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Authors

Maggiotto, Liesbeth V
Sondhi, Monica
Shin, Bo-Chul
et al.

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Circulating blood cellular glucose transporters – Surrogate biomarkers for neonatal hypoxic-ischemic encephalopathy assessed by novel scoring systems

Liesbeth V. Maggiotto, **Monica Sondhi**, **Bo-Chul Shin**, **Meena Garg**, **Sherin U. Devaskar***

Department of Pediatrics, Division of Neonatology & Developmental Biology, The Neonatal Research Center of the Children's Discovery & Innovation Institute, David Geffen School of Medicine at UCLA and the UCLA Mattel Children's Hospital, Los Angeles, CA 90095-1752, United States of America

Abstract

Objective: We examined Red Blood Cell (RBC) Glucose Transporter isoform 1 (GLUT1) and White Blood Cell (WBC) Glucose Transporter isoform 3 (GLUT3) protein concentrations to assess their potential as surrogate biomarkers for the presence of hypoxic-ischemic encephalopathy (HIE) and response to therapeutic hypothermia (TH), with respect to the neurodevelopmental prognosis.

Study design: A prospective feasibility study of 10 infants with HIE and 8 age-matched control subjects was undertaken. Following parental consent, blood samples were obtained at baseline before institution of TH (< 6 h of life), during TH, at rewarming and post-TH in the HIE group with a baseline sample from the control group. GLUT1 and GLUT3 were measured by Enzyme-linked immunosorbent assay (ELISA) with brain biomarkers, Neuron-Specific Enolase (NSE) and Glial Fibrillary Acidic Protein (GFAP). Novel "HIE-high risk" and "Neurological" scores were developed to help identify HIE and to assess severity and prognosis, respectively.

Results: RBC GLUT1 concentrations were increased at the baseline pre-TH time point in HIE versus control subjects ($p = .006$), normalizing after TH ($p = .05$). An association between GLUT1 and NSE concentrations (which was reflective of the HIE-high risk and the Neuro-scores) in controls and HIE pre-TH was seen ($R^2 = 0.36$, $p = .008$), with GLUT1 demonstrating 90% sensitivity and 88% specificity for presence of HIE identified by Sarnat Staging. WBC GLUT3 concentrations were low and no different in HIE versus control, and GFAP concentrations trended higher during re-warming ($p = .11$) and post-TH ($p = .16$). We demonstrated a significant difference between HIE and controls for both the "HIE-high risk" and the "Neurological" Scores. The latter score revealing the severity of clinical neurological illness correlated with the corresponding RBC GLUT1 (R^2 value = 0.39; $p = .006$).

Conclusion: Circulating RBC GLUT1 concentrations with NSE demonstrate a significant potential in reflecting the severity of HIE pre-TH and gauging effectiveness of TH. In contrast, the low neonatal WBC GLUT3 concentrations make discerning differences between degrees of HIE as

*Corresponding author at: 10833 Le Conte Avenue, MDCC-22-412, Los Angeles, CA 90095-1752, United States of America. sdevaskar@mednet.ucla.edu (S.U. Devaskar).

well as assessing effectiveness of TH difficult. The HIE-high risk and Neurological scores may extend the “Sarnat staging” towards assessing severity and neuro-developmental prognosis of HIE.

Keywords

HIE; Brain injury; Therapeutic hypothermia; Glucose transporters; Red blood cells

1. Introduction

Hypoxic-ischemic encephalopathy (HIE) with an incidence of 1 to 8/1000 in the Western world, is a devastating condition with a high incidence of mortality and long term neurological morbidity [1–4]. To date, the only therapy that has been observed to affect the moderate to severe HIE is whole body therapeutic hypothermia (TH) or selective head cooling if instituted within six hours of birth based on nationally accepted criteria [4,5]. Seizures occur in 40–65% of HIE infants and use of anti-epileptics to control seizures with continuous video-EEG monitoring to assess electrical function of the brain has become the accepted norm in major centers [6]. In addition to monitoring vital signs including blood pressure, peripheral oxygen and brain (near infrared spectroscopy (NIRS)) oxygen saturations, normal circulating glucose concentrations are maintained, since an increase or decrease can detrimentally affect the brain even further. Sarnat et al. developed a scoring system to distinguish between mild, moderate and severe HIE based on physical exam alone [5]. Generally following therapeutic hypothermia and rewarming, diffusion weighted magnetic resonance imaging (MRI) is undertaken to assess structural brain injury while simultaneous magnetic resonance spectroscopy gauges the metabolic function [7,8]. A higher incidence of cerebral palsy, intellectual and learning disabilities are encountered in children with HIE. The development of surviving babies is generally followed over a longer period of time for assessment of their motor and cognitive skills. Yet, despite all these diagnostic modalities that contribute to a significant economic burden on society, physicians still lack in the ability to identify the severity of HIE with much certainty, and none of these studies succeed in predicting the degree of subsequent neuro-developmental impairment.

