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ORIGINAL ARTICLE

Proteomic profiles in cerebrospinal fluid predicted death and disability in term infants with perinatal asphyxia: A pilot study

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Abstract

Aim: Perinatal asphyxia, resulting in hypoxic-ischaemic encephalopathy (HIE), has been associated with high mortality rates and severe lifelong neurodevelopmental disabilities. Our aim was to study the association between the proteomic profile in cerebrospinal fluid (CSF) and the degree of HIE and long-term outcomes.

Methods: We prospectively enrolled 18-term born infants with HIE and 10-term born controls between 2000 and 2004 from the Karolinska University Hospital. An antibody suspension bead array and FlexMap3D analysis was used to characterise 178 unique brain-derived and inflammation associated proteins in their CSF.

Results: Increased CSF concentrations of several brain-specific proteins were observed in the proteome of HIE patients compared with the controls. An upregulation of neuroinflammatory pathways was also noted and this was confirmed by pathway analysis. Principal component analysis revealed a gradient from favourable to unfavourable HIE grades and outcomes. The proteins that provided strong predictors were structural proteins, including myelin basic protein and alpha-II spectrin. The functional proteins included energy-related proteins like neuron-specific enolase and synaptic regulatory proteins. Increased CSF levels of 51 proteins correlated with adverse outcomes in infants with HIE.

Conclusion: Brain-specific proteins and neuroinflammatory mediators in CSF may predict HIE degrees and outcomes after perinatal asphyxia.

KEYWORDS
biomarkers, cerebrospinal fluid, hypoxic-ischaemic encephalopathy, perinatal asphyxia, protein profile

1 | INTRODUCTION

Four million infants experience perinatal asphyxia, leading to hypoxic-ischaemic encephalopathy (HIE), each year. HIE is one of the most common contributors to early neonatal mortality.[1] The incidence of moderate to severe HIE is 1–3 per 1,000 live births in high-income countries.[2] Hypoxic-ischaemic brain damage is a complex process that represents an evolving cascade of harmful events. The

Abbreviations: CSF, cerebrospinal fluid; HIE, hypoxic-ischaemic encephalopathy; IQR, interquartile range.

primary phase of the injury, during exposure to hypoxic ischaemia, is followed by a latent phase, where the cerebral oxidative metabolism may partially or completely recover. Therapeutic hypothermia is the only treatment that is available for moderate to severe HIE. It is applied during the latent phase, to ameliorate the secondary phase of progressive energy failure and brain cell death.[3] However, the neuroprotective effect of therapeutic hypothermia is limited. The mortality rate is still high and infants that survive may face lifelong disabilities, including cerebral palsy, epilepsy and cognitive impairment. Accumulating evidence indicates that inflammation contributes to a prolonged hypoxic-ischaemic brain injury, which may last for months or even years. This, in turn, provides the potential for adjunctive treatment options at later stages.[4]

Reliable biomarkers that reflect the complex pathology of HIE could facilitate the evolution of targeted neuroprotective treatment approaches and provide early identification of the patients that could be at risk of long-term sequelae. It has been suggested that various biomarkers may be useful when it comes to predicting outcomes of neonatal HIE, but none of these have been established in clinical settings.[5] Affinity-based proteomic techniques offer a novel insight into the underlying pathophysiology of brain disease. It enables large numbers of proteins to be simultaneously analysed in small samples. Protein arrays have been used in preclinical and clinical studies of adults with traumatic brain injuiries.[6,7] They have also been used for protein profiling of cerebrospinal fluid (CSF) from preterm infants.[8] The present study used antibody suspension bead array technology to assess the levels of brain enriched proteins and known inflammatory mediators[9] in the CSF of infants with HIE. We then compared these results with non-asphyxiated infants, who formed the control group.

The study had two aims. First, we aimed to evaluate the use of protein arrays in predicting long-term outcomes following perinatal asphyxia. Secondly, we wanted to discover novel biomarkers for bedside use when treating these patients.

