Neonatology

Original Paper

Neonatology
DOI: 10.1159/000533473

Received: December 30, 2022
Accepted: July 13, 2023
Published online: September 29, 2023

Neuro-Specific and Immuno-Inflammatory Biomarkers in Umbilical Cord Blood in Neonatal Hypoxic-Ischemic Encephalopathy

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Keywords
Perinatal asphyxia · Umbilical cord blood · Biomarkers · Hypoxic-ischemic encephalopathy

Abstract
Objectives: The aim of the study was to evaluate neuronal injury and immuno-inflammatory biomarkers in umbilical cord blood (UCB) at birth, in cases with perinatal asphyxia with or without hypoxic-ischemic encephalopathy (HIE), compared with healthy controls and to assess their ability to predict HIE. ***Study Design:*** In this case-control study, term infants with perinatal asphyxia were recruited at birth. UCB was stored at delivery for batch analysis. HIE was diagnosed by clinical Sarnat staging at 24 h. Glial fibrillary acidic protein (GFAP), the neuronal biomarkers tau and neurofilament light protein (NFL), and a panel of cytokines were analyzed in a total of 150 term neonates: 50 with HIE, 50 with asphyxia without HIE (PA), and 50 controls. GFAP, tau, and NFL concentrations were measured using ultrasensitive single-molecule array (Simoa) assays, and a cytokine screening panel was applied to analyze the immuno-inflammatory and infectious markers. ***Results:*** GFAP, tau, NFL, and several cytokines were significantly higher in newborns with moderate and severe HIE compared to a control group and provided moderate prediction of HIE II/III (AUC: 0.681–0.827). Furthermore, the levels of GFAP, tau, interleukin-6 (IL-6), and interleukin-8 (IL-8) were higher in HIE II/III cases compared with cases with PA/HIE I. IL-6 was also higher in HIE II/III compared with HIE I cases. ***Conclusions:*** Biomarkers of brain injury and inflammation were increased in umbilical blood in

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cases with asphyxia. Several biomarkers were higher in HIE II/III versus those with no HIE or HIE I, suggesting that they could assist in the prediction of HIE II/III.

Introduction

Perinatal asphyxia is one of the most common causes of neonatal morbidity and mortality. Approximately 2% of deliveries in high-income countries have clinical or biochemical signs of asphyxia [1, 2], and about 0.15% develop some degree of hypoxic-ischemic encephalopathy (HIE) [3].

The use of therapeutic hypothermia (TH) has significantly reduced mortality and adverse neurodevelopmental outcome in infants with moderate and severe HIE [4]. To identify the infants with moderate and severe HIE who are eligible to TH, clinical examination including signs of HIE as well as the infant's pH, base deficit, Apgar score, and in settings where it is available, EEG are assessed. These standard screening methods are not always reliable [5, 6] and clinical grading systems are most accurate beyond the 6-h time window necessary for effective TH [7, 8].

Previous reports show that MRI [9] as well as multiple biochemical biomarkers including brain injury markers glial fibrillary acidic protein (GFAP), tau protein (tau), and neurofilament light protein (NFL) in cerebrospinal fluid or neonatal blood, hours to days after birth, are elevated after moderate and severe asphyxia at least if there is development of HIE [10–12]. GFAP is a protein primarily localized in astroglia, while tau and NFL are structural axonal proteins in neurons. Novel biomarkers continue to be explored in studies, but no marker has been discovered that is superior to the current measures [13]. Biomarkers, detectable in readily accessible body fluids such as umbilical cord blood, to guide diagnoses and prediction, would be a valuable support to clinical decision-making, within the crucial timeframe of starting TH.

The role of inflammation/infection in neonatal HIE is increasingly recognized as being a critical contributor to adverse outcome. Studies show that experimental hypoxia-ischemia or asphyxia in newborns is associated with a marked increase of inflammatory mediators, and exposure to bacterial fragments before hypoxia-ischemia in animal models markedly enhances the injury [14].

There is ongoing research regarding the benefit of TH in mild HIE. Recent studies have shown that survivors of mild HIE have higher rates of disability than expected and have cognitive outcomes similar to those of children with moderate encephalopathy in an uncooled cohort [15], and this could be an indication to distinguish mild HIE as well as moderate and severe HIE.

The aims of this study were first to utilize sensitive analytical assays, to explore if brain injury biomarkers GFAP, tau, and NFL were elevated in umbilical cord blood at birth, in cases with asphyxia with or without HIE, compared with a control group of healthy newborns. Second, we aimed to investigate to what extent immuno-inflammatory and infectious markers in umbilical cord blood are related to the degree of asphyxia.