Previous studies have undertaken Positron Emission Tomography (PET) scanning in infants and revealed that most of the glucose uptake is found infra-tentorial in neonates, increasing to the supra-tentorial structures around infancy when cerebral cortical functions begin developing [9]. PET scanning cannot be routinely offered to infants with HIE during TH as it adds risk of exposure to radiation. In parallel with these findings, we have shown that in autopsy brain samples, facilitative glucose transporters predominantly expressed in the brain increase during infancy as well. In fact, there are two major isoforms in the neonatal and infant brain: GLUT1 (Michaelis Constant (Km) = 1 mM) and GLUT3 (Km = 0.8 mM). While GLUT1 is uniformly expressed in the blood brain barrier (endothelial cells of the micro-vessels) and in glial components [10], GLUT3 is primarily expressed in neurons, particularly at the mature synapses [11]. During development, GLUT1 is reported as early as in the neonatal period, remaining constant throughout infancy [12–14], and GLUT3 is found in low amounts in the neonatal period only increasing in infancy mimicking the PET results of glucose uptake [9]. Further, in pre-clinical mouse studies, postnatal hypoxic-ischemia produced by unilateral carotid artery ligation and exposure to hypoxic conditions, revealed

an early increase in brain GLUT1 concentrations followed by an increase subsequently in GLUT3 concentrations, both compensating for the lack of oxygen experienced by the developing brain. In fact, GLUT3 known to mediate neurotransmission [11,15–17], if mutated, is unable to compensate in response to hypoxia-ischemia and results in increased brain injury [18]. Such brain studies are difficult to replicate in human infants, as studies on autopsy specimens have limitations.

Hence, biomarkers of HIE circulating in the blood take on great importance towards early evaluation of HIE and its consequent ability to introduce early interventions. Neuron-Specific Enolase (NSE$\gamma\gamma$) [19,20] and Glial Fibrillary Acidic Protein (GFAP) [21,22] have previously been explored as non-invasive biomarkers of HIE and white and gray matter injury. In our present study, we hypothesized, that in lieu of obtaining brain tissue, circulating blood cells that carry both GLUT1 and GLUT3 could serve as surrogate markers for heralding brain injury, and thereby extend the value offered by NSE and/or GFAP alone. GLUT1 was originally cloned from human red blood cells [23] and GLUT3 is found on white blood cells [24]. Hence, we questioned whether HIE would result in changes in the RBC GLUT1 and WBC GLUT3 concentrations, predicting brain injury, and amelioration with TH. We compared blood cell GLUT1 and GLUT3 concentrations to plasma concentrations of NSE and GFAP in the setting of HIE.

In order to enhance the acumen in assessing the qualifications for TH, we developed an “HIE-high risk score” compiling valuable information from fetal heart rate (prenatal data), mode of delivery, resuscitation events, Apgar score at 5 min of life, pH and base deficit (BD) in the cord or at less than one hour of life. We also developed a “Neurological score” compiling data from the neurological exam using the established Sarnat staging based upon clinical presentation, plus information garnered from vEEG, MRI, MRS and Bayley Scales of Infant and Toddler Development- III (BSID-III) at long term follow-up. The HIE-high risk score allowed heightened vigilance for detection of HIE in the infant, and the Neurological score proved useful in grading the severity of HIE.

2. Methods

2.1. Study subjects

This prospective pilot study evaluated the potential of different biomarkers in infants with HIE. After UCLA Institutional Review Board’s approval and obtaining consent from parents, all infants with HIE qualifying for whole body cooling were identified and respective age-matched control infants were recruited from the Neonatal Intensive Care Unit (NICU) at UCLA Mattel Children’s Hospital. 11 HIE and 8 control infants eligible for this study met the following criteria: > 36 weeks of gestational age (GA), and > 2 kg body weight. Babies’ eligibility for TH was based on national guidelines which included the presence of metabolic acidosis at < one hour of life (pH ≤7.0 on cord blood gas/blood gas within 1 h of life or a base deficit > 12 mEq/L), the presence of a sentinel event surrounding birth or Apgars ≤5 at 10 min of life, and an abnormal neurological examination or seizures (employing the Sarnat grading) [5]. Exclusion criteria were 1) inability to undergo TH by 6 h of age, 2) chromosomal and metabolic abnormality, 3) major congenital anomaly, 4) blood culture proven sepsis, and 5) parent refusing consent for study participation. In addition, the

most important exclusion criteria for the control group was evidence of HIE based on the national criteria described above. Reasons for admission to the NICU in the control group were mild respiratory distress with no requirement for ventilation, abdominal diagnoses (e.g. gastroschisis, jejunal atresia), or other diagnoses (e.g. small for gestational age, transient bradycardia, and small cephalohematoma). All subjects received standard care in the NICU relevant to their diagnoses. After enrollment, 1 HIE infant revealed 16% mosaic trisomy 21, and was therefore excluded from the study.

2.2. Subjects' clinical characteristics

Maternal and infant clinical characteristics collected included fetal monitoring, events surrounding birth along with presence of any sentinel event, timing of initiation of TH, degree of HIE along with findings of the neurological examination, Electroencephalogram (EEG), Magnetic Resonance Imaging (MRI), Magnetic Resonance Spectroscopy (MRS) and all infant blood testing such as serum glucose, electrolytes, Calcium, Magnesium, renal function and blood gases. In addition, the reason for NICU admission, interventions and length of stay details were assessed. Neurological examinations were performed by Neonatologists upon admission to the NICU using the previously described Sarnat staging scoring system [5]. At 4–13 days of life, all HIE subjects had a brain MRI and MRS (except for one subject with mild HIE who did not complete MRS). The MRI T1, T2 and diffusion weighted images were reviewed by a team of experienced readers, one of whom was a neuro-radiologist, and any differences were resolved by consensus. No MRI/MRS studies were performed on control subjects due to lack of parental consents for imaging and spectroscopy examination. Following discharge from the NICU, the infants' neurodevelopment was assessed via Bayley Scales of Infant and Toddler Development- III (BSID-III) by experienced examiners in the outpatient setting at 6, 12, 18 and/or 24 months of life.

2.3. Derivation of HIE-high risk and Neurological scoring system

To provide uniformity to the detected clinical characteristics towards validating inter-subject comparison, instead of using only the clinical neurological exam (Sarnat scoring system), we developed two novel scoring systems compiling clinical, physical, laboratory and imaging variables described in Table 1. The HIE-high risk score grapples with the severity of the initial perinatal insult, and the Neurological score addresses the neurological consequences over time. Missing values/scores were handled by expressing the ultimate sum of scores as a percent of maximum scores achievable for both the HIE-high risk and "Neurological scoring systems. As an example, some subjects who were lost to follow up developmental outpatient visits had a reduced total achievable score from 10 to 8.

