Biomarkers for Severity of Neonatal Hypoxic-Ischemic Encephalopathy and Outcomes in Newborns Receiving Hypothermia Therapy

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Objective To evaluate serum neuronal and inflammatory biomarkers to determine whether measurements of umbilical cords at birth can stratify severity of hypoxic-ischemic encephalopathy (HIE), whether serial measurements differ with hypothermia-rewarming, and whether biomarkers correlate with neurological outcomes.

Study design This is a prospective cohort of inborn term newborns with varying degrees of HIE by neurological assessment. Neuronal glial fibrillary acidic protein (GFAP), ubiquitin carboxyl-terminal hydrolase L1, and inflammatory cytokines were measured in serum from umbilical artery at 6-24, 48, 72, and 78 hours of age. Neurodevelopmental outcomes (Bayley Scales of Infant and Toddler Development-III scales) were performed at 15-18 months.

Results Twenty neonates had moderate (n = 17) or severe (n = 3) HIE and received hypothermia; 7 had mild HIE and were not cooled. At birth, serum GFAP and ubiquitin carboxyl-terminal hydrolase L1 increased with the severity of HIE ($P < .001$), and serial GFAP remained elevated in neonates with moderate to severe HIE. Interleukin (IL)-6, IL-8, and vascular endothelial growth factor were greater at 6-24 hours in moderate to severe vs mild HIE ($P < .05$). The serial values were unaffected by hypothermia-rewarming. Elevated GFAP, IL-1, IL-6, IL-8, tumor necrosis factor, interferon, and vascular endothelial growth factor at 6-24 hours were associated with abnormal neurological outcomes.

Conclusions The severity of the hypoxic-ischemic injury can be stratified at birth because elevated neuronal biomarkers in cord serum correlated with severity of HIE and outcomes. *(J Pediatr 2014;164:468-74)*.

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Hypoxic-ischemic encephalopathy (HIE) is a complex disease process in which injury severity, duration, and timing of the antenatal injury are difficult to discern. This knowledge gap results in hypothermia being delivered to all newborns with moderate or severe encephalopathy in an identical fashion. Although whole body hypothermia therapy has improved outcomes,[1,2] 40% of neonates with HIE have neurological disability at 18-24 months of age despite therapy. This lack of apparent efficacy suggests that neonates treated with hypothermia therapy are a diverse group who need to be further stratified. At present, there are no specific, easily measured serum biomarkers that identify the extent of neurological injury at birth or thereafter.[2,3] The availability of markers of neuronal injury that correlate with disease severity and are predictive of neurodevelopmental disability in childhood would likely facilitate a more targeted therapeutic approach using adjunctive therapies.

The rewarming phase of whole body hypothermia in patients beyond the newborn period has been associated with a secondary reperfusion phenomenon in multiple organs and tissues, resulting in the release of circulating inflammatory mediators that may contribute to additional pathology.[4-8] The rewarming process and the potential surge of inflammatory biomarkers during reperfusion also could result in additional injury remote from the primary insult but have not been evaluated in the newborn.[9]

Due to the complex multifaceted nature of HIE, we selected a combination of serum biomarkers that have been shown to be mechanistically involved in the energy-depleting, free radical, excitotoxic, and inflammatory cascade that results in brain injury. Glial fibrillary acidic protein (GFAP) is a key cytoskeleton intermediate filament protein that is specific to astrocytes. Ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) is a neuron-specific cytoplasmic enzyme that is concentrated in dendrites.[10,11] Both GFAP and UCH-L1 have been used as markers of neuronal apoptosis. We also examined an array of circulating proinflammatory cytokines and growth factors that are mechanistically involved in the pathogenesis of brain injury.[12-14]

BSID-III	Bayley Scales of Infant and Toddler Development-III
GFAP	Glial fibrillary acidic protein
HIE	Hypoxic-ischemic encephalopathy
IL	Interleukin
MRI	Magnetic resonance imaging
NICHD	*Eunice Kennedy Shriver* National Institute of Child Health and Human Development
ROC	Receiver-operating characteristic
UCH-L1	Ubiquitin carboxyl-terminal hydrolase L1
VEGF	Vascular endothelial growth factor

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L.C. is supported by the National Institutes of Health (K23HD069521) and the Gerber Foundation. The authors declare no conflicts of interest.

 http://dx.doi.org/10.1016/j.jpeds.2013.10.067

We addressed 3 objectives: (1) to measure circulating neuronal and inflammatory biomarkers in umbilical cord serum at birth in order to evaluate the severity of antenatal injury in infants with HIE; (2) to assess whether a surge in inflammatory mediators or neuronal biomarkers occurs during the rewarming phase; and (3) to determine if levels of these potential biomarkers soon after birth correlate with abnormal neurodevelopmental outcomes at 15-18 months of age.

