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BIOMARKERS S100B AND NSE PREDICT OUTCOME IN HYPOTHERMIA-TREATED ENCEPHALOPATHIC NEWBORNS

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Abstract

Objective—To evaluate if serum S100B protein and neuron specific enolase (NSE) measured during therapeutic hypothermia are predictive of neurodevelopmental outcome at 15 months in children with neonatal encephalopathy (NE).

Design—Prospective longitudinal cohort study

Setting—A level IV neonatal intensive care unit in a free-standing children's hospital.

Patients—Term newborns with moderate to severe NE referred for therapeutic hypothermia during the study period.

Interventions—Serum NSE and S100B were measured at 0, 12, 24 and 72 hrs of hypothermia.

Measurements and Main Results—Of the 83 infants were enrolled, fifteen (18%) died in the newborn period. Survivors were evaluated by the Bayley Scales of Infant Development (BSID-II) at 15 months of age. Outcomes were assessed in 49/68 (72%) survivors at a mean age of 15.2±2.7 months. Neurodevelopmental outcome was classified by BSID-II Mental (MDI) and

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Psychomotor (PDI) Developmental Index scores, reflecting cognitive and motor outcomes respectively. Four-level outcome classifications were defined a priori: normal= MDI/PDI within 1SD (>85), mild= MDI/PDI <1 SD (70–85), moderate/severe= MDI/PDI <2 SD (<70), or died. Elevated serum S100B and NSE levels measured during hypothermia were associated with increasing outcome severity after controlling for baseline and socioeconomic characteristics in ordinal regression models. Adjusted odds ratios for cognitive outcome were: S100B 2.5 (95% CI 1.3–4.8) and NSE 2.1 (1.2–3.6); for motor outcome: S100B 2.6 (1.2–5.6) and NSE 2.1 (1.2–3.6).

Conclusions—Serum S100B and NSE levels in babies with NE are associated with neurodevelopmental outcome at 15 months. These putative biomarkers of brain injury may help direct care during therapeutic hypothermia.

Keywords

encephalopathy; neuron specific enolase; S100B protein; therapeutic hypothermia; development

Neonatal encephalopathy (NE) is a major cause of death and long-term neurologic disability in children.[1, 2] Therapeutic hypothermia reduces mortality and morbidity,[3–6] and is the current standard of care for newborns presenting with encephalopathy. Although advances in neonatal neurocritical care (i.e. improved neuromonitoring and neuroimaging capabilities) have paralleled the development of effective therapies (i.e. therapeutic hypothermia), acute bedside assessment of brain-injury risk in the critically-ill newborn remains problematic. Biomarkers of brain injury measurable from accessible biological fluids are needed to identify appropriate candidates for therapy, direct clinical (and investigational) interventions, and offer means for prognostication in babies with NE. No serum biomarker is in current clinical use for this high-risk population.

We recently reported data evaluating two candidate biomarkers, neuron specific enolase (NSE) and S100B protein as markers of neuronal and astrocytic injury respectively, in a cohort of encephalopathic newborns treated with systemic hypothermia.[7] S100B is a neurotrophic calcium binding protein concentrated in astroglial cells of the central nervous system.[8, 9] Neuron specific enolase is a glycolytic enzyme concentrated in the cytoplasm of neurons.[10] These brain specific proteins demonstrated promising predictive ability to distinguish newborns with evidence of severe brain injury based on MRI and clinical assessment in the newborn period. The current study describes the secondary longitudinal follow-up of this ongoing cohort study, and was undertaken to determine whether these brain specific proteins are predictive of later neurodevelopmental outcome.

We hypothesized that increased serum NSE and S100B levels measured during hypothermia treatment in the newborn period would be associated with adverse neurodevelopmental outcome at 15 months in children with NE. We aimed to determine if these candidate biomarkers of brain injury have the potential to guide early risk stratification for future neurotherapeutic trials and, ultimately, to direct clinical care in affected newborns.

