CLINICAL RESEARCH ARTICLE

Placental clearance/synthesis of neurobiomarkers GFAP and UCH-L1 in healthy term neonates and those with moderate–severe neonatal encephalopathy

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BACKGROUND: Fetal concentrations of GFAP and UCH-L1 are elevated in umbilical arterial (UmA) blood of neonates with birth asphyxia plus neonatal encephalopathy (NE), but their source and role of placental clearance/synthesis is unknown.

METHODS: Prospective cohort study of term neonates to (a) determine UmA and venous (UmV) blood concentrations of GFAP and UCH-L1 in term uncomplicated pregnancies and their placental synthesis and/or clearance and (b) compare UmA concentrations in uncomplicated pregnancies with those complicated by fetal hypoxia–asphyxia+NE. Three term groups were studied: uncomplicated cesarean delivery without labor (Group 1, $n = 15$), uncomplicated vaginal delivery with labor (Group 2, $n = 15$), and perinatal hypoxia–asphyxia+NE (Group 3, $n = 8$).

RESULTS: UmA GFAP concentrations were lower in Group 1 vs. 2 ($P = 0.02$) and both demonstrated 100% placental clearance. In contrast, UmA and UmV UCH-L1 concentrations were not unaffected by labor. Group 3 UmA GFAP concentrations were 30- and 8-fold higher than Groups 1 and 2, respectively, $P = 0.02$, whereas UmA UCH-L1 concentrations were similar in all groups.

CONCLUSIONS: UmA GFAP is derived from the fetus, and circulating levels, which are modulated by placental clearance, increase during uncomplicated labor and more so in the presence of fetal hypoxia–asphyxia+NE, providing a better biomarker than UCH-L1 for hypoxia–asphyxia+NE.

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INTRODUCTION

Neonatal encephalopathy (NE) is a devastating complication of perinatal hypoxia–asphyxia with an incidence of 1.5/1000 live term births (95% confidence interval, 1.3–1.7) and is associated with substantial morbidity and mortality.¹ Neonates with severe encephalopathy have a greater risk of death, and survivors have an increased risk of cerebral palsy and/or neurodevelopmental impairment.^{2,3} Recent evidence suggests that there is a progression in the severity of the insult in neonates with NE; thus neonates with mild-to-moderate encephalopathy are less likely to die but are at increased risk of significant motor deficits, fine motor disability, memory impairment, visual or visual–motor dysfunction, increased hyperactivity, and delayed school readiness.^{2,4–6} However, the ability to screen for severity of NE remains an issue.

Clinicians generally use the Sarnat scoring system to predict the severity and outcome of neonates with NE; to stratify affected neonates into mild, moderate, or severe categories; and to measure the progression of the neurologic insult in order to predict a neonate's prognosis.^{7,8} Nevertheless, this clinical scoring system is subjective and likely to change over time. Systemic hypothermia treatment of asphyxiated newborns is the standard neuroprotective treatment for NE and initiated within a few hours after birth based on the assigned Sarnat score. In the triage of neonates into a hypothermia protocol, a valid biochemical marker of NE obtained at birth, or a battery of biomarkers, could be a

valuable adjunct to the Sarnat scoring system. Glial fibrillary acidic protein (GFAP) is a cytoskeleton intermediate filament protein specific to astrocyte cells with no relevant extracerebral sources.⁹ Ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) is a cytoplasmic enzyme highly specific to neurons and neuroendocrine cells.^{10,11} Both are promising neurobiomarkers for determining the presence and severity of hypoxic NE at birth.^{12–19} Translational studies suggest that GFAP is specific to cerebral insults, whereas UCH-L1 is produced by several tissues, including murine and primate placental trophoblasts.^{20,21} Although serum concentrations of GFAP and UCH-L1 in neonates following birth are significantly elevated in the presence of moderate-to-severe NE,^{12–18} it is unclear how this compares to uncomplicated pregnancies and whether there is an effect of labor and/or the route of delivery. Furthermore, it is unclear whether the human placenta contributes to the removal and/or synthesis of either protein within the fetal compartment. The objectives of this study were to determine: (a) whether labor and vaginal delivery in uncomplicated term pregnancies modify circulating concentrations of GFAP and UCH-L1 in the fetal compartment, (b) whether there is evidence for placental synthesis or clearance of GFAP and/or UCH-L1 in term uncomplicated pregnancies by measuring umbilical arterial (UmA) and venous (UmV) cord blood concentrations, and (c) whether circulating concentrations of GFAP and UCH-L1 in fetal arterial blood increase in pregnancies complicated by fetal hypoxia–asphyxia plus NE.

