers during TH and post-rewarming has not been well described. This study aims to investigate a panel of serially measured candidate biomarkers and to determine whether they alone or in combination, are predictive of neurological outcomes.

METHODS

Study population

This prospective cohort study was conducted at Children's National Medical Center in a level IV NICU in the United States of America. Infants admitted for initiation of TH between 2010 and 2016 were screened for enrollment. Criteria for enrollment eligibility included term and near-term infants (>35 weeks gestation) with a diagnosis of moderate or severe NE as determined by modified Sarnat staging,[4,15] who were treated with TH according to the National Institutes of Child Health and Human Development (NICHD) Neonatal Research Network (NRN) protocol.[4] This study was approved by the Children's National Institutional Review Board and written informed consent was obtained from the parents of participants. Baseline (at time of cooling initiation) biomarker data and clinical characteristics of a subset of this cohort was previously published as part of a multicenter study.[16]

Specimen collection and biomarker determinations

Plasma samples were collected from salvaged routine clinical laboratory specimens of infants that met study criteria. Per hospital protocol, routine clinical blood samples are processed and centrifuged at 4000 RPM for 5 min. Excess plasma not utilized for biochemical analyses are then stored at −80 °C for a minimum of 48 h in accordance with the College of American Pathologists Laboratory Standards before disposal. Prior to their disposal from the laboratory, the study team identified and retrieved samples from the enrolled patients at each appropriate timepoint. With the initiation of therapeutic hypothermia set as timepoint 0, specimens were collected at 0, 12, 24, 72, and 96 h of TH when available. Upon collection, samples were thawed once for transfer to storage cryovials and then stored at −80 °C prior to analysis. Inflammatory cytokines and neurotrophic factors, BDNF, IL-6, IL-8, IL-10, and VEGF, were measured using a custom multiplex enzyme-linked immunosorbent assay (ELISA) on the Meso Scale Discovery (MSD) platform (Meso Scale Discovery Inc, Rockville, MD). Calibrators for the analytes were produced using a commercially provided diluent (MSD R50AG-2) and a detection antibody cocktail was likewise prepared in another commercially available diluent (MSD R51BA-5). Plasma samples were diluted 5-fold. The lower limits of detection (LLOD) for the BDNF, IL-6, IL-8, IL-10, and VEGF assays were 48.47, 0.47, 0.56, 1.26, and 1.59 pg/mL, respectively, with interassay coefficients of variation (CV) of 9.7%, 4.7%, 1.8%, 1.7%, and 2.7%, respectively. Any samples that were above the ULOQ were reassayed with a greater dilution to obtain the correct concentration. Any sample CV% > 20 were repeated.

Similarly, a custom duplex ELISA was developed to measure GFAP and NRGN (MSD, Rockville, MD) as previously described.[17,18] Plasma samples were diluted 2-fold. The LLOD for the GFAP and NRGN assays were 0.014 and 0.016 ng/mL, with an interassay CV of 2.6% and 2.8%, respectively.

Tau was measured using a commercial ELISA (MSD, Rockville, MD, Human Total Tau Kit, Cat # N451LAA-1). Plasma samples were diluted 4-fold and assayed according to manufacturer instructions. The LLOD for Tau was 82.03 pg/mL, with an interassay CV of 5.1%.

Magnetic resonance imaging

Per institutional protocol, post-cooling MRIs were performed on each neonate at target age 4–7 days of life. All scans were performed on a 3 Tesla scanner (Discovery MR750, GE Healthcare, Milwaukee, WI) and included the following sequences: 3D T1-weighted Spoiled Gradient Recalled, double acquisition axial FSE T2 proton density, axial T2 propeller (in cases of patient motion), axial T2-Star Weighted Susceptibility Imaging, coronal T1 FLAIR propeller, and axial 30-direction DTI. MRIs were reviewed and scored by a single experienced neuroradiologist who was blinded to clinical and biomarker data. Brain injury severity was scored according to Barkovich and severe brain injury was defined as a basal ganglia score of ≥3 or watershed score ≥4.[19]

Neurodevelopmental assessment

Neurodevelopmental outcomes were assessed using the Bayley-III Scales of Infant and Toddler Development according to our institutional protocol for follow-up after TH. The Bayley-III measures cognitive, language, and gross and fine motor domains, providing composite scores where 100 (±15 SD) is the normative mean.[20] Infants are routinely assessed at 9, 15, 18, and 30 months to follow developmental progress. Testing was performed and scored by a certified developmental psychologist who was blinded to biomarker data. Significant neurodevelopmental delay was classified according to the latest follow-up visit available beyond 1 year and defined as Bayley-III Cognitive Composite <85 or Bayley-III Motor Composite <85.[21–24] Infants were considered not to have a significant developmental delay if both scores were ≥85.