2.4. Blood sample collection

Whole blood (2 mL) samples in BD vacutainer with sodium heparinized tubes (#367871) were collected at four time points in the HIE infants: 1) at baseline pre-TH (0–6 h of life), 2) during the TH phase (at 33.5 °C over 72 h), 3) the rewarming phase towards achieving normothermia, and 4) the post-TH phase, and immediately transported to the laboratory on ice. In our healthy control group we were only able to get parental consent for a single blood draw that was obtained in the same pre-TH and TH time frame (6–76 h of life) as the blood

draws in HIE subjects. Thin blood smears were created on slides and cells were separated from each other using a Lymphocyte Separation Medium (LSM).

2.5. GLUT1 and GLUT3 antibody specificity

Adult (control) and infant blood smears on slides were fixed in 4% paraformaldehyde, primary antibodies (Guinea pig-anti-GLUT1, 1:500 dilution, RRID: AB_2737340 from Dr. Takata, Gunma University; rabbit-anti-human GLUT3, 1:250, IBL- America, catalog #18903, RRID: AB_494545) incubation was carried out overnight at 4 °C. Secondary antibodies were raised against the species in which the primary antibodies were raised. FITC-conjugated IgG (Jackson ImmunoResearch catalog #706–585–148, RRID: AB_2340474, green, 1/500 dilution, Donkey-anti-Guinea pig (GLUT1)) and Alexa Fluoro 594 conjugated IgG (Jackson ImmunoResearch Labs, catalog #705-585-003, RRID: AB_2340432 red, 1/250 dilution, Donkey-anti-rabbit (GLUT3)) were incubated with DAPI (specific to the nuclei) at 1/1000 dilution for 30 min. The images were visualized with a Nikon E-600 microscope (Nikon, Melville, NY) equipped with a cooled, charge-coupled device camera (CoolSNAP HQ Monochrome; Roper Scientific, Tucson, AZ).

2.6. RBC and WBC membrane preparation

For RBC membrane preparation, the membrane ghost pellet prepared from RBC-enriched fractions was lysed and subjected to a protein assay using the BCA Protein Assay Reagents (Pierce, Rockford, IL). RBC membranes' protein concentrations ranged from 2.6–5.9 μg/μL.

For WBC membrane preparation, the membrane ghost pellet prepared from a mononuclear layer was lysed and subjected to a protein assay using BCA Protein Assay Reagents (Pierce, Rockford, IL). WBC membranes' protein concentrations ranged from 0.4–4.9 μg/μL.

2.7. GLUT1 and GLUT3 protein concentrations by Western blots

The homogenized samples (10 μg) were subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). These membranes were incubated with anti-GLUT1 (1/1000, Abcam, catalog #ab652, RRID: AB_305540) and GLUT3 (dilution 1:500, IBL Co., Ltd.,) antibodies. After incubation with horseradish peroxidase-labeled anti-rabbit IgG (dilution 1/20,000) the proteins were visualized in Typhoon 9410 Phosphorimager (GE Healthcare Biosciences, Piscataway, NJ) by using enhanced chemiluminescence (ECL) plus detection kit (GE Healthcare BioSciences Corp., Piscataway, NJ). The protein bands were visualized using Image Quant 5.2 software (GE Healthcare Biosciences, Piscataway, NJ) and integrity of the samples further confirmed with vinculin (1/5000; Sigma, catalog #V9131, RRID: AB_477629), which served as the internal loading control.

2.8. Plasma assays and the quantification of GLUT1 and GLUT3 proteins in RBC and WBC membranes by ELISA

RBC membrane (50 μg) GLUT1 and WBC membrane (25 μg) GLUT3 concentrations were measured in duplicate by enzyme-linked immunosorbent assays (ELISAs; Elabscience Biotechnology Inc., Wuhan, China), following the manufacturer's instructions. Plasma GFAP and NSE were measured using Single Molecule Array (SIMOA) Discovery

immunoassays employing the SIMOA HD-a Analyzer and Single Molecule Array technology [25]. The lower level of detection for GFAP and NSE was 0.192 and 2.67 pg/mL respectively. The coefficient of variation for intra- and inter-assay precision was < 10%. The sensitivity of the SIMOA assay was 1000-fold greater than a regular ELISA with the ability to measure femtomolar concentrations [25,26].

2.9. Data analysis

All the data are expressed as mean ± SEM. Initially a power analyses was undertaken to achieve > 80% power at a SD of 1.48 employing the Statmate program (GraphPad, RRID:SCR_000306). This analysis determined a $n = 9$ per group to achieve projected increased difference of about 50% at a $p = .05$. The data generated was subjected to detection of normality employing SigmaStat software (RRID:SCR_010285). Upon confirming normality of distribution, the Student's *t*-test was employed to determine inter-group differences, and analysis of variance (ANOVA) with post-hoc Fisher's PLSD to determine inter-time differences in the HIE group, at a $p < .05$ achieving significance. To determine associations between independent and dependent variables, the linear regression analysis was employed. Specificity and sensitivity of our assays were based on cut-off values from our controls. Cut-off values from controls were derived using the mean + 2× SD.