Key notes

- Cerebrospinal fluid (CSF) proteomes following perinatal asphyxia provide valuable information on the pathogenesis and prognosis of brain injuries.
- This study identified a proteomic profile in CSF following perinatal asphyxia, representing upregulation of neuroinflammatory pathways and various pathological cascades of hypoxic brain injuries.
- Several of these novel proteins correlated with the degree of hypoxic-ischaemic encephalopathy (HIE) and unfavourable outcomes and had a diagnostic and predictive value for HIE.

2 | PATIENTS AND METHODS

2.1 | Study population

We prospectively enrolled 18 term-born infants with perinatal asphyxia from the neonatal intensive care unit at the Karolinska University Hospital in Stockholm, Sweden, between October 2000 and September 2004. The controls were 10 term-born infants without a history of asphyxia from the Hospital's general medical neonatal ward. The infants were included in the asphyxia group if they had undergone clinically indicated lumbar punctures and fulfilled the criteria for perinatal asphyxia, by showing signs of foetal and postnatal distress. These included foetal bradycardia or decelerations on cardiotocographic registration and a pH of <7.1 or a lactate of >4.8 in scalp blood. The Apgar score needed to be under 6 at 5 min and their umbilical arterial blood, or blood collected within an hour of birth, needed to have a pH of ≤7.00 and/or a base deficit of ≥16 mEq. The inclusion criteria included resuscitation for more than 3 min. The infants also had to have clinical signs of encephalopathy within 6 h of birth, in accordance with the National Institute of Child Health and Human Development classification for modified Sarnat staging.[10]

All patients with asphyxia received supportive care under normothermic conditions, which was the standard treatment at the time of recruitment. The exclusion criteria were encephalopathy related aetiologies other than birth asphyxia. These included metabolic diseases and chromosomal abnormalities, as well as confirmed meningitis. The control infants underwent lumber punctures for suspected, but unverified, infections. They all had elevated C-reactive protein in their blood and displayed clinical symptoms that could represent an infection, in conjunction with negative bacterial blood and CSF cultures.

2.2 | Clinical evaluation

Neurological assessments were performed on all patients and controls, according to the Sarnat and Sarnat criteria,[10] before they were enrolled and these were repeated on day 7 of life. The neurological assessment was repeated on the HIE patients at 12, 36 and 72 h of age in the neonatal intensive care unit. All assessments were performed by the same neonatologist.

A neurodevelopmental follow-up was performed by an experienced paediatric neurologist, who examined all the surviving patients at 3, 6 and 18 months of age. The patients who had signs of abnormal neurodevelopment at 18 months of age were assessed with the Bayley Scales of Infant and Toddler Development, Second Edition.[11] Adverse neurological outcomes were defined as: cerebral palsy, a seizure disorder, a mental developmental index of <85 or being deaf or blind at the 18-month assessment. Information was gathered from outpatient paediatric care centres on the outcomes of the control group when they were 18 months of age. Some of the clinical characteristics of a subgroup of the recruited infants have previously been published.[12]

TABLE 1 Clinical characteristics of asphyxiated neonates and non-asphyxiated controls

	n	GA (week)	Birth weight (kg)	Gender (♀:♂)	APGAR5 (score)*	APGAR10 (score)*	Arterial pH*	BE*
Controls	10	40 (38.8 to 41.7)	3.6 (2.8 to 4.6)	5:5	10 (8 to 10)	10 (9 to 10)	7.4 (7.13 to 7.4)	−2.5 (−4 to 0)
HIE, normal outcome	5	41 (40.3 to 41.9)	3.5 (2.8 to 4.2)	2:3	5 (4 to 6)	6.5 (6 to 7)	7.0 (6.9 to 7.1)	−13 (−18 to −8)
HIE, adverse outcome	13	40 (38.6 to 41.6)	3.6 (2.9 to 4.5)	7:6	3 (0 to 6)	4.5 (2 to 6)	6.7 (6.55 to 7.0)	−22 (−30 to −16)

Note: Data are presented as median (IQR). *$p < 0.0001$.