Statistical analysis

Standard descriptive statistics included means (standard deviations) and medians (ranges) as appropriate for continuous variables, as well as counts (percentages) for categorical data. The bivariate association between biomarker levels and outcome groups were assessed with Mann–Whitney *U* tests. Adverse outcomes were defined as (1) death or severe brain injury by MRI and (2) death or significant neurodevelopmental delay. Logistic regression analyses were used to assess the ability of individual biomarkers to predict the adverse outcome at each timepoint. Additionally, in order to assess the value of serial measures within each patient, summary functions including minimum, maximum, early average (of all values within 24 h), late average (of values beyond 72 h), and overall average were evaluated for predictive value. Finally, cumulative models were developed to assess the additive predictive value of multiple biomarkers, selected based on their demonstrated individual predictive ability. Multivariable models were also performed, adjusting for covariates including Sarnat grade (moderate versus severe), Apgar score at 5 min, presenting pH, electrographic seizure, and socioeconomic status (private versus public insurance; for developmental outcome analyses), using step-wise regression. Model fitting was evaluated by the accuracy of prediction, C-statistic (area under the curve—AUC; where values close to 1 represent perfect model prediction), and Akaike information criterion (AIC; where lower values represent a higher quality of the model). Data were log-transformed for statistical analyses and *p*-values < 0.05 were considered statistically significant. Statistical analysis was performed using SAS 9.4 (SAS Institute, Cary, NC).

RESULTS

A cohort of 103 singleton neonates with NE was enrolled in this study. The study population characteristics included infants with a mean birth weight of 3.25 ± 0.69 kg, mean gestational age of 38.7 ± 1.6 weeks, and were 50% male. These infants had a median 5-min Apgar of 4 with an interquartile range (IQR) of 2–5, and a median presenting pH of 6.97 (IQR 6.83, 7.12). Nineteen infants (18%) had severe encephalopathy. The median number of hours from birth to the initiation of cooling was 4.5 h (IQR 3.79, 5.43). Fifteen infants died and nineteen infants had severe injury by MRI. Characteristics of the study population are described in Table 1.

Table 1. Clinical characteristics by outcome group.

Variable	Total ($n = 103$)	Survived with normal/mild MRI ($n = 69$)	Died or severe injury by MRI ($n = 34$)
Gestational age (weeks)[a]	38.7 ± 1.6	38.7 ± 1.6	38.7 ± 1.5
Birthweight (kg)[a]	3.25 ± 0.69	3.31 ± 0.75	3.11 ± 0.55
Sex (male)[b]	52, 50%	39, 56%	13, 38%
Delivery method[b]			
Cesarean	65, 63%	41, 59%	24, 71%
Spontaneous vaginal	38, 37%	28, 41%	10, 29%
APGAR Score[c]			
1 min	1 (1, 2)	2 (1, 2)	1 (1, 2)
5 min	4 (2, 5)	4 (3, 6)	3 (2, 4)
Initial pH[c]	6.97 (6.83, 7.12)	7.02 (6.90, 7.13)	6.91 (6.80, 7.01)
Encephalopathy grade[b]			
Moderate	82, 82%	66, 96%	18, 53%
Severe	19, 18%	3, 4%	16, 47%
EEG confirmed seizures[b]	29, 28%	12, 17%	17, 50%
Age at MRI (days)[c]	5 (4, 7.5)	5 (4, 8)	4 (4, 5.5)

[a]Mean (SD).
[b]*n*, %.
[c]Median (IQR).

Sample sizes available for each assay are available in Supplemental Table 1.

Prediction of death or severe injury by MRI

Biomarker data stratified by outcome group are shown in Fig. 1. All cytokines peaked in the first 24 h of cooling in the majority of patients, 83%, 69%, and 87% for IL-6, IL-8, and IL-10, respectively. Conversely, the majority (56%) of patients had peak Tau measured during or after rewarming. Cytokine levels differed by outcome group in the first 0–12 h, while Tau differed at 12–96 h (Fig. 1). Bivariate model results are summarized in Table 2. For averaged early IL-6 measures, the best fit model included the quadratic term of IL-6 denoting a non-linear relationship (i.e., extreme low and high values are associated with adverse outcomes). Overall, biomarker measurements of Tau were most predictive of adverse outcome across timepoints and summary functions. Observed relationships were not solely driven by levels in patients who died, as levels also generally differed across survivors with and without brain injury (Supplemental Table 2).