3. Results

The clinical characteristics of HIE and control subjects are depicted in Table 2A and show that the HIE and CONTROL groups were statistically comparable with respect to gestational age, birth weight and sex, although trends towards a lower birth weight and fewer females within the control group existed. The HIE subjects uniformly revealed abnormalities on EEG recordings, with only 55% demonstrating abnormal findings on MRI, and 60% presenting with abnormalities on MRS. There was 83% concordance between MRI and MRS (5 of the 6 abnormal MRI's had an abnormal MRS and vice versa). Only 27% of the HIE subjects required anti-epileptic medications which was 1/3 of the subjects with an abnormal MRS. We compared point of care (POC) blood glucose measurements between HIE pre-TH, and control, as serum glucose was not routinely measured in the laboratory neither in the HIE nor control subjects. The glucose concentrations between the HIE group pre-TH and control was not significantly different, however they tended to be higher pre-TH in infants with HIE, compared to control (see Table 2A).

A review of clinical characteristics (Neuro score) revealed that the severity of clinical neurological illness correlated with the corresponding plasma NSE (R^2 value = 0.58; p value = .01). The severely affected HIE subject with feeding difficulty entailing the need for G-tube prior to hospital discharge revealed the highest NSE and GLUT1 concentrations, while no such effect was observed with plasma GFAP concentrations. Table 2B illustrates the ranges, means and SEM of the derived HIE-high risk score and the Neurological score comparing the HIE study group with controls. We demonstrate a significant difference between HIE and controls for both the HIE-high risk and the Neurological scores.

Fig. 1 (panels A–I) demonstrates the results of immunohistochemistry performed on blood smears of an adult (panels A,B,C), a control subject (panels D,E,F), and an infant with HIE (panels G, H, I) to assess cell-specificity of GLUT1 and GLUT3 antibodies. Fig. 2A demonstrates a representative tube with clear separation of the different cellular fractions from the plasma (P). We further authenticated primary antibodies by Western blot analysis (panels B and C). While GLUT1 (B) required pre-treatment with deglycosylating enzyme GNPase F to demonstrate a clear band around ~35 kD (since the enzyme untreated band revealed a heavily glycosylated broad band around ~55 kD), GLUT3 in the absence of deglycosylation demonstrated a relatively clear band at 45–50 kD sized by molecular markers (Fig. 2C). These results collectively attest to the specificity of the antibodies employed in our present study.

Our quantification of RBC GLUT1 concentrations/unit protein concentration by ELISA revealed an increase in HIE subjects (mean ± SD: 5.24 ± 2.82 ng/mL) at the baseline pre-cooling stage when compared to control subjects (2.04 ± 1.48 ng/mL; $p = .0057$). Institution of TH appeared to normalize GLUT1 concentrations (Fig. 3A), achieving significance at the post-TH stage ($p = .05$). In contrast, WBC GLUT3 concentrations were low in controls (0.46 ± 0.19 ng/mL) and HIE (0.38 ± 0.39 ng/mL) subjects and trended towards an increase after TH, although not achieving significance (Fig. 3B). In addition, employing the subjects' RBC and WBC differential counts, we expressed GLUT1 per RBC and GLUT3 per mononuclear cell. Similar increase in GLUT1 concentrations pre-TH, during TH and post-TH when compared to control subjects ($p = .05$ compared to control) was observed (data not shown). In contrast, GLUT3/Mononuclear cell concentrations were reduced pre-TH compared to control values ($p = .04$) (data not shown).

Average neuron-specific enolase concentrations ranged between 4 and 20 ng/mL in the control group (mean 9.21 ± 2.2 ng/mL). NSE concentrations in HIE subjects ranged between 2 and 40 ng/mL, means being 16.3, 7.4, 7.3 and 6.9 ng/mL during pre-TH, TH, rewarming and post-TH respectively. The mean NSE in HIE pre-TH was much lower than previously reported [20,27]. NSE concentrations were significantly increased in the HIE group, especially at the pre-TH baseline compared to control. Therapeutic Hypothermia significantly reduced NSE in the HIE group (Fig. 3C).

GFAP concentrations while trending towards an increase, were not significantly different in our HIE subjects (351 ± 281 pg/mL) compared to the control group (159 ± 60 pg/mL) secondary to large variations encountered with this protein in this HIE population. A relative increase in GFAP was noted during TH compared to control, with an additional increase during rewarming and post-TH (Fig. 3D). Mean GFAP concentrations in the control group were much higher than previously reported [21,27–29].

Linear regression analyses were performed on control and HIE pre-TH measurements. We demonstrated a significant correlation between GLUT1 and NSE concentration ($R^2 = 0.36$, $p = .008$). We noted a weaker association between GLUT1 and GFAP, although not significant ($R^2 = 0.21$, $p = .06$). GLUT1 ($R^2 = 0.3$, $p = .02$), NSE ($R^2 = 0.26$, $p = .03$) and GFAP ($R^2 = 0.24$, $p = .04$) correlated with the HIE-high risk score. In addition, an association existed between GLUT1 ($R^2 = 0.39$, $p = .006$) or NSE ($R^2 = 0.38$, $p = .007$) and the Neurological

score. No such correlation was seen with GFAP and the Neurological score. Further, no significant associations between GLUT3 and NSE, GFAP, GLUT1, or Neuro/HIE-high risk score were evident.

Employing the mean and SD of values from our control population as the cut-off, sensitivity and specificity for HIE were derived and found to be as follows: NSE: (cut-off for normal values was at < 13.63 ng/mL) sensitivity 50%, specificity 88%; GLUT1: (< 2.52 ng/mL) sensitivity 90%, specificity 88%; GFAP: (< 216 pg/mL) sensitivity 50%, specificity 88%; GLUT3: (> 0.17 ng/mL) sensitivity 40%, specificity 100%.