Gestational age, weight, sex and maternal infection didn't differ between groups. As expected, the HIE group had a lower Apgar score and blood gas pH values, compared with non-asphyxiated infants (*$p < 0.001$).

2.3 | CSF analysis

The CSF samples were obtained within the first 3 days of life. The median times and interquartile ranges were 22.5 (15–42) hours after birth for the asphyxiated infants and 26 (13.3–48) hours for the controls. The samples were spun at 3,000 rpm for 10 min and then the supernatants were stored at −80°C until they were analysed.

Antibody suspension bead array technology was used to conduct a comprehensive profiling of the protein expression in the CSF samples taken following perinatal asphyxia. The suspension bead was created from 220 antibodies, which were the affinity reagents, and these targeted the 178 unique proteins, selected from the Human Protein Atlas (Science for Life Laboratory, Stockholm, Sweden)[13] (Table S1). The proteins that were selected had known associations with hypoxic brain injuries and there were previous indications that they had been used as brain injury biomarkers. The selected proteins all provided high tissue enrichment in the central nervous system and were involved in different brain functions.[6,7] The FlexMap3D instrument (Luminex Corp, Texas, USA) was used to analyse cross-linked interacting proteins in the antibody suspension bead. The relative abundance of proteins is reported as the median fluorescent intensities for each sample and bead identity. Further methodological details can be found in Lindblad et al[7] and Appendix S1.

2.4 | Statistics

Clinical and laboratory variables are presented as medians and IQRs. The Mann–Whitney U test was used to compare the independent groups. The results of the protein array analysis have been reported as median fluorescent intensities and IQRs for each sample and antibody. We also used the Mann–Whitney U test to analyse the differences in CSF protein profiles between the patients and controls. No normalisation was performed, due to the low numbers of samples, and this meant that raw median fluorescent intensity data were used. To simplify, we further calculated log2- transformed fold changes of protein levels, visualised as a Volcano plot, to analyse the differences between patients and controls.

We performed principal component analysis to reduce the number of dimensions spanned by the 178 proteins that we measured. The analysis was carried out in R, version 4.0.3 (R Foundation, Vienna, Austria), with the FactoMineR package, version 1.34 (R Foundation).[14] The patients were grouped by their HIE grades and outcomes. The projections of loadings onto the line of best fit were used as a measure of the contribution of each protein to the separation between patients, according to their HIE grade and outcome, respectively.

We compared the groups of patients with adverse outcomes, patients with normal outcomes and controls, using the Kruskal-Wallis test and then used Dunn's multiple comparison test to show differences in the rank sums. The sequentially rejective Bonferroni was used to control for the false discovery rate of multiple testing.[15] The

differences were considered statistically significant if the p value was <0.05. The results are presented on scatter plots that show the differences in protein levels. Pathway analysis was conducted using the R pathfinder package (R Foundation).[16] All proteins were eligible for analysis and the threshold for being included in the input was $p < 0.05$. The enrichment analysis was performed using the BioCarta gene set (BioCarta, Charting pathways of life. http://www.biocarta.com) and results were filtered at $p < 0.05$ after Bonferroni adjustment. The BioGRID (Biological General Repository for Interaction Datasets, thebiogrid.org) protein-protein interaction network was used.

2.5 | Ethics

This study was performed in accordance with European Community guidelines and the Declaration of Helsinki. It was approved by the regional ethics committees at the Karolinska Institute and Stockholm County (Dnr 98–246, 2003–174, 2011/1891-31). Informed, written consent was obtained from the parents of the enrolled patients.