Several multivariable models were derived considering early data alone (within 24 h) as well as cumulative data over time to assess the combinative value of biomarkers. When early measurements of IL-8, and Tau were combined, the model demonstrated improved predictive ability (AUC = 0.737, AIC = 91.92, accuracy 77.3%) when compared to early Tau alone (AUC = 0.677, AIC = 92.43, accuracy 72%). The combination of early IL6, IL8, and IL10 best-predicted death or severe brain injury by MRI (AUC = 0.826, AIC = 81.48, $n = 75$, Table 3) with an accuracy of 78.7%. The results remained consistent after adjusting for clinical covariates in the model (severe encephalopathy grade OR 25.26, 95% CI: 2.22–287.96, $p = 0.009$ and electrographic seizure OR 1.83, 95% CI: 1.83–47.38, $p = 0.007$ were selected for inclusion in the final adjusted model). In the patients who had both early and late measures available, late measures of Tau alone was a better single predictor of outcome compared to early combined cytokines (OR 4.37, 95% CI: 1.99–9.59, $p < 0.001$, AUC = 0.883, AIC = 48.91, Accuracy 82.5%, $n = 57$). The best cumulative model (AUC = 0.915, AIC 46.05, Accuracy 86%, $n = 57$, Table 3) including early and late biomarker data included early IL8 (OR 3.05, 95% CI: 1.01–9.26, $p = 0.048$) and late Tau measures (OR 3.81, 95% CI: 1.57–9.22, $p = 0.016$). After evaluating clinical covariates via stepwise regression, no clinical variables were selected for inclusion in the final model, suggesting that Tau measures were the strongest independent predictor of outcome.

Prediction of death or significant neurodevelopmental delay

A subset ($n = 48$, 54.5% of survivors) of subjects had available neurodevelopmental outcome after 1 year of age. Of these infants with neurodevelopmental outcomes, 43 had moderate HIE and 5 had severe HIE. Eleven infants met the study definition of significant developmental delay with either poor cognitive outcomes ($n = 6$) and/or poor motor outcomes ($n = 9$). The cohort lost to follow-up was similar with regards to baseline and clinical characteristics including gestational age, sex, presenting pH/ base deficit, Apgar score at 5 min, Sarnat grade of encephalopathy, and presence of electrographic seizures ($p > 0.05$). More infants lost to follow-up had public insurance compared to private insurance, but this difference was not statistically significant (57% of lost to follow-up vs 42% retained cohort; $p = 0.058$). In the cohort with follow-up data, late biomarker measurements (72 and 96 h of TH) of IL10 and Tau were consistently predictive of neurodevelopmental outcome (Table 4). Measures of GFAP and NRGN at 96 h of life were inversely related to adverse outcome (Fig. 2). Late Tau alone was the strongest predictor of neurodevelopmental outcome with an accuracy of 81% (aOR 2.59, 95% CI: 1.36–4.95, β 0.953, SE 0.330, AUC = 0.81, AIC = 45.17, $n = 42$) and the addition of clinical covariates, other biomarkers or early Tau measures did not improve prediction.

DISCUSSION

The results from this study illustrate the importance of serial measurements of multiple biomarkers to predict outcomes in infants with NE. As a stand-alone biomarker, Tau protein was consistently a predictive marker for brain injury on MRI, although later measurements of Tau at 72 and 96 h of life had the best predictive ability compared to earlier measurements within the first 24 h. Evidence for Tau protein as a putative biomarker of brain injury in NE was also observed in its ability to predict adverse neurodevelopmental outcomes when measured after 72 h. However, when considering biomarkers measured within the first 24 h of life, early measurements of cytokines IL-6, 8, and 10 in combination were most predictive of death or severe brain injury by MRI. These data support the use of a serial biomarker panel to assess brain injury over the time course of disease in NE. Cytokines have high predictive value when measured within the first day of presentation, which is helpful in making timely treatment decisions. The later predictive value of Tau is helpful in confirming the accuracy of prediction and to give appropriate counseling and rehabilitative interventions. That these relationships remained independently predictive of outcomes even in the context of common clinical indicators of severity (encephalopathy grade, Apgar score, initial pH, EEG seizures) further supports the potential for biomarkers to aid in the clinical care of neonates with NE.

Previous studies have examined the correlation of blood-based biomarker measurements and neurological outcome in newborns with NE. These studies identified important candidate biomarkers of brain injury including inflammatory cytokines, Tau, GFAP, VEGF, BDNF, and S-100B as indicators of clinical severity and adverse sequelae.[6–9,11–14] Although the relationship between biomarkers and the neurological outcome has been suggested, the temporality of these markers and their combinative value for predicting

