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# Umbilical cord blood biomarkers of neurologic injury and the risk of cerebral palsy or infant death☆

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ABSTRACT

To evaluate the association between cerebral palsy (CP) or infant death and putative cord blood biomarkers of neurologic injury, we performed a nested case–control secondary analysis of a multicenter randomized trial of magnesium sulfate ($MgSO_4$) versus placebo to prevent CP or death among offspring of women with anticipated delivery from 24 to 31 weeks' gestation. Cases were infants who died by 1 year ($n = 25$) or developed CP ($n = 16$), and were matched 1:2 to a control group ($n = 82$) that survived without developing CP. Umbilical cord sera concentrations of S100B, neuron-specific enolase (NSE) and the total soluble form of the receptor for advanced glycation end-products (sRAGE) were measured by ELISA in duplicates. Maternal characteristics were similar between the 2 groups. Cases were born at a lower gestational age (GA) and had lower birth weight compared with controls. There were no differences in concentrations of the three biomarkers and the composite outcome of CP or infant death. However, S100B was higher (median 847.3 vs. 495.7 pg/ml; $P = 0.03$) in infants who had CP and total sRAGE was lower (median 1259.3 vs. 1813.1 pg/ml; $P = 0.02$) in those who died compared with the control group. When corrected for delivery GA and treatment group, both differences lost statistical significance. In conclusion, cord blood S100B level may be associated with CP, but this association was not significant after controlling for GA and $MgSO_4$ treatment.

*Abbreviations:* CP, cerebral palsy; $MgSO_4$, magnesium sulfate; NSE, neuron-specific enolase; sRAGE, soluble form of the receptor for advanced glycation end-products; ELISA, enzyme-linked immunosorbent assay; GA, gestational age; pPROM, preterm premature rupture of membranes; HIE, hypoxic ischemic encephalopathy; DAMP, damage-associated molecular pattern molecules.

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## 1. Introduction

Cerebral palsy (CP) is characterized by aberrant control of movement or posture appearing early in life, and not the result of recognized progressive disease (ACOG & AAP, 2003). Currently, it is estimated that the prevalence of CP is 1.5–2.5 per 1000 live births (Paneth et al., 2006). These numbers have been stable despite the advances in neonatal and perinatal care which resulted in reductions in mortality of extremely premature infants (Paneth et al., 2006; Winter et al., 2002). The leading risk factor for CP as well as perinatal and infant death is prematurity with both CP and death rates inversely proportional to the gestational age (GA) at birth (Saigal and Doyle, 2008).

A definite etiology for CP is never found for the majority of cases. It has been hypothesized that CP is the result of neuronal injury or insult to the developing brain secondary to inflammatory, hypoxic, excitatory, or oxidative injury; and that the timing of that insult is in the prenatal or perinatal period in about 70% of the cases in infants born preterm and 85% in those born at term (Hagberg et al., 2001). Although CP is generally not diagnosed until the second year of life or later, it is possible that biomarkers present at birth could predict the risk of its development (or non-development). If such prediction were possible, neonates who are at highest risk and who would benefit most from any neuroprotective therapies might be identified (Perlman, 2006).

Some of these potential markers are brain-specific and are elevated in the serum after brain injury. Examples include the calcium binding protein S100B and neuron-specific enolase (NSE) (Marchi et al., 2004; Nagdyman et al., 2001; Nguyen et al., 2006; Park et al., 2004; Ramaswamy et al., 2009; Thorngren-Jerneck et al., 2004). Others, such as the advanced glycation endproducts (AGEs) or their receptor (RAGE), are associated with the activation of the fetal inflammatory processes that potentiate tissue injury (Buhimschi et al., 2009; Ramaswamy et al., 2009). Direct inflammatory mediators such as the various interleukins, TNF-α, and others are also involved (Buhimschi et al., 2009; Kaukola et al., 2004; Ramaswamy et al., 2009). The biology of the RAGE pathway is complicated and dependent on its ligands as well as on a soluble truncated form of the receptor (sRAGE) which acts as a decoy receptor and an inhibitor of the native receptor (Buhimschi et al., 2009). It has been shown that S100B, a brain specific marker of neurologic injury, interacts with RAGE to amplify its downstream signal leading to accelerated cellular injury, and that activation of the RAGE pathway in the setting of fetal inflammation is associated with reduced levels of its soluble truncated form (i.e. sRAGE) (Buhimschi et al., 2009).

We hypothesized that, in neonates subsequently diagnosed with CP, select biomarkers of brain injury in umbilical cord blood could differentiate neonates with CP from those who survived without CP. Because death would preclude the diagnosis of CP, we also included death in the outcome. Thus, the objective of our study was to evaluate the association between infant death by 1 year of corrected age or the diagnosis of CP and potential umbilical cord blood markers of fetal neurologic injury. Additionally, we intended to test whether the relationship between these biomarkers and the outcomes (CP, death, and the two combined) was modified by antenatal exposure to magnesium sulfate ($MgSO_4$).