4. Discussion

This is the first study examining circulating blood cell glucose transporters with the assumption that they can serve as surrogates to those present on brain cells. Our study determined the usefulness of blood cell glucose transporters in indirectly gauging the state of HIE. Even though our study was limited by small numbers given that the incidence of this condition is only 0.1–0.8%, our results show that HIE at baseline pre-TH increases RBC GLUT1 concentrations when compared to non-HIE control infants that were age-matched. This increase is suppressed during the cooling phase only to return during the re-warming phase settling to control values after the therapeutic phase of cooling. These changes in GLUT1 parallel the changes in plasma NSE concentrations, which increased in HIE infants pre-TH compared to control and diminished to control values with therapeutic hypothermia. NSE is a glycolytic enzyme found in the cytoplasm of neurons [19]. The content of NSE in blood is 30× lower than that in the brain [30]. Previously plasma NSE was shown to increase and could only be measured upon neuronal death and disruption of the blood-brain-barrier (BBB) [31]. Celtik et al. reported that NSE > 45 ng/mL distinguishes HIE infants with a normal prognosis from an abnormal/poor prognosis [20]. In contrast, GLUT1 being a membrane associated protein is not known to and may not leak out of the brain into plasma upon damage to the endothelial blood-brain-barrier and the white matter. Instead, GLUT1 changes may reflect a compensatory protective phenomenon mediating increased glucose transport across the BBB towards adequately fueling brain cells during HIE. This is perhaps why TH, while decreasing NSE immediately, only gradually reduced the RBC GLUT1 concentrations to control values, perhaps reflecting the gradual reduction of a need for fuel with TH as cellular metabolism and demand diminished.

In contrast, we found little difference in the white blood cell GLUT3 in HIE versus controls and no further changes during the different phases of cooling and re-warming, unless the pre-TH values were expressed per mononuclear cell and then noted to be lower than controls. This finding may suggest that neonatal neuronal GLUT3 is low and does not change with HIE, or that lower values of GLUT3 with HIE reflect neuronal/synaptic damage. Alternately, GLUT3 is found in rather low amounts in neuronal synapses and WBCs in the neonatal period [11] reflecting the fact that the gray matter is not fully developed at this stage, only to begin developing during infancy.

Our findings in the neonatal period reflect the findings of pre-clinical studies during the postnatal period, where brain GLUT1 increases prior to GLUT3 in response to hypoxic-

ischemia [11,32]. These changes in the RBC GLUT1 concentrations did not reflect any changes in plasma glucose concentrations which were maintained in the normal range for all babies with HIE and in controls, despite initial brief changes in some of the babies. Besides, an increasing trend in blood glucose noted in HIE versus Control would lead to downregulation of RBC GLUT1 concentrations, as we have previously reported in diabetic children [33], which was not seen in our present study. If anything, the opposite was observed with HIE increasing GLUT1 pre-TH.

Previous investigations in term HIE infants have employed plasma GFAP as a non-invasive prognosticating biomarker. GFAP is a major constituent of the astroglial cytoskeleton and is involved in the maintenance of the BBB. Ennen et al. noted a GFAP > 150 pg/mL following hypothermia to be associated with abnormal MRI studies [21]. GFAP in increasing amounts were noted not only in HIE infants' circulation but also suggestive of white matter injury. Our study showed much higher concentrations of GFAP and much lower NSE concentrations than reported previously [20,21,27–29,34], which may reflect the different technology and increased sensitivity (> 1000-fold) of the SIMOA immunoassays (Banyan Inc.) [25]. In our present study, GFAP demonstrated significant variability despite the sensitivity of the assay. This may be related to the timing of the antecedent sentinel event leading to HIE. In HIE, it is often unknown when exactly the insult occurred; whether it was within the last few minutes, hours or days and weeks ago. Some biomarkers may reflect more the severity of the insult, while others reflect the duration of the injury. Alternately, this may reflect wide variation in timing as to when astrogliosis towards repair occurs in response to brain injury. GFAP trended towards high levels in our study, and may become more significant at later postnatal ages of study than that included in our present study.

In contrast, NSE, an enzyme found to be high in premature infants with periventricular leukomalacia, is a neuronal marker which was significantly perturbed in our HIE neonates. The periventricular region is rich with neural stem cells and progenitors that subsequently differentiate into neurons predominantly and radial glial cells that form a scaffold upon which neurons migrate to the destination within the cerebral cortex [35,36]. These previous studies provide additional credence to our present observations of significant changes more in circulating blood cellular GLUT1 and plasma NSE rather than GLUT3 concentrations in response to HIE at this early neonatal age. These findings support an early response by circulating GLUT1 in keeping with NSE. Thus consideration may be given to improved accuracy of detection and prognostication by combining the results of GLUT1 and NSE in newborn infants with suspected HIE.

While isolation of WBC can prove to be cumbersome, isolation of RBC and ELISA based quantification of GLUT1 should be technically feasible in most clinical laboratories with a turnaround time that can prove to be clinically useful. During the early stages of HIE, particularly when the infant is undergoing TH, it is often difficult to gauge the severity of HIE other than the clinical history, neurological examination. Even after EEG findings of disruption in the background activity and the presence of seizures, and after a post-TH MRI/MRS obtained for prognostication [7,8] it continues to be hard to assess the degree of HIE. To overcome these difficulties, we developed the HIE-high risk score to enhance the initial detection of HIE in infants, and the Neurological score to assess the severity of HIE.