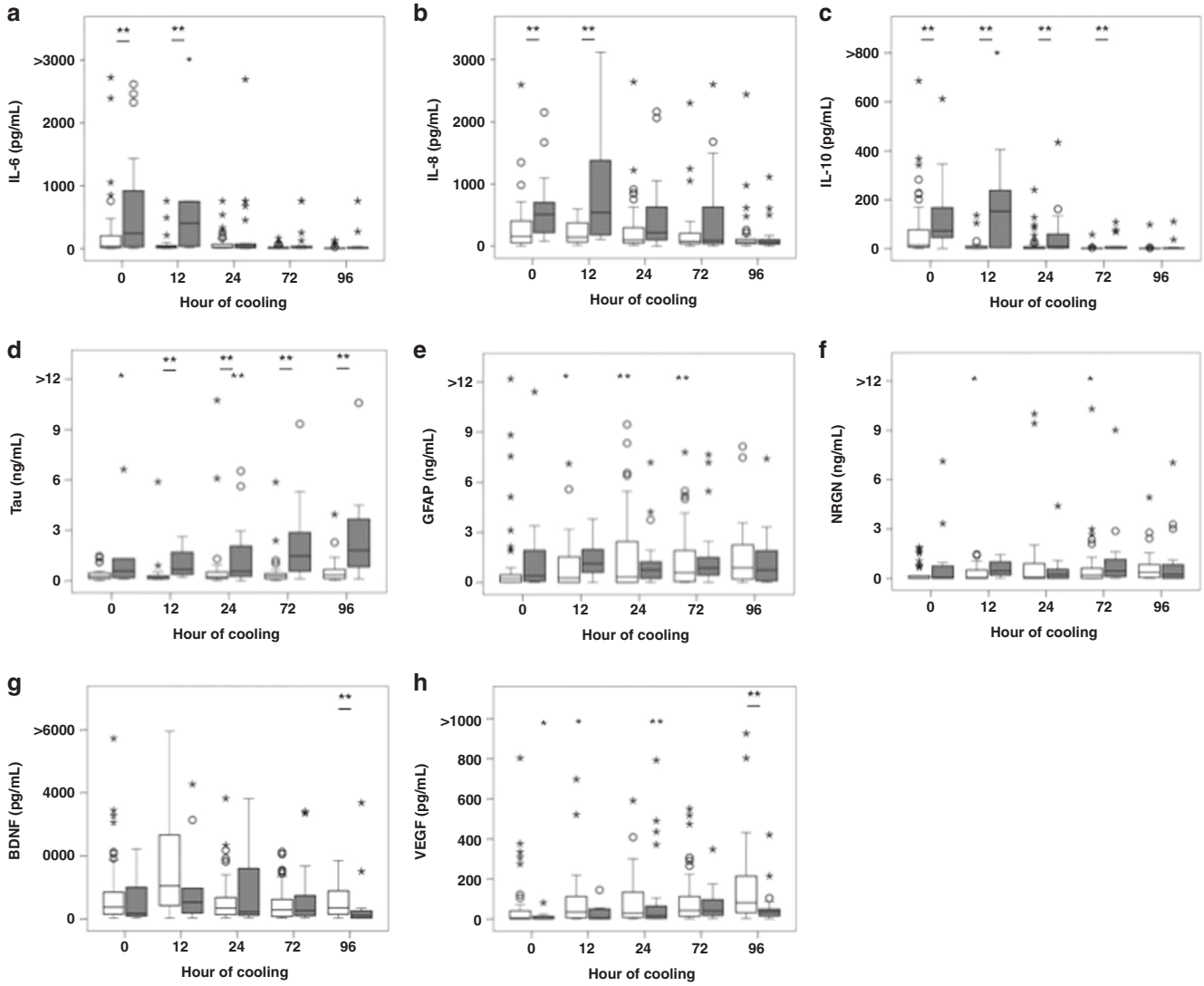

Fig. 1 Box plots depicting medians and interquartile ranges of raw biomarker data over time. Data for **a** interleukin (IL)-6, **b** IL-8, **c** IL-10, **d** Tau, **e** glial fibrillary acidic protein (GFAP), **f** neurogranin (NRGN), **g** brain-derived neurotrophic factor (BDNF), and **h** vascular endothelial growth factor (VEGF) are presented over time. Data from infants who died or had severe brain injury by MRI (gray boxes) are compared to those who survived with normal or mild injury by MRI. Outliers and extremes represented by open circles and asterisks respectively. § denotes significance ($p < 0.05$) by logistic regression analyses.

outcomes has not been well established. Most studies have measured biomarker values as single measures or within limited time-frames, such as just within the first 24 h of life.[8,9,11–14] While other studies measured values for up to 1 week after birth, few have attempted to compare alternate summary functions or evaluate combinative values across biomarkers.[7–9] Additionally, only recent studies have focused on cohorts of infants with NE that have been treated with TH, which is the current standard of care.[6,7,9,13,14]