## 2. Materials and methods

### 2.1. Study design

This was a secondary analysis and a nested case–control study of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD) Maternal–Fetal Medicine Units Network "Randomized Clinical Trial of the Beneficial Effects of Antenatal Magnesium Sulfate", in which women who had singleton or twin gestations between 24 and $31^{6/7}$ weeks' gestation and at high risk for imminent preterm birth were randomized to receive either magnesium sulfate ($MgSO_4$) infusion or placebo (Rouse et al., 2008). Women were enrolled at 20 institutions between December 1997 and May 2004. For this analysis, cases were infants who died by 1 year of corrected age or were diagnosed with CP at or beyond 2 years of corrected age. The control group included infants who survived until 2 years of age without CP. Subjects enrolled in the trial and who had available umbilical cord blood serum and 2-year data on neurological outcomes or death were included. We excluded cases of fetal demise and neonates with major congenital malformations or genetic syndromes. Cases were matched at a ratio of 1:2 to controls for ethnicity/race, infant gender, and number of fetuses. The primary outcome of the study was a composite of infant death by 1 year or development of CP (any severity) among survivors as assessed at 24–28 months of life (corrected for prematurity). The secondary outcomes analyzed included infant death by 1 year of age and CP (any severity). Because of the limitation in our sample size, we did not perform a subgroup analysis for moderate-severe or mild CP. Details about the definitions and diagnoses of these selected outcomes are described elsewhere (Rouse et al., 2008).

### 2.2. Laboratory testing

Venous umbilical cord blood was obtained in a red top tube and centrifuged for 10 min (3400 RPM) within 2 h of birth. Serum was aliquoted and stored at −70 C. All samples available from the original study were shipped on dry ice for batch analysis in a single lab at the University of Texas Medical Branch in Galveston, TX. Personnel performing the laboratory analysis were blinded to case/control status.

Umbilical cord serum neuron-specific enolase (NSE), S100 calcium binding protein B (S100B) and the total soluble form of the receptor for advanced glycation end-products (sRAGE) were measured with commercially available enzyme-linked immunosorbent assay (ELISA) kits and according to the manufacturer's recommendations. Grossly hemolyzed samples ($n = 12$) were not included in NSE analysis. Immunoassay kits for NSE, S100B and sRAGE were purchased from Alpha Diagnostic International (San Antonio, TX, USA), BioVendor, LLC (Chandler, NC, USA) and R&D Systems (Minneapolis, MN, USA) respectively. The detection limits for the 3 assays are 1 μg/L, 5 pg/mL, and 4.1 pg/mL respectively. The inter- and intra-assay coefficients of variation were <10% for all analytes. All samples were run in duplicate and the mean values used in analyses.

### 2.3. Statistical analysis

Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC). Maternal and neonatal continuous variables were compared with the use of the Wilcoxon rank-sum test, and categorical variables with the chi-square or Fisher's exact test. Biomarker concentrations were analyzed both as continuous and categorical variables. Data were divided into categories using the 75th percentile (S100B and NSE) or the 25th percentile (sRAGE) of control samples from patients in the placebo group ($n = 43$). We had nearly 80% power to detect an odds ratio of 3.0 in the primary outcome of interest using these thresholds and the available sample size. Logistic regression was used to analyze the association between biomarker concentrations and neonatal outcomes, while including the effect of delivery GA and study treatment group. Similarly, we studied the effect of latency after preterm premature rupture of membranes (pPROM) on the relationship between neonatal outcomes and biomarker concentrations. We did not adjust for multiple comparisons. A two-sided *P*-value of less than 0.05 was considered to indicate statistical significance, and no adjustments were made for multiple comparisons.

## 3. Results

41 infants who developed CP ($n = 16$) or infant death ($n = 25$) and met all study criteria were matched by race, infant gender, and number of fetuses to 82 controls. Infants with serum available for testing were similar for maternal characteristics, but were on average 204 g heavier at birth and were born 1 week later in gestation. This difference is likely related to the difficulty of obtaining sufficient cord blood from the smallest neonates. Maternal and neonatal characteristics for the analyzed cohort are summarized in Tables 1 and 2. Neonates who died or had CP weighed less at birth and were born at earlier gestational ages compared with the control group. In addition they had lower Apgar scores at one and 5 min and were more likely to have had neonatal sepsis. On the other hand, there were no significant differences between cases and controls in such maternal characteristics as preterm premature rupture of membranes (pPROM), clinical chorioamnionitis, mode of delivery or treatment allocation (i.e. $MgSO_4$ or placebo).

There were no differences in S100B, NSE or total sRAGE concentrations between infants who died or developed CP and controls, nor in the percentage of them who had highest quartile concentrations of S100B (above 871.5 pg/mL) or NSE (above 20.8 μg/L),