METHODS

Study Population

All patients referred for therapeutic hypothermia to a neonatal intensive care unit in a free-standing children's hospital between May 2008 and March 2011 were approached for enrollment in this prospective longitudinal study. Encephalopathic newborns are treated with whole-body hypothermia according to the NICHD Neonatal Research Network protocol,[3] based on established criteria (i.e. infants were greater than 36 weeks gestational age, greater than 1800 grams at birth, demonstrated metabolic acidosis by cord/first hour pH <7 OR base deficit >16, and/or low Apgar scores, and exhibited signs of moderate to severe clinical encephalopathy). Infants were excluded if they were small for gestational age or had a known or suspected chromosomal abnormality. Parents of eligible participants provided initial verbal agreement with this minimal risk study in-person or via telephone, followed by written informed consent for all patients continuing in the longitudinal aspect of the study. The study was approved by the Institutional Review Board and a Waiver of Documentation of Informed Consent was obtained to allow for the initial verbal consent used at enrollment. All data were collected in compliance with Health Information Portability and Accountability Act regulations.

Data Collection

Clinical and demographic data were collected from birth hospital and study site medical records. Presenting characteristics were noted including umbilical cord or first-hour of life blood gas, Apgar scores and presence of seizures. Initial clinical grade of encephalopathy was classified according to modified Sarnat criteria used in the NICHD trial.[3, 11] Socioeconomic status (SES) was classified by eligibility for medical assistance and maternal education level.

S100B and Neuron Specific Enolase Determinations

Additional one milliliter blood specimens were collected at 0 (on admission), 12, 24, and 72 hours of cooling coinciding with routine clinical laboratory monitoring. After remaining at room temperature for 30 minutes, samples were refrigerated and further processed within 24 hours (centrifuged at 500 rpms for 10 minutes and supernatants stored at -70 °C). If parents could not be initially reached for consent, stored serum from specimens obtained for clinical purposes were used. Salvaged clinical specimens, which are routinely frozen and retained up to one week after collection in the clinical laboratory, were collected and stored at -70 °C after consent was obtained. S100B and NSE levels were determined by enzyme-linked immunosorbent assay (ELISA) via commercially available kits according to the manufacturer's instructions (International Point of Care Inc., Toronto, Canada). The coefficient of variation for inter and intra-assay variability is <10% for both measurements. Assays were performed in duplicate and results were averaged for analysis.

Neurodevelopmental Assessment

Neurodevelopmental outcomes are assessed with the Bayley Scales of Infant Development-Second Edition (BSID-II)[12] at 15, 21 and 30 months of age in infants enrolled in this

prospective study. These time points were selected to coincide with the end of key developmental periods in infancy. This report describes data from the first assessment at 15 months of age. The BSID-II is a standardized assessment that includes a Mental Developmental Index (MDI) that assesses the child's level of cognitive, language and personal-social skills, as well as a Psychomotor Developmental Index (PDI) that evaluates fine and gross motor development. BSID-II scores of 100 ± 15 represent the mean $\pm$ 1sd. Although the third edition of the Bayley Scales (BSID-III) became available in 2006,[13] we did not transition to the BSID-III until 2011, after enrollment of the current cohort. BSID-II was performed in order to have comparable data to previously evaluated babies with NE [3–5]. Evaluations were performed by an experienced developmental psychologist who was blinded to the clinical history and biomarker data of the child. Additionally, presence of a cerebral palsy diagnosis made by a child neurologist and sensory (hearing and vision) deficits were collected from parental report.