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METHODS

Study design

We conducted a prospective cohort study that included 38 term pregnant women admitted to the Delivery Service at Parkland Hospital between June 2015 and April 2017. They were divided into three groups: Group 1 included non-laboring women admitted for repeat elective cesarean delivery in the absence of any pregnancy complications ($n = 15$), Group 2 included laboring women who underwent spontaneous vaginal delivery ($n = 15$) in the absence of any pregnancy or intrapartum complications, and Group 3 ($n = 8$) included pregnancies complicated by perinatal hypoxia-asphyxia resulting in moderate-to-severe NE in neonates who subsequently received hypothermia treatment per NICHD guidelines.²² Pregnancies in Groups 1 and 2 were selected randomly from term pregnant women without pregnancy complications who were admitted to the "Low Risk" Delivery Service at Parkland Hospital. They had to have competed at least 37 weeks of gestation; a singleton pregnancy; and no evidence of infection, fetal anomalies, or abnormal fetal growth. The first author (I.N.M.) randomly selected these women and attended these "low risk deliveries" in order to collect UmA and UmV blood samples in the delivery room. In contrast, Group 3 included emergently delivered pregnancies complicated by perinatal hypoxia-asphyxia. Thus they occurred throughout the day and were not attended by the first author; nonetheless, UmA blood was collected as described below.

Blood collection

Immediately after delivery of the neonate and before delivery of the placenta, i.e., with an intact placental circulation, a segment of intact umbilical cord was double clamped (~25 cm), resected, and blood separately withdrawn within 1–2 min from the UmA and UmV with separate sterile 5-ml plastic syringes. Thus the blood samples represent the intact fetal-placental circulation. Samples were allowed to clot, centrifuged for 10 min at 10,000 rpm, and the serum was brought to the laboratory and stored at -80°C until analyzed. An UmA sample (0.5 ml) was immediately sent to measure fetal arterial blood gases. As noted above, Group 3 pregnancies were acutely diagnosed and emergently delivered throughout the day, preventing the presence of an investigator. However, as standard of care, UmA blood samples are routinely collected at >90% of deliveries from a segment of double clamped umbilical cord before delivery of placenta and analyzed for blood gases. These blood samples were available for analysis.

GFAP and UCH-L1 assays

At the time of assay, blood samples from all the groups were thawed and 50 μl analyzed in duplicate for measurement of GFAP and UCH-L1 using an Immunoassay Kit (EnCor Biotechnology Inc., Gainesville, FL). The coefficient of variation for all assays was <15% for all the analysts.

Statistical analyses

Placental clearance was calculated using the following equation: $(\text{UmA concentration} - \text{UmV concentration})/\text{UmA concentration} \times 100$.

A descriptive analysis of GFAP, UCH-L1, and other variables was conducted using frequency distributions and differences between the concentrations in UmA and UmV in vaginal and cesarean-section groups were assessed by a two-way analysis of variance (ANOVA) followed by Tukey's post hoc test. One-way ANOVA followed by post hoc test was also used to compare UmA concentrations of GFAP and UCH-L1 in Groups 1, 2, and 3.

The relationship or association between categorical variables and the groups was determined by χ^2 analysis or Fisher exact tests. Linear regression was used to determine whether placental clearance was saturable. Statistical analyses were performed using the statistical package GraphPad Prism 7.03.

Data are presented as means $\pm$ standard error of the mean (SEM) unless otherwise noted.

Study approval

The study met the Health Insurance Portability Accountability Act requirements and was approved by the Institutional Review Board of the University of Texas Southwestern Medical Center and Parkland Health and Hospital Systems.

RESULTS

Patient population

There were no significant differences in maternal age, gestational age (GA) at delivery, or race/ethnicity across study Groups (Table 1). Within Group 3, there were 3 vaginal and 5 emergent cesarean deliveries, all associated with a nonreassuring fetal heart rate pattern and 7/8 with meconium-stained amniotic fluid. Male neonates accounted for >55% of study patients, and neonates were predominantly White Hispanic, mirroring the delivery population at Parkland Hospital. As per study design, Group 3 had significantly lower Apgar scores and evidence of severe fetal acidemia and intrapartum complications, including fetal heart rate abnormalities.