Cytokines have been widely investigated as a surrogate of disease severity and outcome in infants with NE. In hypoxic–ischemic injury, as well as many other types of brain injury, cytokines are an important mediator of the neuroinflammatory cascade.[25] They can have pro- or anti-inflammatory properties, contributing to immune cell chemotaxis, cellular adherence, and other immune-modulating properties.[25] These cytokines usually peak around the first 12–24 h after injury, indicating the possible role of different cytokines in the initial inflammatory cascade and repair processes.[26] We previously described that higher levels of IL-6, 8, and 10 at 24 h of TH was associated with adverse neurological outcomes, whereas by 72 h most cytokine responses had dissipated and no longer distinguished outcome groups.[6] Other studies have reported comparable findings, indicating that both the measured levels and timing of peak concentrations of cytokines are important considerations in evaluating neurologic severity.[7,11] Our study is the first to include and compare serial measurements of inflammatory cytokines IL-6, 8, and 10 at several points both within the first 24 h and at 72 to 96 h after an injury to evaluate for brain injury and neurodevelopmental outcome. This comparison of early versus late measurements showed that cytokine measurements within the first 24 h have the best predictive value of subacute brain injury on MRI and supports the use of these biomarkers in initial therapeutic decisions. That the combination of cytokine measures did not predict neurodevelopmental outcomes may be related to the limited sample size entering these analyses due to loss to follow-up. Given the trends of higher early cytokine levels in the adverse outcome group (Supplemental Table 3) and reports from others relating cytokine levels to adverse neurodevelopmental outcomes in HIE,[7,12,25] the relationship between early cytokine measures and long-term functional outcomes warrants further study.

Table 2. Bivariate analysis of biomarkers by timepoint predicting death or severe brain injury on MRI.

Timepoint	Biomarker	OR	Cutoff value[a]	Cutoff value (in original scale)	AUC	*p*-value	Accuracy (%)	Sensitivity (%)	Specificity (%)	Sample size (*n*)	Event (*n*)
T0	IL6	1.84	6.631	758.24	0.683	0.021	75.76	42	89	66	19
	IL8	2.23	6.236	510.81	0.741	0.006	77.27	53	87	66	19
	IL10	1.55	3.704	40.61	0.714	0.012	72.72	79	70	66	19
T12	IL6	3.77	5.401	221.63	0.792	0.007	83.33	60	92	36	10
	IL8	3.93	6.447	630.81	0.812	0.009	86.11	50	100	36	10
	IL10	2.14	4.969	143.88	0.827	0.002	88.89	60	100	36	10
	Tau	2.87	6.291	539.69	0.818	0.024	80.65	67	82	31	9
T24	IL10	1.38	4.085	59.44	0.665	0.019	72.29	27	93	83	26
	Tau	1.74	6.862	955.28	0.742	0.010	77.42	48	93	62	21
T72	IL10	1.99	1.464	4.32	0.718	0.002	80.00	45	93	80	22
	Tau	3.67	6.314	552.25	0.864	0.001	87.04	86	88	54	14
T96	Tau	3.36	6.603	737.30	0.847	0.003	81.40	86	79	43	14
	BDNF	0.51	4.168	64.59	0.737	0.005	76.19	40	93	63	20
	VEGF	0.61	3.269	26.29	0.698	0.023	73.02	45	86	63	20
Early average	IL8	1.72	6.332	562.28	0.721	0.016	76.84	42	94	95	31
	IL10	1.46	3.656	38.71	0.667	0.007	76.84	52	89	95	31
	Tau	1.74	6.229	507.25	0.687	<0.001	75.33	58	84	77	26
Late average	IL10	1.64	1.464	4.32	0.682	0.008	74.44	36	92	90	28
	Tau	3.84	6.513	673.84	0.880	<0.001	84.29	89	83	70	22
	BDNF	0.78	3.689	40.00	0.603	0.153	70.00	25	90	90	28
	VEGF	0.85	1.845	6.33	0.563	0.309	67.78	18	90	90	28
Minimum	IL6	1.44	3.795	44.48	0.642	0.027	73.81	19	98	84	26
	IL10	1.90	0.555	1.74	0.676	0.005	78.57	50	91	84	26
	Tau	2.71	5.725	306.43	0.811	0.004	79.66	74	83	59	19
Maximum	Tau	4.68	7.514	1833.53	0.886	0.000	86.44	74	93	59	19

[a]Cutoff value selected based on highest accuracy (correct classification).

Table 3. Multivariable models for prediction of death or severe brain injury by MRI.