Statistical analysis

Descriptive statistics included standard measures of central tendency and variability for continuous data and frequencies for categorical variables. Analyses were performed to evaluate the association of biomarker with a four-level outcome classification defined as: 1) Normal= MDI or PDI within 1sd (>85), 2) Mild/moderate impairment= MDI or PDI >1 sd ($70-85$), 3) Severe impairment= MDI or PDI >2 sd (<70), or 4) Died. Ordinal regression models were used to evaluate the relationship between outcome category and biomarker levels over time, including interactions between time and biomarker levels. This approach enabled the inclusion of all data from each subject, even if a measurement at a given timepoint was missing. These analyses controlled for differences across groups in baseline birthweight, gestational age, race, gender, and SES. Results were also expressed as standardized odds ratios expressing the increase in odds of a higher ordinal outcome level associated with a one standard deviation change in biomarker level. This approach enabled us to account for differences in metrics between S100B and NSE when assessing comparative effects. Expanded models were then developed via a backwards step-wise approach to evaluate potential clinical covariables including presenting pH, base deficit, grade of encephalopathy (moderate vs. severe), Apgar score at 5 minutes, hour of life on cooling. Variables were included in the final expanded model if their presence altered (by at least 10%) the magnitude of the association between biomarker and outcome. Receiver operating curve (ROC) analyses were secondarily developed to evaluate the sensitivity and specificity of alternative cut-off values at each timepoint to predict the most severe outcome categories (BSID-II MDI/PDI <70 or Death). ROC area under the curve (AUC) is presented as the parameter estimate where values range from 0.5 (no better than chance) to 1 (perfect discrimination of outcome).

Sample Size and Power

We planned to recruit 90 subjects over a 3-year period. Accounting for parental refusal and attrition, a priori power calculations were based on analysis of data from 80 subjects. This sample size would provide 80% statistical power to detect an odds ratio of 3.2, considered to be a moderate effect size. Statistical analyses were performed with STATA 11.0 software. [14]

RESULTS

Of the 92 infants referred for therapeutic hypothermia during the study period, three were excluded due to being small for gestational age (n=1), having a congenital malformation (n=1), and failing to meet encephalopathy criteria by examination (n=1). Of the 89 eligible subjects, 83 (93%) were enrolled. Six patients were not enrolled due to parental refusal (n=4) or inability to reach parents for consent (n=2). Demographic and clinical characteristics of the study population are presented in Table 1. Fifteen of the 83 infants enrolled (18%) died. Of these, 14/15 (93%) of deaths were secondary to familial withdrawal of care due to poor neurological prognosis (based on persisting severe encephalopathy by clinical exam[15] that could not be attributable to sedation/anticonvulsants and EEG[16–19]/neuroimaging[20, 21] (CT/MRI) evidence of brain injury consistent with profound hypoxic-ischemic encephalopathy). Infants who died had more profound metabolic acidosis at presentation, higher frequency of severe encephalopathy and higher incidence of electrographic seizures compared to surviving infants. Outcomes were assessed in 49/68 (72%) of surviving children. Baseline and clinical characteristics of the infants who were lost to follow-up were similar to those infants who were assessed at 15 months except for indices of socioeconomic status. Participants who were lost to follow-up were more likely to be recipients of medical assistance and less likely to have mothers who received higher-level education. Of the infants with available follow-up data, 9 were diagnosed with cerebral palsy, 2 had hearing deficits requiring aids, and 6 had cortical visual impairment.

Serial blood specimens were collected at all timepoints in 64/83 patients. In 7 patients, informed consent was received after the T0 timepoint and stored clinical specimens were inadequate for assay. All of these patients had the remaining timepoints collected. Four patients did not have a T12 hour specimen due to collection error. Eight patients died prior to the completion of rewarming and therefore did not have specimens collected at either 72 hours (n=7) or at both 24 and 72 hours (n=1). S100B and NSE levels over time differed by outcome category for both motor and cognitive outcomes (Figure 1). The association between time-dependent S100B and NSE levels and neurodevelopmental outcome remained statistically significant after controlling for baseline characteristics, with comparable standardized odds ratios for S100B (OR= 2.5–2.6) and NSE (OR= 2). These associations remained statistically significant, although NSE achieved borderline significance, after the inclusion of expanded covariates that met inclusion criteria for each model (for cognitive outcomes selected covariates included encephalopathy grade and hour of life on cooling; for motor outcomes covariates included encephalopathy grade and Apgar score at 5 minutes). Results of the multiple regression models are summarized in Table 2. Apart from S100B and NSE levels, encephalopathy grade at presentation was the only other factor that was statistically significantly associated with outcome category across all models (OR 15–90, $p<0.01$).