Materials and Methods

Infants were recruited from two prospective HIE cohorts: the Validation of BIomarkers in Hypoxic Ischemic Encephalopathy (BiHiVE1) study, Ireland (2009–2011), and the BiHiVE2 study, Ireland and Sweden (2013–2015) (Fig. 1; please see detailed description in online supplementary material; for all online suppl. material, see https://doi.org/10.1159/000533473) [16–18].

Sample Collection

Mixed arterial/venous umbilical cord blood was drawn from all infants within 20 min of birth and the samples were processed and stored in a biobank. The samples probably represent mostly venous blood since it is much more difficult to sample sufficient amounts from the artery and arterial blood is often used for acid base/blood gas analysis. Sampling, processing, and storage of samples were conducted in adherence to strict standard operating procedures. In brief, cord blood was collected at delivery and placed in an EDTA tube and kept on ice. The sample was centrifuged at 2,400 *g* for 10 min at 4°C and the plasma was stored at −80°C within 3 h of delivery. We used the HIE cases that had sufficient sample volume remaining in the biobank and then selected the first PA case and control case with available sample that matched with the sex and birthweight of the case in the database. The matching was made manually, +/− 3 days and +/− 300 g. Samples were transported at constant −80°C to Gothenburg for analyses of neuro-specific markers and inflammation/infection markers.

Analysis of GFAP, tau, and NFL

GFAP, tau, and NFL concentrations were measured using commercially available ultrasensitive single-molecule array (Simoa) assays on an HD-X Analyzer according to instructions from the manufacturer (Quanterix, Billerica, MA, USA). The measurements were performed in one round of experiments using one batch of reagents by board-certified laboratory technicians who were blinded to clinical information. Lower limits of quantification were around 1 pg/mL and intra-assay coefficients of variation were below 10%.

Analysis of Cytokines, Chemokines, and Other Protein Biomarkers

A Bio-Plex Pro™ Human Cytokine Screening 48-plex Panel (#12007283, Bio-Rad) was applied according to the manufacturer's instructions. The serum samples were diluted 1:4 in Sample Diluent HB (Bio-Rad). The cytokines were measured on a Bio-Plex 200 Systems (Bio-Rad). Standard series were included in all experiments and used to determine the protein concentrations. Five