Variable name	Regression coefficient	Standard error	*p*-value	Odds ratio	Lower OR	Upper OR
Best early model						
Log IL6 early average	−2.65	0.740	<0.001			
Log IL6 early average	0.28	0.085	0.001			
Log IL8 early average	0.84	0.240	0.042	1.63	1.02	2.62
Log IL10 early average	0.49	0.350	0.016	2.33	1.17	4.61
Best cumulative model						
Log IL8 early average	0.91	0.460	0.048	3.05	1.01	9.26
Log Tau late average	1.43	0.452	0.016	3.81	1.57	9.22

Perhaps one of the most promising candidate biomarkers of brain injury in NE is Tau, a microtubule-associated protein found in the axons of both neurons and oligodendrocytes.[8] The initial injury caused by hypoxic conditions in the brain contribute to the phosphorylation of Tau, leading to neuronal damage and increased levels of Tau detected in the blood.[26] Thus, it has been proposed that Tau protein can serve as a marker of the extent neuronal injury and has a role in predicting outcomes in NE. A study by Takahashi et al. measured serum Tau levels in non-cooled neonates with asphyxia on postnatal days 0, 3, and 7 and found that only Tau measurements on days 3 and 7 could predict neurological outcome. Few studies have investigated Tau protein measurements in babies receiving TH. A subset of this cohort was included in a recently reported multicenter study evaluating candidate biomarkers measured in the first 24 h of life in babies with NE who received TH.[16] Plasma Tau was the only biomarker that related to MRI abnormalities and Bayley-III cognitive and motor outcomes. In another previous cohort of cooled babies receiving Epo, Tau was selected among a panel of other biomarkers as the most significant in predicting brain injury on MRI and neurodevelopmental outcome.[11] This study evaluated Tau at baseline (randomization to Epo versus placebo within 24 h of life) and day 5, however, lacked more frequent measurements of Tau between 48 and 72 h to provide more information on its trajectory over time.[11] The current study addresses this knowledge gap by investigating the role of Tau more granularly in the evolution of brain injury in NE. Interestingly, this study found that of all the biomarkers included in our analysis, Tau was the best

Table 4. Bivariate analysis of biomarkers by timepoint for prediction of death or significant neurodevelopmental delay.

Timepoint	Biomarker	OR	Cutoff value[a]	AUC	*p*-value	Accuracy (%)	Sensitivity (%)	Specificity (%)	Sample size (*n*)	Event (*n*)
T72	IL10	1.89	0.850	0.67	0.021	76.60	63	86	47	19
	Tau	3.07	7.284	0.82	0.009	86.67	70	95	30	10
T96	IL10	1.76	0.560	0.72	0.039	79.07	67	86	43	15
	Tau	2.13	6.710	0.77	0.034	77.42	89	73	31	9
	GFAP	0.64	−2.767	0.72	0.032	70.00	31	96	40	16
	NRGN	0.65	−4.140	0.71	0.039	72.50	38	96	40	16
Late average	IL10	1.74	0.660	0.68	0.026	75.47	67	81	53	21
	Tau	2.51	7.007	0.80	0.004	79.55	80	79	44	15
Minimum	Tau	1.94	7.284	0.70	0.044	72.97	31	96	37	13
Maximum	Tau	1.79	7.514	0.71	0.042	72.97	69	75	37	13

[a]Cutoff value selected based on highest accuracy (correct classification).