ROC analyses were performed to identify optimal cut-points for prediction of death or BSID-II MDI or PDI <70 (Figure 2). ROC AUC was statistically significantly greater than 0.5 for both S100B and NSE at each timepoint. Cut-points selected from the coordinates of the ROC curve and their associated sensitivities and specificities are presented in Table 3. Cut-points were selected to optimize overall predictive ability (i.e. % correctly classified).

Additionally, alternative S100B cut-points were selected from the ROCs at each timepoint that provided optimal specificity, given certainty of poor prognosis (i.e. few false positives) is important for directing care and consideration of experimental therapies.

DISCUSSION

Biomarker identification and validation is an important adjunct to advancing neonatal neuroprotection. Incorporation of biomarkers can optimize future clinical investigations by providing better means to identify the most appropriate candidates for treatment and provide surrogate outcome measures in order to prioritize resources towards long-term evaluation of only the most promising interventions. Ultimately, validated biomarkers can be used in clinical care to gauge treatment efficacy and guide therapeutic decision-making. The current study supports consideration of S100B and NSE as leading biomarkers warranting further large-scale validation.

Current methods to assess brain injury risk in the newborn have inherent limitations. Although classification of moderate versus severe encephalopathy grade remained a significant predictor in the current study, it provides less granular classification of outcome than a biomarker that is measured across a continuous range of values and can therefore provide a more nuanced assessment of outcome risk. Additionally, clinical exam is often confounded by neuroactive medications and has been reported to be less reliable early in the setting of hypothermia.[22] The predictive ability of brain MRI appears to be unaffected by hypothermia treatment,[20, 21] however it has limited predictive value in the first 24 hours of life[23, 24] when therapeutic decisions are often made. Conventional[16, 17] and amplitude integrated[18, 19] electroencephalography also provide useful predictive information, but, as with MRI, they require specialized equipment and interpretive expertise. Thus, easily measurable and interpretable serum biomarkers, once validated, are an attractive means to improving bedside diagnostics in newborns at risk for brain injury.

Brain-specific proteins, in particular, represent a class of neurobiological markers that can reflect the severity and progression of brain injury regardless of insult etiology.^{8, 9, 10} Both NSE and S100B proteins are released into the periphery in the setting of cell death and have been evaluated in other neurological diseases,[25, 26] including small cohorts of encephalopathic newborns in the pre-cooling era.[27–30] Other brain-specific proteins, including ubiquitin carboxyl-terminal hydrolase L1 (UCHL1),[31, 32] glial fibrillary acidic protein (GFAP),[32, 33] phosphorylated neurofilament H (pNF-H),[31] and Activin A[34] have also been proposed and investigated in small series of babies with NE. However, this is the first study to establish the association of brain-specific proteins with later neurodevelopmental outcome in babies with NE treated with hypothermia. This work corroborates our prior findings relating these biomarkers to structural injury by MRI[7]. Further work is needed to establish whether a combination of these and/or other biomarkers will provide improved predictive ability over any single biomarker in isolation, particularly since the limited predictive ability of NSE over clinical covariables makes it unlikely to stand alone as a viable biomarker. It is possible that future intervention paradigms will be directed by a neurodiagnostic panel of biomarkers that can be used to tailor therapies to an individual's biological profile and ongoing response (or non-response) to treatment.