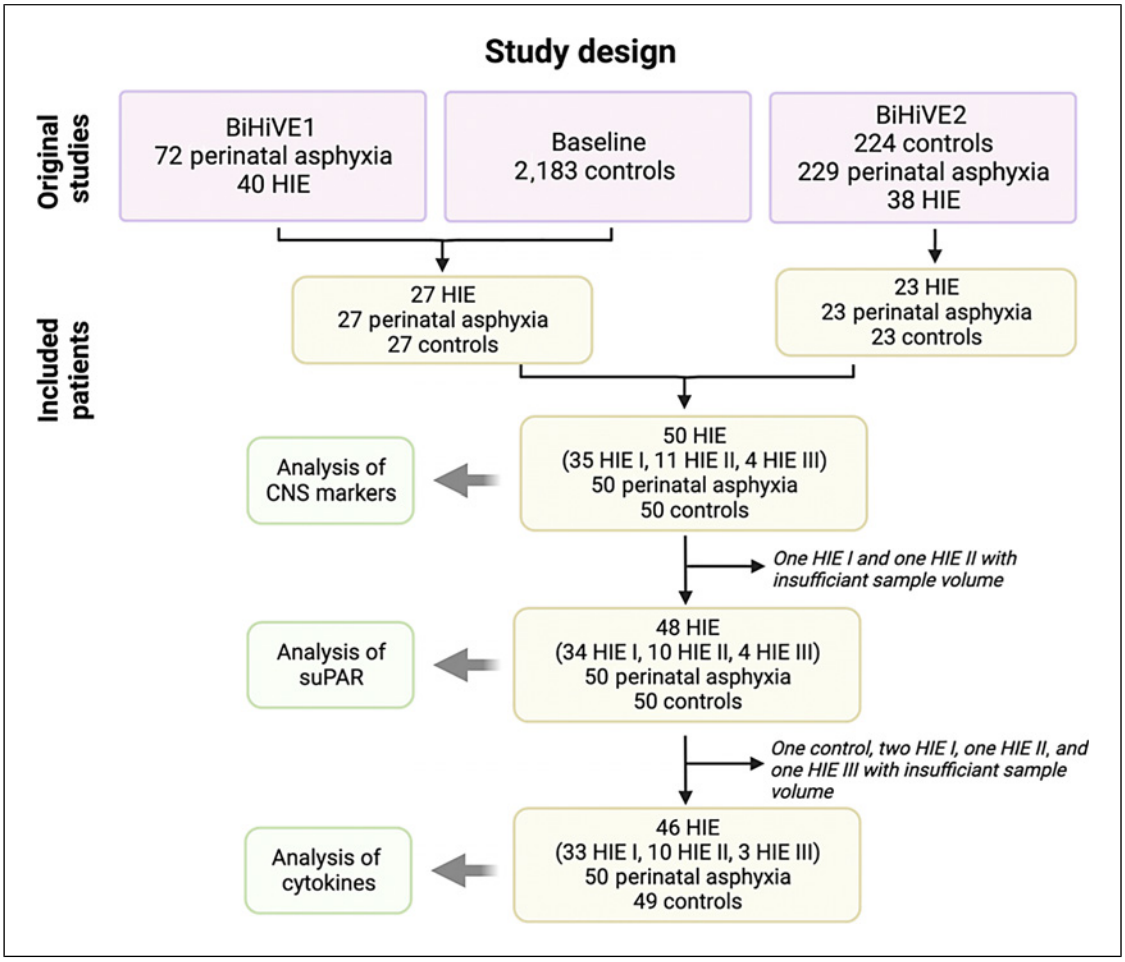

Fig. 1. Flowchart of study design.

cases (one control, two mild HIE, one moderate HIE, and one severe HIE) had insufficient amount of sample volume to allow for analysis of cytokines (Fig. 1). Markers with >25% values under detection limit were excluded. One outlier in hepatocyte growth factor (HGF) and interleukin-6 (IL-6) (defined as +/− 4 SD) were excluded. Six samples in interleukin-16 (IL-16) were excluded due to values below detection limit.

Analysis of suPAR

The suPARnostic®AUTO Flex ELISA (E001, ViroGates) was performed following manufacturer instructions. Two cases had insufficient amount of sample volume to allow for analysis of soluble urokinase plasminogen activator receptor (suPAR) (one mild HIE and one moderate HIE). One outlier in suPAR (defined as +/− 4 SD) was excluded.

Statistical Analysis

Descriptive statistics were carried out using mean and SD for parametric data, median and IQR for nonparametric data for continuous variables, and number and percentage for categorical variables. For group-wise comparisons, the Kruskal-Wallis test (*n* groups) was used. Mann-Whitney test (2 groups) was used as appropriate for continuous variables. The predictive ability of the individual markers for HIE II/III was assessed using area under the receiver operating characteristic curve analysis and calculation of sensitivity and specificity. Statistical analysis was performed using IBM SPSS Statistics 26 and Prism v 8.0.

Results

In total, umbilical cord blood samples from 150 infants were analyzed in this cohort study, 50 term newborns with any grade of HIE were matched for sex, gestational age, and birthweight with 50 PA and 50 control newborns. There were 35 cases with mild HIE (HIE I), 11 cases with moderate HIE (HIE II), and 4 cases with severe HIE (HIE III). From the BiHiVE1 study, there were 27 cases with HIE, 27 cases with asphyxia, and 27 control

Table 1. Demographic details

BiHiVE1 and 2 cohorts	HIE (*n* = 50)	PA (*n* = 50)	Control (*n* = 50)
Gestational age, median (IQR), weeks	40.4 (39.1–41.2)	40.4 (39.7–41.1)	40.0 (38.9–40.9)
Gender (M/F)	29/21	29/21	29/21
Birthweight, median (IQR), kg	3.50 (3.21–3.96)	3.55 (3.22–3.99)	3.52 (3.24–3.97)
Method of delivery, *n* (%)			
VD	8 (16)	15 (30)	21 (42)
Instrumental VD	30 (60)	25 (50)	19 (38)
Elective LSCS	1 (2)	1 (2)	4 (8)
LSCS in labor	0 (0)	0 (0)	3 (6)
Emergency LSCS	11 (22)	8 (16)	3 (6)
Unknown*	0 (0)	1 (2)	0 (0)
First pH, mean (SD)	7.00 (0.135)	7.04 (0.110)	7.26 (0.089)
Missing, %	4	4	52
1-min Apgar median (min/max)	3 (0/8)	4.5 (1/9)	9 (1/10)
5-min Apgar median (min/max)	6 (0/10)	8 (2/10)	10 (7/10)
Sentinel events, *n* (%)	8 (16)	4 (8)	1 (2)
HIE I total *n* = 35	5 (14)		
HIE II total *n* = 11	2 (18)		
HIE III total *n* = 4	1 (25)		
TH treatment, *n* (%)	11 (22)	0 (0)	0 (0)
HIE I total *n* = 35	0 (0)		
HIE II total *n* = 11	9 (82)		
HIE III total *n* = 4	2 (50)		

LSCS in labor: a non-elective caesarean section during labor. EmLSCS: a caesarean section which is done within a few minutes. Sentinel events: prolapsed cord or shoulder dystocia. M, male; F, female; HIE, hypoxic-ischemic encephalopathy; VD, vaginal delivery; LSCS, lower segment cesarean section. *One delivery was noted as unknown in the database.

patients originated from the BASELINE study. From the BiHiVE2 study, there were 23 cases with HIE, 23 cases with asphyxia, and 23 control patients. The results from BiHiVE1 and BiHiVE2 were pooled in the analysis. Demographic data for each group are provided in Table 1.