Both these scores are novel and demonstrate associations with GLUT1 and NSE. Thus, further development of the RBC GLUT1 testing such as impregnation of the GLUT1 antibody on a filter paper linked to a colour change reaction when exposed to a drop of blood could prove useful, since a POC test with a quick turnaround time may help more accurately to categorize these newborn infants into mild, moderate and severe categories. In addition, the effectiveness of TH may be forthcoming. However, prior to taking on further refinement of this test along with NSE uniformly in clinical practice, the limitation of our present feasibility study should be overcome. Future recruitment of larger numbers of subjects in a multicenter clinical trial will support retrospective categorization into mild, moderate and severe HIE, based upon the HIE-high risk and Neurological scores. In addition, sub-group analysis focused on the effect of sex, birth weight and gestational age upon RBC GLUT1 concentrations can be undertaken. Further, blood collection at longitudinal time points in control subjects, with RBC GLUT1 and plasma NSE concentrations, will provide robust time-matched comparisons.

5. Conclusion

We have demonstrated that circulating RBC GLUT1 concentrations in conjunction with plasma NSE has the potential to serve as a surrogate biomarker for HIE prior to the institution of TH, and to further gauge the effectiveness of TH going forward. In contrast, WBC GLUT3 may not be useful as a marker for HIE during this immediate neonatal period of life. We have developed the HIE-high risk and Neurological scores to help extend the “Sarnat staging” towards assessing severity and neuro-developmental prognosis of HIE. Defining and developing testing for highly specific and sensitive non-invasive biomarkers and scoring systems will pave the future for improved diagnostic modalities, thereby allowing for timely interventions targeting HIE with detrimental outcomes.

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Abbreviations:

HIE	Hypoxic-ischemic encephalopathy
TH	Therapeutic Hypothermia
RBC	Red Blood Cell
WBC	White Blood Cell
GLUT1	Glucose transporter isoform 1
GLUT3	Glucose transporter isoform 3

NSE	Neuron-specific Enolase
GFAP	Glial Fibrillary Acidic Protein
NICU	Neonatal Intensive Care Unit
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy
EEG	Electroencephalogram
SIMOA	Single Molecule Array
ELISA	Enzyme-linked immunosorbent assay

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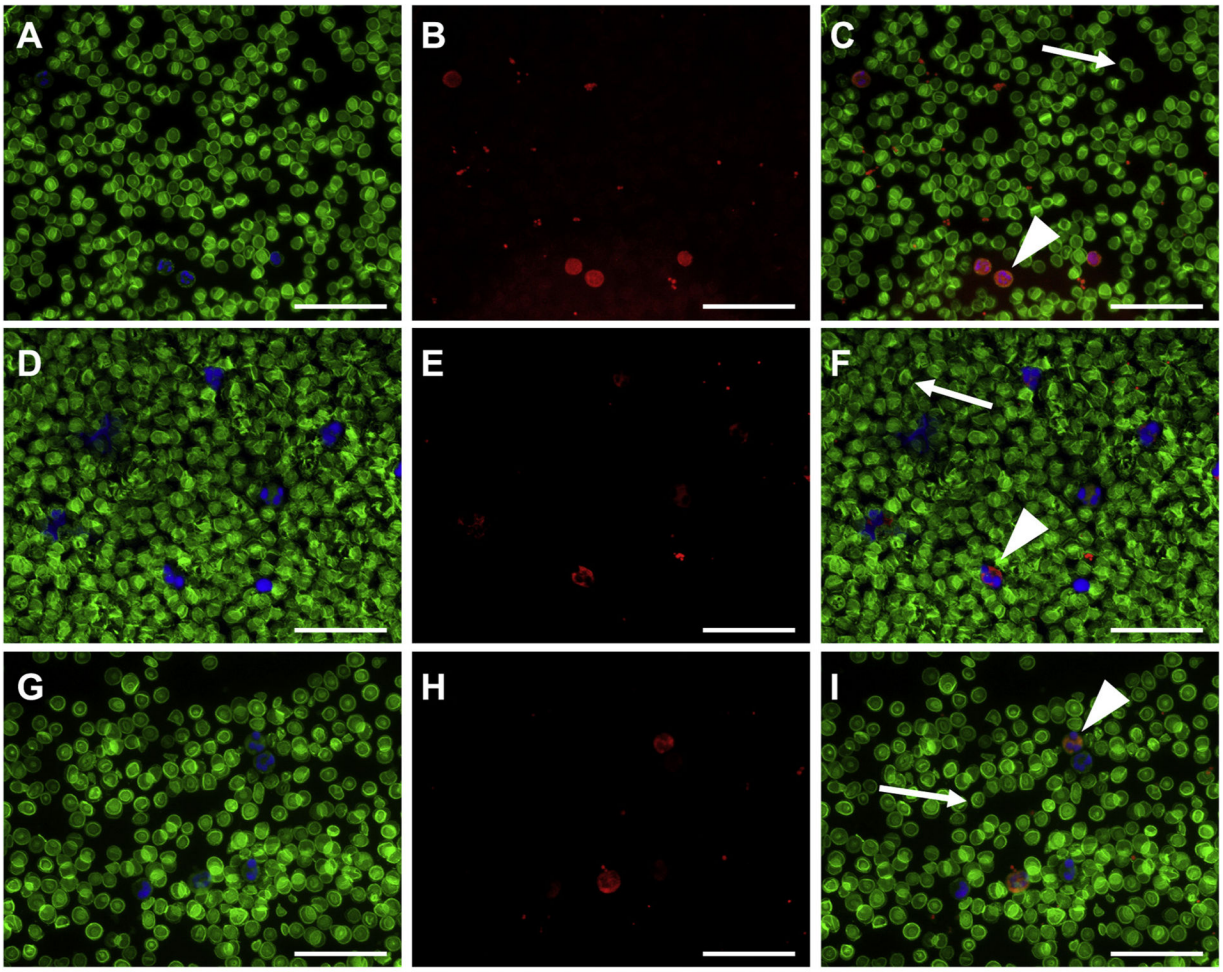

Fig. 1.