Limitations of the current study should be acknowledged. As with many longitudinal studies, attrition due to loss to follow-up was a concern. While it is reassuring that infants who were lost to follow-up were similar to those retained with regards to baseline and clinical characteristics, that they may have represented a higher SES risk-profile remains a possible source of bias. Our future studies will aim to address this attrition by ideally providing more financial support to enable families of lower SES to return for follow-up. Sample size limitations necessitated the use of the stepwise approach to explore potential covariates during regression model building. That some patients did not have specimens for determination at all timepoints is also acknowledged, although the use of the ordinal regression approach enabled us to evaluate biomarker data over time from each subject as long as 2 or more serial measurements were available. These results require large-scale validation before the added utility of S100B and NSE determinations over clinical covariables can be established. Although we could not evaluate a non-encephalopathic control group for comparison, it is notable that serum levels in our study population were higher than normative values previously reported for healthy newborns (normal range for S100B 0.68 ± 0.29 ng/mL [35] and NSE 21 ± 5.3 [30]). By design, normal and/or mild babies were not included in this study as our aim was to evaluate the ability of the biomarkers to differentiate outcome amongst at-risk patients (i.e. all babies undergoing standard therapy with hypothermia). Technical confounders should also be considered, such as time to processing, effect of hemolysis, and effects of temperature on assay results as they may affect reproducibility and reliability. [36] Correction for the effects of hemolysis on neuron specific enolase measurements have been reported, [37] however these require quantification of hemolysis which was not performed in this study. Integrity of the blood brain barrier and other factors that control release of proteins into the periphery in the setting of brain injury may be another source of variability. Finally, extra-neural sources of S100B (including muscle, kidney, heart and adipose tissue) [38] and NSE (platelets, hemolysis) [39] have been reported and could affect reliability of results in infants with multi-organ systemic disease.

An important aspect of this study was selection of the primary outcome of interest, defined as a four-level outcome classification in order to provide clinically meaningful inferences. This also enabled inclusion of infants who died in the neonatal period in addition to survivors assessed at 15 months. Exclusion of this group would have eliminated an important cluster of patients with severe outcomes, since the vast majority of deaths resulted from withdrawal of care and rates of withdrawal are variable amongst different institutions, cultures, and families. Should these infants have survived it is presumed that assessment would have revealed severe disability, justifying their inclusion as the most severe outcome category in these analyses. Larger validation studies, when performed, will need to evaluate the relationship of biomarkers and outcome independent of mortality. Also of note, this study used the BSID-II to assess developmental outcomes, as this was the tool used clinically in our follow-up program during the study period. Although the third edition of the Bayley Scales (BSID-III) became available in 2006, [13] we continued to use the BSID-II in order to have comparable data to previously evaluated babies with NE, [3–5] and because of concerns that the BSID-III has been reported to overestimate developmental progress. [40, 41] Future studies will need to confirm if these relationships are demonstrated when the BSID-III is used for routine developmental assessment. Additionally, whether biomarker

levels are associated with later outcome is an important future aim of this ongoing cohort study, as it is acknowledged that the stability of the BSID-II in early infancy is limited.

CONCLUSIONS

Increased levels of serum S100B and NSE measured in encephalopathic newborns during hypothermia treatment were associated with adverse neurodevelopmental outcomes at 15 months. These biomarkers warrant further study for large-scale validation to assess their role in identifying appropriate candidates for therapy, gauging treatment response and offering prognosis for later outcome after injury and intervention.

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Figure 1.

Biomarker levels (S100B upper, NSE lower graphs) are presented by outcome category for both cognitive (left) and motor (right) domains. Boxplots represent medians and interquartile ranges. Whiskers represent range with outliers depicted by circles. Adjusted p-values are shown representing significant differences in biomarker levels over time across outcome category groups.

Figure 2. Receiver operating curves for (A) S100B and (B) NSE and prediction of death or BSID-II <70. Each time point has a representative curve (legend). TPR= true positive rate. FPR= false positive rate.