CNS-Specific Biomarkers

Glial Fibrillary Acidic Protein

The GFAP levels in all HIE cases (HIE I, II, and III) were significantly higher ($p = 0.0005$) than in controls (Table 2). There was an association between the degree of HIE and the level of GFAP ($p = 0.003$; Fig. 2a). The GFAP levels were higher in the HIE II/III group than in controls ($p = 0.001$). GFAP levels in the umbilical cord predicted HIE II/III with an AUC of 0.773 (Table 3). There was a significant difference ($p = 0.02$) in the levels of GFAP in all HIE cases compared to PA cases (Table 2). GFAP levels were also higher in HIE II/III compared to PA/HIE I ($p = 0.03$; Table 2).

Tau Protein

Tau levels were significantly higher ($p = 0.03$) in HIE II/III versus PA/HIE I (Table 2). There was an association with the degree of HIE and the levels of tau ($p = 0.048$; Fig. 2b). Tau levels were higher in HIE II/III versus controls ($p = 0.004$; Table 2). Tau levels in the umbilical cord predicted HIE II/III with an AUC of 0.742 (Table 3).

Neurofilament Light Protein

NFL levels were higher in all HIE cases versus controls ($p = 0.0001$; Table 2). There was an association with degree of HIE and levels of NFL ($p = 0.0005$; Fig. 2c). The NFL levels were higher in HIE II/III versus controls ($p < 0.0001$). NFL levels in the umbilical cord predicted HIE II/III with an AUC of 0.825 (Table 3).

Biomarkers of Infection and Inflammation

Interleukin-6

IL-6 levels were higher in all HIE cases versus controls ($p = 0.008$). There was an association with the degree of HIE and levels of IL-6 ($p = 0.002$; Fig. 2d). The IL-6 levels were higher in HIE II/III versus controls ($p = 0.0002$; Table 2). IL-6 in the umbilical cord predicted HIE II/III with an AUC of 0.827 (Table 3). IL-6 levels were higher ($p = 0.001$) in HIE II/III cases versus PA/HIE I and IL-6 levels were also higher ($p = 0.007$) in HIE II/III versus HIE I (Table 2).

Interleukin-8

Interleukin-8 (IL-8) levels were higher in all HIE cases versus controls (p = 0.04). There was an association with degree of HIE and levels of IL-8 (p = 0.03; Fig. 2e). The IL-8 levels were higher in HIE II/III versus controls (Table 2). IL-8 levels in the umbilical cord predicted HIE II/III with an AUC of 0.794 (Table 3). IL-8 levels were higher (p = 0.01) in HIE II/III cases versus PA/HIE I (Table 2).

Interleukin-16

The IL-16 levels were higher in HIE II/III versus controls (p = 0.03; Table 2). IL-16 levels in the umbilical cord predicted HIE II/III with an AUC of 0.696 (Table 3).

Soluble Urokinase Plasminogen Activator Receptor

The suPAR was higher in all HIE cases versus controls (p = 0.03). There was an association with degree of HIE and levels of suPAR (p = 0.03; Fig. 2f). The suPAR levels were higher in HIE II/III versus controls (p = 0.01; Table 2). The suPAR levels in the umbilical cord predicted HIE II/III with an AUC of 0.720 (Table 3).

For the results of interleukin-2ra (IL-2ra), HGF, macrophage colony-stimulating factor human (M-CSF), platelet-derived growth factor BB (PDGF-bb), and stem cell factor (SCF), please see Tables 2 and 3. There were no differences in TNFα, TNFβ, eotaxin, CTACK, GRO-α, GM-CSF, G-CSF, IFNα2, IFNγ, IL-1β, IL-2, IL-3, IL-4, IL-7, IL-9, IL 10, IL-12, IL-13, IL-17, IL-18, IL-1α, IL-1ra, IL-2ra, PDGF, MIP-1β, MIP-1α, MIG, FGF-β, SDF-1α, SCGF-1β, VEGF, TRAIL, MCP-1, MCP-3, LIF, IP-10, MIF, RANTES, and β-NGF between controls and any subgroups of asphyxia. IL-5, IL-15, and IL-12 (p40) were excluded due to >25% of values were below lowest detection limit.