Effect of labor on umbilical cord blood GFAP concentrations

In Group 1, UmA concentrations of GFAP were 6.1 ± 2.4 ng/ml; UmV levels, however, were below detection of the assay, demonstrating 100% placental clearance. UmA concentrations of GFAP in Group 2 were ~4 times greater than values in Group 1 (23.4 ± 7.1 vs. 6.1 ± 2.4 ng/ml, respectively; Fig. 1a, $P = 0.01$); and as in Group 1, UmV concentrations were below detection of the assay, again demonstrating 100% placental clearance and thus a nonsaturable mechanism at the UmA concentrations observed, $R = 1.0$.

Effect of labor on umbilical cord blood UCH-L1 concentrations

In Group 1, UmA concentrations of UCH-L1 were 6 times greater than GFAP, but there was no UmA–UmV concentration difference in Groups 1 and 2 (70.2 ± 12 and 70.7 ± 7.2 , 96 ± 32 and 123 ± 29 ng/ml, respectively; Fig. 1b, $P = 0.6$) and thus no evidence of placental clearance. Although UCH-L1 appears to rise in the presence of labor and vaginal delivery (Group 2), this is not significant, $P = 0.09$.

We also calculated the estimated metabolic clearance rate (MCR) for GFAP in Groups 1 and 2 using birth weight and the assumption that UmA blood flow at term gestation is ~170 ml/min-kg.^{23–25} Notably, the MCR was 1037 ± 348 and 3910 ± 120 ng/min-kg for Groups 1 and 2, respectively, increasing nearly 4 times in the presence of labor and vaginal delivery, $P = 0.01$.

Effect of birth asphyxia and NE on umbilical cord blood concentrations of GFAP and UCH-L1

UmA concentrations of GFAP in neonates with NE were nearly 8 times greater than uncomplicated vaginal deliveries and 30 times greater than uncomplicated cesarean deliveries, 181 ± 113 , 23.4 ± 7.1 , and 6.1 ± 2.4 ng/ml, respectively (Fig. 2a; $P = 0.02$, one-way ANOVA). In contrast, UmA UCH-L1 concentrations did not differ in the three groups (Fig. 2b; $P = 0.4$, one-way ANOVA). In Group 3 with NE, UmA concentrations of GFAP and UCH-L1 did not differ in neonates born by emergent cesarean delivery vs. vaginal delivery following labor. As noted earlier, owing to the urgent nature of the deliveries, UmV blood samples were not available for comparison in NE, thus placental clearance could not be assessed.

DISCUSSION

The validity of the use of neurobiomarkers, in particular GFAP and UCH-L1, in the evaluation of the severity of the brain insult in NE

Table 1. Comparison of maternal and neonatal characteristics at term pregnancy for neonates delivered by cesarean section in the absence of labor and vaginally in the presence of labor without encephalopathy and those with encephalopathy

Characteristics	Cesarean delivery (N = 15)	Vaginal delivery (N = 15)	Neonatal encephalopathy (N = 8)
Maternal age (years)	29 ± 5	28 ± 5	32 ± 6
Obstetric gestational age (weeks)	39.3 ± 0.6	39.6 ± 1.2	38.6 ± 1
Gravidity	3 ± 1	2 ± 1	3 ± 1
Race/ethnicity			
White Hispanic	13 (90)	14 (94)	7 (88)
Black	0	1 (6)	1 (12)
White non-Hispanic	2 (10)	0	0
Pregnancy complications	0	0	2 (25)
Birth complications			
Meconium in amniotic fluid	1 (6) ^a	1 (6) ^a	7 (88) ^{b*}
PROM	0 ^a	1 (6) ^a	2 (25) ^{b*}
NRFHP	0 ^a	0 ^a	8 (100) ^{b*}
Pediatric gestational age (weeks)	39.3 ± 0.6	39.6 ± 1.2	38.6 ± 1
Male	8 (65)	6 (55)	6 (75)
Birth weight (g)	3464 ± 407	3422 ± 372	3203 ± 245
Fetal growth classification			
AGA	11 (75)	11 (75)	8 (100)
LGA	4 (25)	4 (25)	0
Apgar score			
1 min	8 (8.9) ^a	8 (8.9) ^a	2 (0.4) ^{b*}
5 min	9 (9.9) ^a	9 (9.9) ^a	3 (2.6) ^{b*}
Resuscitation > PSDS	0 ^a	0 ^a	8 (100) ^{b*}
Umbilical arterial gases			
pH	7.24 ± 0.05 ^a	7.28 ± 0.07 ^a	6.9 ± 0.14 ^{b*}
PCO ₂	59.8 ± 6.7 ^a	48.4 ± 8.4 ^a	89.3 ± 23.4 ^{b*}
Base deficit	-5.62 ± 0.5 ^a	-5.9 ± 0.7 ^a	-20.8 ± 2.3 ^{b*}