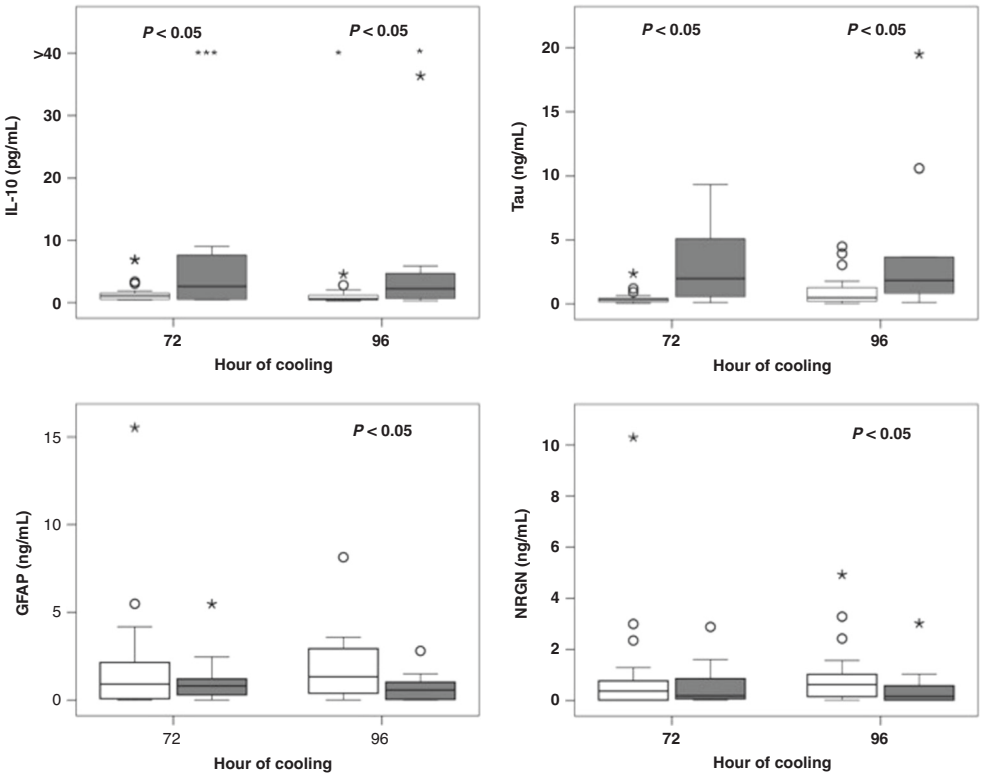

Fig. 2 Box plots depicting medians and interquartile ranges of raw biomarker data over time. Data for **a** interleukin (IL)-10, **b** Tau, **c** glial fibrillary acidic protein (GFAP), and neurogranin (NRGN) are presented at 72 and 96 hours after cooling initiation. Data from infants who died or had a significant developmental delay (gray boxes) are compared to those who survived with normal outcomes at 15–30 months. Outliers and extremes represented by open circles and asterisks respectively. § denotes significance ($p < 0.05$) by logistic regression analyses.

predictor of both MRI injury and neurodevelopmental outcome especially when the measurements were taken at 72 and 96 h after injury. This supports the previous findings that Tau is a marker of the later biological consequences of hypoxic–ischemic brain injury and that it is a strong predictor for later functional outcomes.

This study included several other candidate biomarkers that have been previously shown to correlate with disease severity and outcome in babies with NE such as NRGN, GFAP,[7,9] BDNF,[11] and VEGF.[7] While our analyses indicated limited evidence for these analytes as brain injury biomarkers, we recognize that additional studies are needed to confirm the associations (or lack of associations) observed in this cohort of babies with NE given that there are limitations to this study. First, the samples collected for the biomarker assays were salvaged from clinical samples which meant that we were unable to obtain a sample at every timepoint for each subject, given some samples did not have adequate volume remaining for analysis. Thus, the use of a convenience sample based on available plasma (with varying sample sizes by the analyte and by timepoint) precluded the calculation of an a priori determined sample size. While we included all available specimens in analyses for each individual timepoint, we only included subjects with two or more timepoints in the calculation of summary functions, thus excluding those with incomplete data

which can lead to potential selection bias. Second, variable collection and storage processes in the clinical setting (i.e., the time between collection and processing, degree of hemolysis, etc) and the introduction of freeze–thaw cycles in the storing and analyzing of samples can provide a source of technical variability. Given the goal of this work was to evaluate the individual and combinative value of the time-dependent biomarkers, we analyzed data separately for each timepoint as well as overall summary functions. Given our limited sample size and the exploratory nature of this study, we did not adjust for multiple comparisons and thus these results require confirmatory investigation. Lastly, many of our subjects were lost to follow-up, which may introduce selection bias and additionally limited our sample size for the neurodevelopmental outcome analyses.

CONCLUSIONS

Based on the results of this study, serial measurements of Tau and inflammatory cytokines show differences in their ability to predict brain injury by MRI and neurodevelopmental outcomes based on the time of measurement over the first 96 h of life. These data support the use of a serial biomarker panel in newborns with moderate to severe NE treated with TH to accurately guide intervention and counseling strategies. Tau protein may have the most utility as a stand-alone biomarker of brain injury and neurodevelopmental impairment in NE. Large-scale validation of early cytokine and serial Tau measures are needed prior to integration into the clinical setting for monitoring of disease progression and directing care in newborns with NE.

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AUTHOR CONTRIBUTIONS

Substantial contributions to conception and design (T.C., A.D.E, P.G., J.B., A.N.M.), acquisition of data (M.M.M., A.C.O., G.V., T.C., N.B., P.G., A.N.M.), or analysis (J.G., J.B.) and interpretation of data (G.V., T.C., P.G., J.B., A.D.E., A.N.M.); drafting the article (M.M.M.) or revising it critically for important intellectual content (all authors); and final approval of the version to be published (all authors).