Discussion

We have shown that the brain injury markers GFAP, tau, and NFL were significantly higher in newborns with moderate and severe HIE compared to a control group. We also found that the levels of IL-6, suPAR, HGF, M-CSF, and PDGF-bb were significantly higher in cases with any grade of HIE compared with a control group. GFAP was able to differentiate between HIE and PA, and GFAP, tau, IL-6, and IL-8 were higher in moderate and severe HIE compared with PA and/or mild HIE.

Table 2. CNS-specific and inflammatory proteins

Biomarker, median (IQR), pg/mL**	Controls	PA	All asphyxia*	HIE I	HIE II	HIE III	All HIE	Controls/ HIE I/HIE II/HIE III, p value	Controls/ all asphyxia, p value	Controls/ PA + HIE I, p value	Controls/ all HIE, p value	Controls/ HIE II + III, p value	PA + HIE I/HIE II + III, p value	HIE I/ HIE II + III, p value	PA/ HIE all, p value	PA/HIE I, p value	HIE I/ HIE II, p value
GFAP	363.2 (155.7–511.7)	400.1 (293.0–533.4)	429.2 (308.1–615.8)	505.8 (282.3–714.6)	560.3 (388.3–698.8)	818.9 (450.1–1114)	525.7 (349.2–720.1)	**0.003**	**0.006**	**0.027**	**0.0005**	**0.001**	**0.03**	0.24	**0.02**	0.13	0.56
TAU	41.5 (21.2–63.1)	47.4 (30.1–93.8)	46.7 (30.6–112.4)	40.0 (29.2–97.3)	114.0 (38.9–179.6)	167.5 (48.3–858.7)	49.8 (31.1–122.9)	**0.048**	**0.04**	0.14	0.05	**0.004**	**0.03**	0.06	0.39	0.81	0.17
NFL	20.4 (15.6–27.5)	27.2 (19.9–37.2)	28.3 (19.8–38.5)	28.0 (18.8–38.6)	32.8 (23.3–34.2)	51.4 (29.9–76.7)	29.6 (19.5–39.2)	**0.0005**	**0.0001**	**0.0008**	**0.0001**	**<0.0001**	0.11	0.11	0.58	0.92	0.33
IL-2ra	103 (91.3–142.6)	125.0 (95.7–146.8)	123.1 (97.2–146.4)	111.9 (89.3–141.4)	126.7 (116.8–158.6)	138.0 (95.2–162.6)	118.7 (97.9–142.9)	0.26	0.15	0.29	0.28	**0.048**	0.19	0.10	0.70	0.31	0.12
IL-6	10.3 (6.2–14.1)	10.9 (8.0–18.7)	12.3 (8.4–22.2)	11.3 (7.4–22.5)	18.0 (14.2–27.2)	68.0 (40.0–89.1)	14.4 (9.3–25.1)	**0.002**	**0.02**	0.12	**0.008**	**0.0002**	**0.001**	**0.007**	0.11	0.82	0.07
IL-8	23.4 (18.6–28.8)	23.4 (18.8–32.0)	25.8 (19.5–34.7)	24.8 (18.0–41.6)	35.7 (27.9–52.5)	34.1 (31.2–43.2)	29.8 (20.4–43.4)	**0.03**	0.14	0.44	**0.04**	**0.0008**	**0.01**	0.09	0.13	0.66	0.14
IL-16	72.3 (44.7–103.9)	80.0 (48.0–141.2)	88.3 (54.9–142.1)	75.6 (53.6–142.2)	95.8 (83.2–138.4)	448.6 (49.1–555.3)	91.9 (56.3–143.4)	0.19	0.19	0.37	0.10	**0.03**	0.14	0.24	0.37	0.76	0.46
HGF	439.8 (351.0–637.8)	571.5 (452.1–670.9)	613.3 (457.1–778.5)	675.3 (449.8–857.6)	620.5 (475.0–804.1)	1,345.9 (624.6–4,955.3)	647.6 (475.8–878.2)	**0.002**	**0.0006**	**0.002**	**0.0002**	**0.004**	0.22	0.65	0.054	0.12	0.77
suPAR**	3.70 (3.24–4.27)	4.05 (3.42–4.48)	4.05 (3.50–4.64)	3.94 (3.45–4.60)	3.94 (3.47–5.22)	4.65 (4.45–6.16)	4.05 (3.55–4.80)	**0.03**	**0.04**	0.11	**0.03**	**0.01**	0.11	0.20	0.41	0.88	0.65
M-CSF	32.9 (26.6–39.7)	36.3 (29.3–45.9)	35.6 (30.0–45.5)	34.7 (30.0–43.6)	38.8 (34.0–55.5)	40.4 (28.2–66.0)	35.2 (30.5–46.1)	0.10	**0.03**	0.08	**0.04**	**0.015**	0.16	0.16	0.76	0.77	0.17
PDGF-bb	2,069.2 (882.8–3,436.6)	2,701.3 (1,380.6–4,384.2)	2,787.4 (1,546.8–4,535.5)	2,936.3 (1,852.2–4,650.9)	2,408.4 (1,280.0–3,829.4)	3,221.0 (1,593.9–5,120.5)	2,861.2 (1,619.0–4,595.8)	0.17	**0.04**	**0.04**	**0.046**	0.47	0.68	0.48	0.53	0.38	0.37
SCF	107.7 (93.1–130.2)	119.6 (102.8–145.6)	120.3 (96.7–151.1)	117.8 (94.9–149.4)	135.7 (98.3–189.5)	171.1 (94.7–182.2)	120.3 (94.9–156.0)	0.24	0.08	0.15	0.17	**0.045**	0.13	0.12	0.94	0.55	0.21