Representative photomicrograph demonstrating immunohistochemistry showing GLUT1 on RBCs (green) and nuclear DAPI (blue; A,D,G) and GLUT3 on WBCs (red; B,E,H) in peripheral blood smears of an adult (A,B,C), control infant (D,E,F) and infant with HIE (G,H,I). GLUT1/GLUT3/DAPI overlay shown in C,F,I; scale bars 50 μm; white arrows points to RBC stained with GLUT1 antibody; arrowheads point to WBCs that are doubly stained with DAPI and GLUT3 antibody.

Abbreviations: GLUT: Glucose Transporter; DAPI: 4′,6-diamidino-2-phenylindole; RBC: Red blood cell; WBC: White blood cell; kD: kilo Dalton.

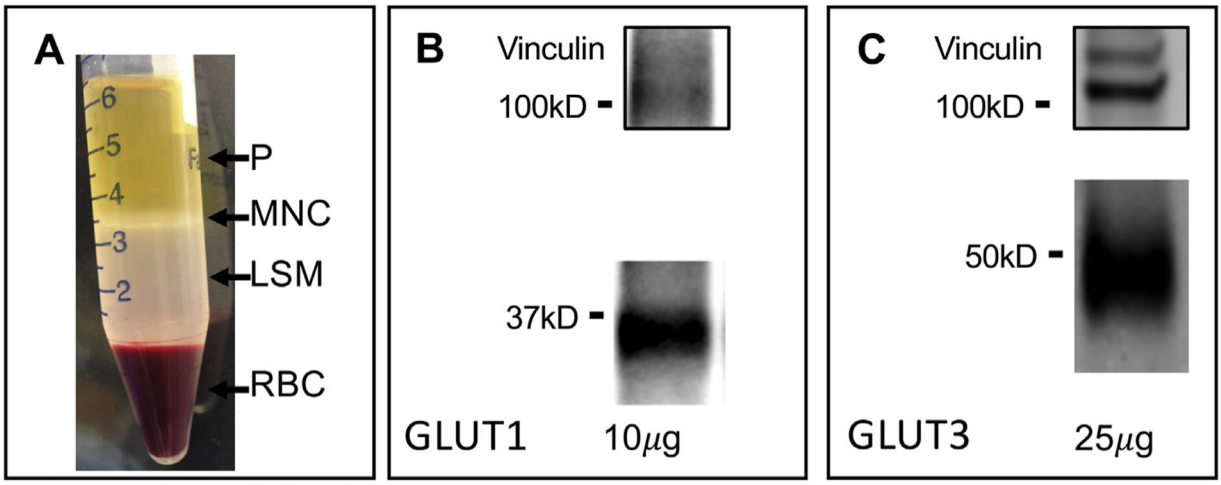

Fig. 2.

A: Representative tube demonstrating separation of plasma (P), MNC (Mononuclear cells-white blood cells), LSM (Lymphocyte Separation Medium) and RBCs. B: Representative Western Blot demonstrating the GLUT1 protein band (~35 kD) in 10 μg of RBC protein after deglycolization with enzyme GNPase F with Vinculin loading control on top. C: Representative Western Blot demonstrating the GLUT3 protein band (45–50 kD) in 25 μg of WBC protein with Vinculin loading control.

Abbreviations: GLUT: Glucose Transporter; RBC: Red blood cell; WBC: White blood cell; kD: kilo Dalton.