Data are presented as means ± SEM or Median (25th, 75th centile)
Numbers in parenthesis are percentage of neonates in each column
*Data across the row were analyzed by one-way ANOVA, $P < 0.05$. Different superscripts (a, b) across rows designate significant differences after one-way ANOVA with Bonferroni correction
AGA appropriate for gestational age, LGA large for gestational age, PROM prolonged rupture of membranes, NRFHP non-reassuring fetal heart pattern, PSDS positioning, suction, drying, and stimulation

due to fetal hypoxia-asphyxia remains unclear as no one has assessed their site of synthesis in the fetal-placental compartment, including the role of the placenta as a potential site of removal and/or synthesis. Key findings in this study are that GFAP is of fetal origin and the placenta is the site of fetal clearance as demonstrated by significant UmA-UmV concentration differences in uncomplicated term cesarean and vaginal deliveries. Moreover, UmA concentrations of GFAP increase in the presence of labor and even more so in pregnancies complicated by moderate-to-severe NE with evidence of fetal asphyxia. Notably, UmA UCH-L1 was

Fig. 1 Concentration differences of glial fibrillary acidic protein (GFAP **(a)** and ubiquitin carboxy-terminal hydrolase L1 (UCH-L1 **(b)**) in umbilical arterial (UmA) and venous (UmV) blood after term uncomplicated cesarean delivery in the absence of labor ($n = 15$) and term vaginal delivery in the absence of complications ($n = 15$). Data analyzed by a two-way analysis of variance, followed by Tukey's post hoc test. GFAP, $P = 0.01$; UCH-L1, $P = 0.09$

unaffected by labor and vaginal delivery as well as fetal asphyxia and there was no evidence of placental clearance. Thus our study findings suggest that UmA concentrations of GFAP may serve as a neurobiomarker for fetal hypoxia-asphyxia and the occurrence of moderate-to-severe NE.

GFAP is considered to be a specific biomarker for neuronal injury and thus a potential measure of the magnitude of severity of brain injury.⁹ In order for a biomarker to have specificity for a disease process or injury, it is essential to determine whether there are non-pathologic elevations and, if so, to determine what they are and the range of those elevations.²⁶ To address this, we examined the effects of labor and vaginal delivery on UmA concentrations of GFAP. To the best of our knowledge, this is the first study demonstrating that GFAP is present in the fetal arterial circulation in the absence of labor and that concentrations increase substantially in the presence of uncomplicated term labor and vaginal delivery. Moreover, it is solely derived from the fetus with no contribution by placental synthesis or maternal transfer since UmV concentrations are below detection of the assay. In fact, in uncomplicated pregnancies the placenta serves as a site for complete removal and thus acts to modulate circulating fetal concentrations through a non-saturable mechanism. This is strikingly similar to interleukin (IL)-6 and IL-8 in uncomplicated vaginal deliveries, which also are fetal in origin, increase during vaginal delivery, and placental clearance modulates circulating concentrations in the fetal compartment.²⁷ Notably, in this instance placental removal may be protective. The mechanism responsible for the placental clearance of GFAP is not known but could reflect transplacental transport, as with IL-6,^{28,29} or placental

Fig. 2 Comparison of umbilical arterial concentrations of glial fibrillary acidic protein (GFAP) and ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) in term neonates delivered by cesarean section ($n = 15$) and vaginal delivery without encephalopathy ($n = 15$) and neonates with encephalopathy ($n = 8$). Data were analyzed by one-way analysis of variance, followed by post hoc test; GFAP, $P = 0.02$; UCH-L1, $P = 0.4$

metabolism as reported for angiotensin II.^{30,31} Further studies are needed to address this.