*All asphyxia (PA, HIE I, HIE II, HIE III). **suPAR ng/mL.

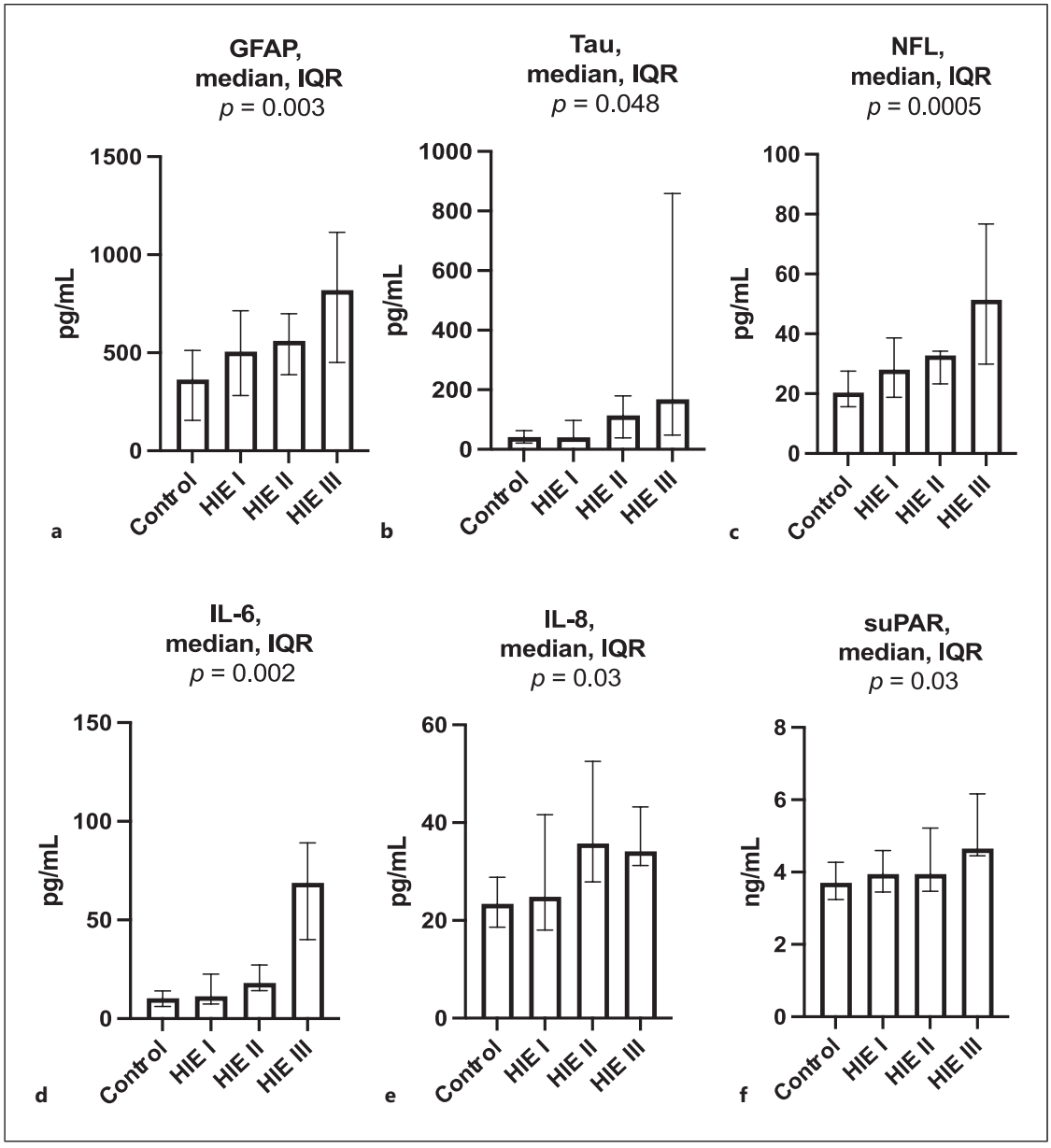

Fig. 2. a–f Biomarker levels in controls, HIE I, HIE II, and HIE III expressed as medians and interquartile range. The difference between medians in the groups was analyzed with Kruskal-Wallis test.