Methods

This prospective cohort pilot study included all inborn infants ≥36 weeks of gestation and birth weight ≥1800 g who were admitted to the neonatal intensive care unit at Parkland Memorial Hospital, Dallas, TX, from June 2010 to June 2011 and had perinatal asphyxia with metabolic acidosis. Exclusion criteria included the presence of congenital anomalies or if comfort care was planned. The study was approved by the Institutional Review Board of the University of Texas Southwestern Medical Center. Written informed consent was obtained from each mother after delivery.

Perinatal acidemia was determined by measuring blood gases in umbilical arterial cord plasma that is evaluated routinely on all deliveries from a double-clamped section of umbilical cord.[15] Criteria for an infant to qualify for a detailed neurological examination were as described in the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD) Neonatal Research Network study of whole body hypothermia[1]: (1) pH ≤7.00 or base deficit ≥16 mEq/L in umbilical arterial cord plasma or (2) history of an acute perinatal event and either no arterial blood gas available or a pH from cord arterial serum ranging from 7.01 to 7.15 or a base deficit from 10 to 15.9 mEq/L, in addition to a 10-minute Apgar score ≤5 or assisted ventilation initiated at birth. To establish the diagnosis and severity of encephalopathy, a neurological examination was performed by 1 of 2 certified examiners (L.C., P.S.) within 6 hours of birth according to the NICHD classification for modified Sarnat staging.[16] The hypothermia group was composed of newborns with a composite exam (at least 3 of 6 abnormal categories) consistent with a diagnosis of moderate or severe encephalopathy who received hypothermia therapy. The mild encephalopathy group was composed of newborns with 1-2 abnormal categories in the neurologic assessment but who did not meet criteria for hypothermia and received supportive care that included an indwelling umbilical catheter. This group of newborns was used in the time-series analyses for comparisons with the hypothermia group.

Whole body hypothermia was started within 6 hours after birth by placing the newborn on a cooling blanket (Blanketrol II; Cincinnati Sub-Zero, Cincinnati, Ohio) and maintaining the esophageal temperature at 33.5°C with the blanket servomechanism for 72 hours.[1]

Then, 1 mL of blood was collected from each newborn at birth (umbilical arterial serum), and serially. Time zero was representative of initiation of hypothermia: sample A (6-24 hours), sample B (48-72 hours), sample C (immediately at the end of rewarming and on achieving normothermia at 78 hours), and sample D (6-12 hours after completion of rewarming: 84-90 hours). Testing of umbilical cord arterial plasma that had been refrigerated and stored in the Transfusion Services Department was performed with informed consent. We used serial samples from the lower aorta via an umbilical arterial catheter to reflect changes during hypothermia (samples A and B) and rewarming (samples C and D). Neonates with mild HIE had serial sampling after 24 hours of age only while indwelling arterial catheters were in place. Samples were centrifuged at 2700 rpm for 10 minutes, aliquoted to subsamples, and stored at −80°C until time of immunoassay. Samples underwent enzyme-linked immunosorbent assay for interleukin (IL)-1, IL-6, IL-8, vascular endothelial growth factor (VEGF), tumor necrosis factor-α, interferon-γ, Regulated on activation, normal T cell expressed and secreted (Bio-Plex Pro Human Cytokine, Bio-Rad, Hercules, California), UCH-L1 (Banyan Biomarkers, Gainesville, Florida), and GFAP (Banyan Biomarkers). The lower level of detection for the GFAP assay was 0.03 ng/mL. All samples were assayed in duplicate with the average recorded. The coefficient of variation for intra- and inter-assay precision was <10%.

Magnetic resonance imaging (MRI) was performed from 7 to 14 days of age. Conventional T1 and T2 images were assessed using the NICHD MRI classification[17] by a pediatric neuroradiologist who had no knowledge of the outcomes.

All infants who received hypothermia were seen after hospital discharge at Children's Medical Center Dallas at 15-18 months of age for neurodevelopmental assessment using the Bayley Scales of Infant and Toddler Development-III (BSID-III). An abnormal neurological outcome was defined a priori by BSID-III developmental scores <85 on any of the 3 cognitive, language, or motor domains or the presence of deafness, blindness, or cerebral palsy.[18]

Statistical Analyses

Statistical analysis was performed using Sigma Plot 11.0 (Systat Software, San Jose, California) and SAS 9.2 (SAS Institute, Cary, North Carolina). The results are reported in a tabular format as medians, 25th-75th quartiles, mean ± SD, or number and percentage. The Wilcoxon rank sum test was used to compare newborns with moderate to severe HIE who received hypothermia to newborns with mild HIE and no hypothermia treatment. Trends across mild, moderate, and severe HIE newborns were assessed with use of the Jonckheere-Terpstra test. After log transformation, serial serum biomarkers were compared using mixed-model repeated-measures analysis. Serial cytokine data were plotted and analyzed using geometric means ($\log_{10}$). Data for samples (C and D) collected 6-12 hours apart during rewarming were comparable so the average values are summarized in **Table I** (available at www.jpeds.com) to represent the rewarming interval. The predictive values for development of abnormal neurological outcome were calculated using sensitivity, specificity, and positive and negative predictive values. Receiver-operator characteristic