Table 1

Demographic and Clinical Characteristics of the Study Population

	Total (n=83)	Died (n=15)	Survivors With Outcome (n=49)	Lost To Follow-up (n=19)
Birthweight (mean±SD Kilograms)	3.39±0.64	3.49±0.58	3.40±0.67	3.35±0.37
Gestational Age (mean±SD weeks)	38.7±1.8	38.6±1.9	38.8±1.8	38.9±1.3
Gender (n, %male)	39 (47)	8 (53)	31 (63)	10 (53)
Race (n, %)				
- Black	41 (49)	5 (33)	23 (47)	13 (68)
- White	39 (47)	9 (60)	24 (49)	6 (32)
- Other	3 (4)	1 (7)	2 (4)	0
Presenting pH	6.92 (6.44–7.35)	6.78 (6.5–7.15)*	6.94 (6.5–7.35)	6.99 (6.44–7.35)
Presenting BD	18.9 (9–36)	23 (12–33)*	18 (9–36)	16.2 (8–26)
Encephalopathy Grade (n,%)				
- Moderate	61 (73)	2 (13)	41 (84)	18 (95)
- Severe	22 (27)	13 (87)*	8 (16)	1 (5)
Apgar				
- 5 minutes	3 (0–7)	2 (0–6)	3 (0–7)	4 (1–7)
- 10 minutes	5 (0–9)	3 (0–7) ^a	5 (0–9) ^b	6 (3–8) ^c
Age at Hypothermia Start (hours:minutes)	4:47 (1:35–6:06)	4:07 (3:34–6:06)	4:46 (1:35–6:04)	4:50 (2:58–5:57)
EEG Seizure (n, %)	30 (36)	10 (67)*	16 (33)	4 (21)
SES (n, %)				
- Medical assistance	17 (20)	2 (13)	7 (14)	8 (42)**
- Maternal education				
• Grade school	19 (23)	7 (47)	8 (16)	4 (21)
• High school graduate	34 (41)	6 (40)	16 (33)	12 (63)
• Higher level education	30 (36)	2 (13)	25 (51)	3 (16)**

Data presented as median (range) unless otherwise noted.

* Significantly differs from surviving infants (p<0.05)

** Significantly differs from survivors with outcome group (p<0.05)

10 minute Apgar data available in

^a 14/15,^b 40/49, and^c 17/19 subjects

Table 2

Summary of Multiple Regression Analyses

	Outcome	Odds Ratio	95% CI	P value
S100B	Cognitive			
	• Basic ^a	3.5	1.4 – 8.5	0.007
	• Standardized ^b	2.5	1.3 – 4.8	0.007
	• Expanded ^c	3.0	1.3 – 6.6	0.008
	Motor			
	• Basic	3.7	1.3 – 10.4	0.012
	• Standardized	2.6	1.2 – 5.6	0.012
	• Expanded ^d	3.7	1.3 – 10.5	0.013
NSE	Cognitive			
	• Basic	1.0	1.0 – 1.0	0.010
	• Standardized	2.1	1.2 – 3.6	0.010
	• Expanded ^c	1.0	1.0 – 1.0	0.044
	Motor			
	• Basic	1.0	1.0 – 1.0	0.010
	• Standardized	2.1	1.2 – 3.6	0.010
	• Expanded ^d	1.0	1.0 – 1.1	<0.001

^a Model covariates: time of measurement, birth weight, gender, race, need for medical assistance, maternal education level^b Standardized per unit standard deviation change in biomarker level^c Basic model plus selected covariates (encephalopathy grade, hour of life on cooling)^d Basic model plus selected covariates (encephalopathy grade, Apgar score at 5 minutes)

Table 3

Predictive Abilities of Selected Cut-points

	Timepoint	Cutpoint	Sensitivity	Specificity	LR+ ^b	AUC ^c
NSE	0	80	63	74	2.4	0.684
	12	73	85	68	2.8	0.825
	24	73	65	72	2.3	0.714
	72	53	67	71	2.3	0.771
S100B	0	0.6 1 ^a	67 52	69 81	2.1 2.8	0.681
	12	0.4 0.8 ^a	73 59	75 89	2.5 5.2	0.788
	24	0.3 0.8 ^a	65 49	66 98	1.9 21.0	0.715
	72	0.2 0.3 ^a	68 53	69 95	2.2 11.0	0.732

^a Alternative cutpoints for optimal specificity^b Likelihood ratio positive^c AUC= Area under the receiver operating curve