Previous reports of GFAP in umbilical cord blood have shown contradictory results [19, 20]. Chalak et al. [19] reported significantly raised levels of GFAP in umbilical cord blood in neonates with moderate and severe HIE (median 0.05 ng/mL) compared to mild HIE (median 0.008 ng/mL). Looney et al. [20] demonstrated no significant difference in GFAP levels between control cases and HIE in cord blood. In our study, we found that GFAP was significantly higher versus both controls and PA. One reason for the contradictory results could be that Looney et al. used different methods for analyzing GFAP (enzyme-linked immunosorbent assay), whereas we used ultrasensitive Simoa.

We found that the neuronal injury biomarker tau was significantly higher in the group with moderate and severe HIE. A pilot study from our research group has shown that tau was significantly higher in cord blood in a group of neonates with asphyxia compared to a control

Table 3. Area under the curve

Biomarker	AUROC controls versus HIE II + III (95% CI)	Threshold level	Sensitivity, %	Specificity, %
GFAP	0.773 (0.647–0.900)	>348.2	93	44
TAU	0.742 (0.574–0.911)	>37.3	80	44
NFL	0.825 (0.701–0.949)	>30.1	73	88
IL-6	0.827 (0.723–0.932)	>14.4	85	78
IL-8	0.794 (0.650–0.937)	>28.7	85	76
IL-16	0.696 (0.550–0.842)	>66.3	77	45
HGF	0.757 (0.625–0.890)	>446.7	92	52
suPAR	0.720 (0.559–0.881)	>3.6	79	43
M-CSF	0.718 (0.583–0.854)	>32.7	85	47
SCF	0.681 (0.503–0.859)	>103.4	69	45

group [21]. Interestingly, tau was one of the few markers which showed a trend to differ between mild and moderate HIE, making it potentially useful in aiding clinical decision-making in the first 6 h of life.

The other neuronal injury biomarker NFL was significantly higher in the group with moderate and severe HIE and in the PA group compared to the control group. NFL has been shown to be elevated in CSF of asphyxiated infants [11] as well as in cord blood in neonates with asphyxia [21]. We are the first group to examine NFL levels in infants with PA who did not progress to HIE. We did not find any difference between PA and HIE indicating that NFL will not be as helpful as a stand-alone marker for determining eligibility for TH.

Previous studies on cytokines in cord blood report that IL-6 and IL-16 have been elevated in newborns with HIE compared to controls [22–24]. We confirmed these results in our study. However, the greatest rise in IL-6 and IL-16 was seen in the infants with severe HIE and less differentiation was seen between mild and moderate infants.

The biomarker suPAR is the soluble form of the cell membrane-bound protein uPAR. uPAR is expressed mainly on immune cells, endothelial cells, and smooth muscle cells. and it is released during immune activation rather than hypoxia/ischemia. To the best of our knowledge, this is the first report that correlates the level of suPAR with HIE. The greatest increase was again seen in those with severe HIE. Indeed, both experimental and clinical studies suggest that infections could be an important antecedent to the development of asphyxia and sensitize to the development of brain injury and increase the risk of cerebral palsy [25–27].

The strength of this study is the relatively large number of neonates with HIE, in total 50 cases, prospectively recruited, with matching umbilical cord blood samples from infants with both a group of asphyxia without HIE (PA) and healthy controls. Another strength is the high sensitivity of the analytical methods used detecting pg levels in μL amounts of plasma allowing us to accurately detect alterations in protein markers. Limitations are the moderate sample size and lack of a validation cohort. In particular, the number of cases with severe HIE (n = 4) was limited. Since this was an exploratory study, limited information was available upon which to base a power calculation. In a real world setting, the ratio of PA to HIE would be closer to 4:1, rather than 1:1. The ratio of controls to HIE would be 1,000:1–2, so it is not really possible to examine real-world prediction in an exploratory biomarker study. Another limitation is that chorioamnionitis was not an exclusion criterion. The majority of infants were screened for sepsis during their stay, but placental histology was not available. Sepsis screening was based on clinician discretion. However, only one of the cases in the PA group had a diagnosis of culture-negative sepsis. None had culture-confirmed sepsis.