Increased fetal concentrations of circulating GFAP have previously been reported in neonates with hypoxia–asphyxia^{13,15,17,19}; however, they were measured predominantly in mixed UmA–UmV blood, which does not allow one to determine either the source or the role of the placenta as a site for synthesis and/or clearance. Of interest, in the current study, the estimated GFAP MCR increased nearly three times in the presence of labor and vaginal delivery in uncomplicated pregnancies, demonstrating an association between fetal production in the presence of the stresses of labor and vaginal delivery and possibly alterations in fetal oxygen delivery. More importantly, in the neonates with hypoxia–asphyxia and moderate-to-severe NE, UmA levels of GFAP rose an additional eight-fold, which is consistent with our previous observations of elevated UmA levels in neonates with NE.¹⁵ These observations could reflect a substantial rise in fetal synthesis, a fall in placental clearance due to decreases in fetal–placental blood flow that contribute to poor fetal oxygen uptake and hypoxia–asphyxia, or a combination of the two. This cannot be addressed in the absence of UmV blood to assess clearance or maternal uterine venous blood to assess transplacental transfer. Nonetheless, the fetal brain is most likely the source of UmA GFAP since it is a cytoskeleton intermediate filament protein “specific” to astrocytes and has no relevant extra-cerebral sources.⁹ Thus our data demonstrate that fetal stresses associated with vaginal delivery increase fetal GFAP synthesis and that hypoxia–asphyxia in neonates with moderate-to-severe NE does so in even greater magnitude, suggesting that it may be an excellent biomarker of fetal stress. In particular, these observations suggest that GFAP release may be dependent on cerebral oxygen delivery. However, further studies are needed to confirm this. Notably, we¹⁵ previously observed that serum GFAP at birth increased with increasing severity of NE and that the rise in serum GFAP at 6–24 h after birth was associated with a greater likelihood of neurodevelopmental impairment at 18–24 months. Thus GFAP appears to be an excellent biomarker for the severity of NE.

UCH-L1 is a member of the UCH family and one of the most abundant soluble proteins in the brain, accounting for 1–5% of total soluble protein.^{21,32–35} It also is used as a biomarker for brain injury.^{12,14–19} However, translational studies have documented expression in the decidua of the mouse placenta²⁰ and cytotrophoblasts of the cynomolgus monkey placenta.²¹ In addition, it is found in murine spermatogonia and Sertoli cells^{35–37} and oocytes.³⁴ Thus its source within the fetal compartment may not be limited to the fetal brain. Fetal UCH-L1 is poorly studied in uncomplicated human pregnancy and circulating concentrations, as with GFAP, are primarily based on mixed UmA–UmV blood samples.^{17,19} In contrast to GFAP, there were no UmA–UmV concentration differences for UCH-L1 in the absence or presence of labor in uncomplicated pregnancies, thus no evidence of either

placental clearance or synthesis or maternal transfer. In contrast to GFAP, circulating levels were unchanged in the presence of labor and vaginal delivery as well as moderate-to-severe NE. The latter is consistent with our previous report, wherein we noted highest levels of UCH-L1 in the umbilical cord blood, which did not differ postnatally with severity of NE.¹⁵ Thus UCH-L1 is not a reliable measure of the severity of fetal hypoxia–asphyxia and its source is unclear.

Our study has several strengths, including a well-characterized patient cohort in randomly selected uncomplicated term pregnancies without and with labor that included measurements of placental clearance and consideration of multiple maternal and neonatal variables. In addition, blood samples were collected, prepared, and processed in a manner that minimized any alterations in measurements of the neurobiomarkers, e.g., the serum samples were thawed only for the assay. Limitations of the design include our inability to evaluate the mechanisms that regulate placental clearance of GFAP (placental transfer or metabolism) or the site of UCH-L1 synthesis. Other limitations include the lack of UmV samples in neonates with NE, so we cannot address whether placental GFAP clearance is saturable at the concentrations associated with birth asphyxia. Future studies should address these questions.

We conclude that, although GFAP and UCH-L1 are derived from neuronal tissues, GFAP appears to be a more specific marker of fetal hypoxia–asphyxia and moderate-to-severe NE. Our prior study observed a relationship between levels of GFAP at birth and postnatal that correlated with neurodevelopmental outcome, suggesting that the responses to fetal hypoxia–asphyxia may indeed be dose dependent¹⁵, but that needs further study.

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AUTHOR CONTRIBUTIONS

All authors contributed to drafting the article or revising it critically for important intellectual content and final approval of the submitted version. Specifically, I.N.M. participated in concept, study design, sample and data acquisition and interpretation, performed the statistical analysis and drafted the first version of the manuscript, and finalized the manuscript for submission after comments from the other authors. L.S.B. performed the statistical analysis, participated in data interpretation and review, revision of the manuscript, and reviewed the final version. C.R.R. and L.F.C. participated in concept, study design, data interpretation and review, revision of the manuscript, and participated in finalizing the manuscript after comments from the other authors.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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