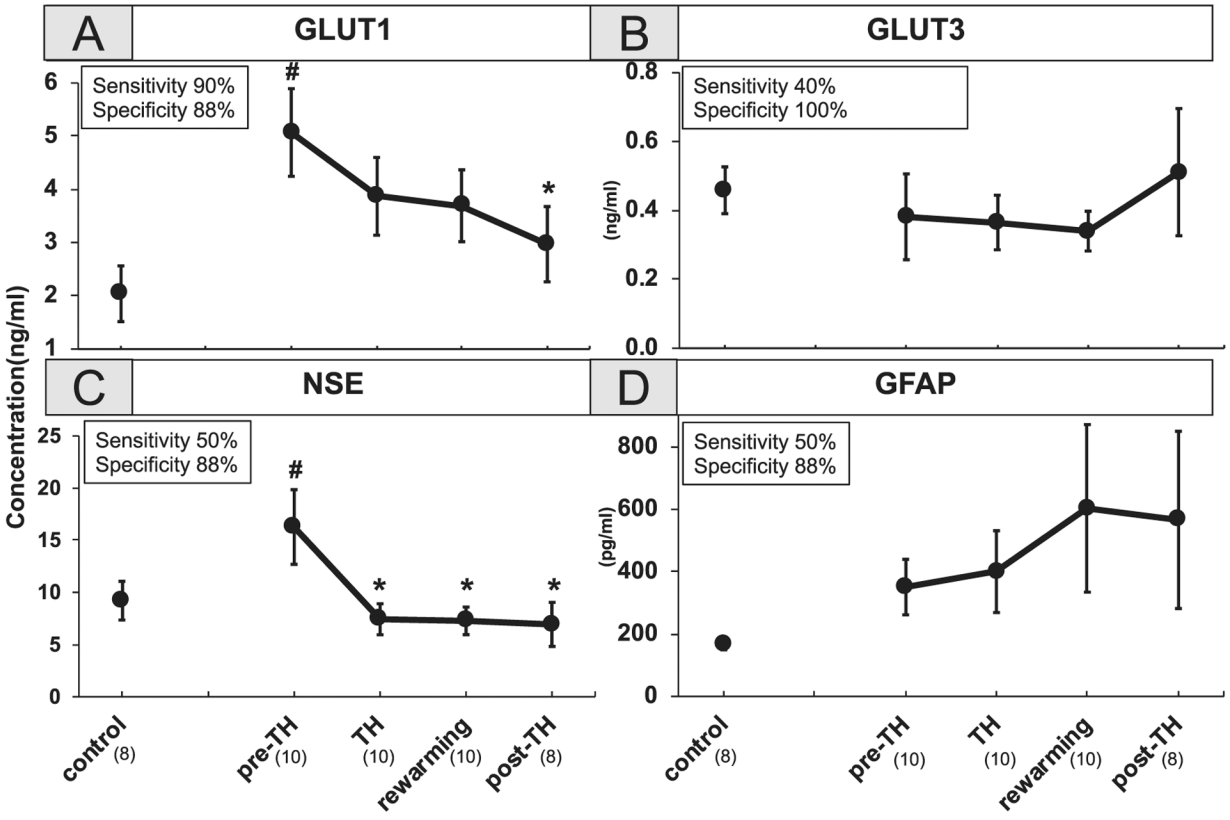

Fig. 3.

Biomarker concentrations in HIE subjects pre-HT, during HT, warming and post-TH. RBC GLUT1 (A), WBC GLUT3 (B), NSE (C) concentrations in ng/mL (mean ± SEM) and plasma GFAP (D) concentrations in pg/mL measured by ELISA. **GLUT1**: # $p = .006$: HIE pre-TH vs baseline control; * $p = .05$: HIE post-TH vs pre-TH. **NSE**: # $p < .05$: pre-TH vs the baseline control; * $p < .01$ various stages of TH versus pre-TH values in HIE subjects. Abbreviations: RBC red blood cell; WBC white blood cell; GLUT glucose transporter; HIE hypoxic-ischemic encephalopathy; TH therapeutic hypothermia; NSE Neuron-Specific Enolase; GFAP Glial Fibrillary Acidic Protein.

Table 1

Criteria defining the HIE-high risk score and Neurological score.

HIE-high risk score (0–2)	**0**	**1**	**2**
Fetal heart rate and mode of delivery	Normal vaginal spontaneous delivery or elective caesarean section. No fetal heart rate decelerations	Fetal decelerations but no emergent delivery	Fetal decelerations and/or emergent/crash delivery
Resuscitation	No chest compressions or epinephrine	Chest compressions no epinephrine	Chest compressions and epinephrine
5 min Apgar	≥ 7	> 3 < 6	< 3
pH at < 1 h age	≥ 7.1	6.9–7.0	≤ 6.8
BD at < 1 h age	≥ −10	−10 to −20	≤ −21
Neurological score (0–2)	**0**	**1**	**2**
Neurological examination	Normal	Mild HIE/Sarnat stage 1	Mod-severe HIE/ Sarnat stage ≥ 2
EEG	Normal	Mild discontinuity, IBI < 10s, no seizures	Excess discontinuity, IBI ≥ 10s, and/or seizures
MRI	Normal	Focal gray or white matter injury only	1 plus basal ganglia/thalamic and/or watershed lesions with/ without more extensive hemispheric injury [7]
MRS	Normal	X	Lactate and other abnormal peaks
BSID III	Normal, total score ≥ 85	total score 70–84, or subtest < 7	Total score < 70

Focal gray or white matter injury and basal ganglia/thalamic and/or watershed lesions with/without more extensive hemispheric injury [7]. Abbreviations: EEG Electroencephalogram; MRI Magnetic Resonance Imaging; MRS Magnetic Resonance Spectroscopy; BSID III Bayley Scales of Infant and Toddler Development III; BD Base Deficit; HIE Hypoxic Ischemic Encephalopathy.

Table 2

Clinical characteristics of subjects.

A. Clinical characteristics at birth			
Characteristics	**HIE (*n* = 11)**	**Control (*n* = 8)**	***p* value**
GA (wks) [mean ± SEM]	38 4/7 ± 3.5/7	38 1/7 ± 4/7	0.39
Birth weight (g) [mean ± SEM]	3170 ± 139	2861 ± 252	0.92
pH [mean ± SEM]	6.98 ± 0.05	7.27 ± 0.02	0.001
Glucose (mg/dL) [mean ± SEM]	107 ± 16	71 ± 4	0.06
Apgar score at 1 min [mean (range)]	2 (0–6)	6 (3–8)	0.0001
Apgar score at 5 min [mean (range)]	5 (2–8)	8 (7–9)	< 0.0001
Female sex [n (%)]	5 (45%)	2(25%)	0.39
Resuscitation			
Supplemental Oxygen [n (%)]	10 (91)	0	< 0.0001
CPAP [n (%)]	5 (45)	2(25)	0.15
IPPV [n (%)]	10 (91)	3(37)	0.01
Intubation [n (%)]	6 (55)	0	0.01
Chest compressions [n (%)]	2 (18)	0	0.2
Neuro exam abnormal [n (%)]	11 (100)	0	< 0.001
MRI abnormal [n (%)]	6/11 (55)	n/a	n/a
MRS abnormal [n (%)]	6/10 (60)	n/a	n/a
EEG abnormal [n (%)]	11 (100)	n/a	n/a
B. HIE-high risk and Neurological score			
Characteristics	**HIE (*n* = 10)**	**Control (n = 8)**	***p* value**
HIE-high risk score [mean ± SEM (range)]	4.9 ± 0.8 [1–9]	0.75 ± 0.3 (0–2)	0.001
Neurological score [mean ± SEM (range)]	6.1 ± 0.62 (3.75–10)	0	0.0000002

A: Clinical Characteristics at birth. B: HIE-high risk and Neurological scores showing the mean ± SEM, range and *p* value for comparisons between HIE and control groups. Abbreviations: GA Gestational Age; CPAP: Continuous Positive Airway Pressure; IPPV: Intermittent Positive Pressure Ventilation.