In exploring the clinical utility of these markers, several markers were higher in the cases with the moderate-severe HIE compared to healthy controls, and in comparison, to mild HIE. Interestingly, we found in this study that IL-6 and to some extent tau showed the greatest potential to separate those groups. IL-6 and tau could therefore be interesting to study in the future. GFAP levels showed the best ability to differentiate between PA and all grades of HIE suggesting that GFAP in umbilical cord blood could be helpful in settings where TH is offered to all grades of HIE. Further studies are needed with a greater number of HIE and PA cases before we can prove that these biomarkers are clinically useful. Once a clinically useful marker is established, translation to clinical use will require the development of testing methods with a rapid turnaround or ideally point-of-care potential.

Acknowledgment

Dr. Karin Sävman is gratefully thanked for advice in the design of the study.

Statement of Ethics

The Clinical Ethics Committee of the Cork Teaching Hospitals approved both studies (ECM-4 (Q)05/05/09 and ECM-4 09/01/2013) and the Regional Ethical Review Board in Stockholm also approved the multicentre validation study. Written informed consent was obtained from parents of all study participants.

Conflict of Interest Statement

H.Z. has served at scientific advisory boards and/or as a consultant for AbbVie, Alector, ALZPath, Annexon, Apellis, Artery Therapeutics, AZTherapies, CogRx, Denali, Eisai, NervGen, Novo Nordisk, Pinteon Therapeutics, Red Abbey Labs, reMYND, Passage Bio, Roche, Samumed, Siemens Healthineers, Triplet Therapeutics, and Wave; has given lectures in symposia sponsored by Cellectricon, Fujirebio, AlzeCure, Biogen, and Roche; and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work). K.B. has served as a consultant, at advisory boards, or at data monitoring committees for Abcam, Axon, BioArctic, Biogen, JOMDD/Shimadzu. Julius Clinical, Lilly, MagQu, Novartis, Ono Pharma, Pharmatrophix, Prothena, Roche Diagnostics, and Siemens Healthineers, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program, outside the work presented in this paper.

Funding Sources

This work was supported by Rune and Ulla Almlövs stiftelse SEB (Y.C., 2019), Mary Von Sydow (Y.C., 2019-2219), Frimurarfonden Barnhusdirektionen Foundation (Y.C., GLS 7000991), Wilhelm and Martina Lundgren Foundation (Y.C., 2019-2941), Linnea and Josef Carlsson Foundation (Y.C., 2019), and VR 2019-01320 (H.H.). The study was also financed by grants from the Swedish state under the agreement between the Swedish government and the county councils, the ALF-agreement (Y.C.: ALFGBG-77860, ALFGBG-75710, H.H.: ALFGBG-965174, H.Z.: ALFGBG-71320). The BiHIVE study was funded by the Molecular Medicine Ireland Clinical Translational Scholars Award. The BiHIVE2 study was funded by the Irish Health Research Board (ref CSA 2012/40). H.Z. is a Wallenberg Scholar supported by grants from the Swedish Research Council (#2018-02532), and the European Research Council (#681712 and #101053962). K.B. is supported by the Swedish Research Council (#2017-00915), Hjärnfonden, Sweden (#FO2017-0243 and #ALZ2022-0006) and the Swedish state under the agreement between the Swedish government and the County Councils, the ALF-agreement (#ALFGBG-715986 and #ALFGBG-965240).

Author Contributions

Hanna Toorell has contributed to the methodology, investigation, data curation, formal analysis, and writing of the initial manuscript. Ylva Carlsson has contributed to design of the study, investigation, supervision, data curation, formal analysis, and writing and reviewing the manuscript. Boubou Hallberg has contributed to design of the study, funding acquisition, and reviewing the manuscript. Mairead N. O'Riordian has contributed to data curation. Brian Henry Walsh has contributed with design of the study, methodology, funding acquisition, and reviewing of the manuscript. Marc Paul O'Sullivan has contributed to the methodology, data curation, and reviewing of the manuscript. Geraldine B. Boylan has contributed to the methodology, investigation, funding acquisition, data curation, and reviewing of the manuscript. Henrik Zetterberg has contributed to design of the study, methodology, investigation, funding acquisition, formal analysis, and reviewing of the manuscript. Kaj Blennow has contributed to the methodology, supervision, funding acquisition, and reviewing of the manuscript. Deirdre Murray has contributed to design of the study, investigation, supervision, funding acquisition, data curation, formal analysis, and reviewing of the manuscript. Henrik Hagberg has contributed to design of the study, investigation, supervision, funding acquisition, data curation, formal analysis, and writing and reviewing of the manuscript.

Data Availability Statement

The data that support the findings of this study are not publicly available due to their containing information that could compromise the privacy of research participants but are available pseudonymized from the corresponding author (H.T.).

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