ADDITIONAL INFORMATION

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REFERENCES

1. Shankaran, S., Woldt, E., Koepke, T., Bedard, M. P. & Nandyal, R. Acute neonatal morbidity and long-term central nervous system sequelae of perinatal asphyxia in term infants. *Early Hum. Dev.* **25**, 135–148 (1991).
2. Gluckman, P. D. et al. Selective head cooling with mild systemic hypothermia after neonatal encephalopathy: multicentre randomised trial. *Lancet Lond. Engl.* **365**, 663–670 (2005).
3. Kurinczuk, J. J., White-Koning, M. & Badawi, N. Epidemiology of neonatal encephalopathy and hypoxic-ischaemic encephalopathy. *Early Hum. Dev.* **86**, 329–338 (2010).
4. Shankaran, S. et al. Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy. *N. Engl. J. Med.* **353**, 1574–1584 (2005).
5. Wu, Y. W. et al. High-dose erythropoietin and hypothermia for hypoxic-ischemic encephalopathy: a Phase II Trial. *Pediatrics* **137**, e20160191 (2016).
6. Orrock, J. E. et al. Association of brain injury and neonatal cytokine response during therapeutic hypothermia in newborns with hypoxic-ischemic encephalopathy. *Pediatr. Res.* **79**, 742–747 (2016).
7. Chalak, L. F. et al. Biomarkers for severity of neonatal hypoxic-ischemic encephalopathy and outcomes in newborns receiving hypothermia therapy. *J. Pediatr.* **164**, 468–474.e1 (2014).
8. Takahashi, K. et al. Serum tau protein level serves as a predictive factor for neurological prognosis in neonatal asphyxia. Brain Dev. **36**, 670–675 (2014).
9. Ennen, C. S. et al. Glial fibrillary acidic protein as a biomarker for neonatal hypoxic-ischemic encephalopathy treated with whole-body cooling. *Am. J. Obstet. Gynecol.* **205**, 251.e1–7 (2011).
10. Kubota, Y., Putkey, J. A. & Waxham, M. N. Neurogranin controls the spatiotemporal pattern of postsynaptic Ca2+/CaM signaling. *Biophys. J.* **93**, 3848–3859 (2007).
11. Massaro, A. N. et al. Plasma biomarkers of brain injury in neonatal hypoxic-ischemic encephalopathy. *J. Pediatr.* **194**, 67–75.e1 (2018).
12. Ramaswamy, V. et al. Systematic review of biomarkers of brain injury in term neonatal encephalopathy. *Pediatr. Neurol.* **40**, 215–226 (2009).
13. Massaro, A. N. et al. Biomarkers of brain injury in neonatal encephalopathy treated with hypothermia. *J. Pediatr.* **161**, 434–440 (2012).
14. Massaro, A. N. et al. Biomarkers S100B and neuron-specific enolase predict outcome in hypothermia-treated encephalopathic newborns. *Pediatr. Crit. Care Med.* **15**, 615–622 (2014).
15. Sarnat, H. B. & Sarnat, M. S. Neonatal encephalopathy following fetal distress. A clinical and electroencephalographic study. *Arch. Neurol.* **33**, 696–705 (1976).
16. Dietrick, B. et al. Plasma and CSF candidate biomarkers of neonatal encephalopathy severity and neurodevelopmental outcomes. *J. Pediatr.* https://doi.org/10.1016/j.jpeds.2020.06.078 (2020).
17. Yang, J., Korley, F. K., Dai, M. & Everett, A. D. Serum neurogranin measurement as a biomarker of acute traumatic brain injury. *Clin. Biochem.* **48**, 843–848 (2015).
18. Bembea, M. M. et al. Glial fibrillary acidic protein as a brain injury biomarker in children undergoing extracorporeal membrane oxygenation. *Pediatr. Crit. Care Med.* **12**, 572–579 (2011).
19. Barkovich, A. J. et al. Prediction of neuromotor outcome in perinatal asphyxia: evaluation of MR scoring systems. *Am. J. Neuroradiol.* **19**, 143–149 (1998).
20. Michalec, D. in *Encyclopedia of Child Behavior and Development* (eds Goldstein, S. & Naglieri, J. A.) 215–215 (2011).
21. Anderson, P. J. et al. Underestimation of developmental delay by the new Bayley-III Scale. *Arch. Pediatr. Adolesc. Med.* **164**, 352–356 (2010).
22. Vohr, B. R. et al. Are outcomes of extremely preterm infants improving? Impact of Bayley assessment on outcomes. *J. Pediatr.* **161**, 222–8.e3 (2012).
23. Chalak, L. F. et al. Neurodevelopmental outcomes after hypothermia therapy in the era of Bayley-III. *J. Perinatol. Off. J. Calif. Perinat. Assoc.* **34**, 629–633 (2014).
24. Moore, T., Johnson, S., Haider, S., Hennessy, E. & Marlow, N. Relationship between test scores using the second and third editions of the Bayley Scales in extremely preterm children. *J. Pediatr.* **160**, 553–558 (2012).
25. Jenkins, D. D. et al. Serum cytokines in a clinical trial of hypothermia for neonatal hypoxic-ischemic encephalopathy. *J. Cereb. Blood Flow. Metab.* **32**, 1888–1896 (2012).
26. Wu, H. et al. SBDPs and Tau proteins for diagnosis and hypothermia therapy in neonatal hypoxic ischemic encephalopathy. *Exp. Ther. Med.* **13**, 225–229 (2017).