3 | RESULTS

The patient characteristics are summarised in Table 1. This shows that 7 patients had severe HIE (HIE-III), 7 had moderate HIE (HIE-II) and 4 had mild HIE (HIE-I), according to the Sarnat et Sarnat classification of clinical signs.[10] Five patients died during the neonatal period, 8 patients had survived with adverse neurological outcomes by the time of the 18-month follow-up evaluation and 5 patients had normal outcomes. All the non-asphyxiated infants in the control group had normal outcomes.

The relative protein abundance detected in the CSF samples was measured as median fluorescent intensities for each antibody. A distinct CSF proteome, which reflected hypoxic-ischaemic brain injury characteristics, was observed following asphyxia, as several unique proteins were altered in CSF compared with controls (Figure 1A). A differential analysis was performed to compare the outcome groups with regard to the clinical importance of the protein signature in the CSF following perinatal asphyxia. This found that 51 unique CSF proteins correlated with unfavourable outcomes (p value <0.05) (Figure 1B, Table S2). Furthermore, there was an upregulation of neuroinflammatory pathways in CSF following asphyxia, which demonstrated a distinct inflammatory profile compared to the non-asphyxiated controls. Pathway analysis confirmed the importance of immune related proteins (Figure 1C-D). The complement pathway was the most important pathway when it came to discriminating between both HIE grades and outcomes.

A principal component analysis was applied to the data to reduce the number of dimensions spanned by all of the proteins (Figure 2A-B). When the data were grouped by HIE grade and outcomes, both revealed almost identical paths and these created similar gradients from favourable to unfavourable HIE grades and outcomes, respectively. The projections of loadings onto a line of best fit of the centroids are outlined in Table S3. This effectively measured the contribution of each protein to the separation of the data along the favourable to unfavourable gradient. These have been expressed as alpha coefficients. A strong correlation was observed between the principal component analysis alpha coefficients for the proteins that contributed strongly to the differences in data. These were evident in both the fold changes and the p-values on the volcano plot, (Figure 2C-D). Several proteins made a high contribution to the separation between the groups we examined. These included structural proteins, like myelin basic protein and alpha spectrin-II. They also included proteins related to the energy turnover of cells and hypoxic regulation, like neuron-specific enolase, Aldolase C and the ATPase H^+ transporting V1 subunit G2. The list also comprised several synaptic regulating proteins. Table 2 displays the proteins that exhibited the biggest changes in median fluorescent intensities and fold changes between the patients with unfavourable outcomes and control infants (p value <0.005). No differences in median fluorescent intensities were seen between the proteins in patients with normal outcomes and the control infants, apart from beta-synuclein, which was higher in patients than in controls (data not shown). The four proteins that differed most between outcome groups are show in Figure 3A-D.

4 | DISCUSSION

We used an antibody array to analyse 178 proteins related to the central nervous system and inflammation in CSF samples from infants with perinatal asphyxia and non-asphyxiated controls infants. This sensitive measure of the composition CSF proteins identified differences in the concentrations of 51 proteins that correlated with death or adverse neurological outcomes following perinatal asphyxia. The protein profiles that we observed reflected biochemical changes in the CSF, which is in direct contact with the extracellular matrix of the brain, as opposed to blood analyses, which may not reflect events in the central nervous system.[17] Proteins that indicated brain injuries were identified and a clear relationship was determined between the protein concentrations and both the HIE grades and outcomes in patients.

4.1 | Metabolic proteins

We confirmed previous studies that highlighted the importance of proteins that are related to the metabolism of brain cells in hypoxic brain injuries, including neuron-specific enolase, Aldolase C and ATPase H^+ transporting V1 subunit G2. Neuron-specific enolase, which is involved in glycolytic energy metabolism, is an established brain-specific marker of neuronal damage[18] and has been correlated with the risk of death or severe neurological impairment in HIE.[19] It is a commonly used biomarker for traumatic brain injuries[20] and is used in guidelines for managing cerebral anoxia following cardiopulmonary resuscitation in adults, where increasing levels in serum predict an

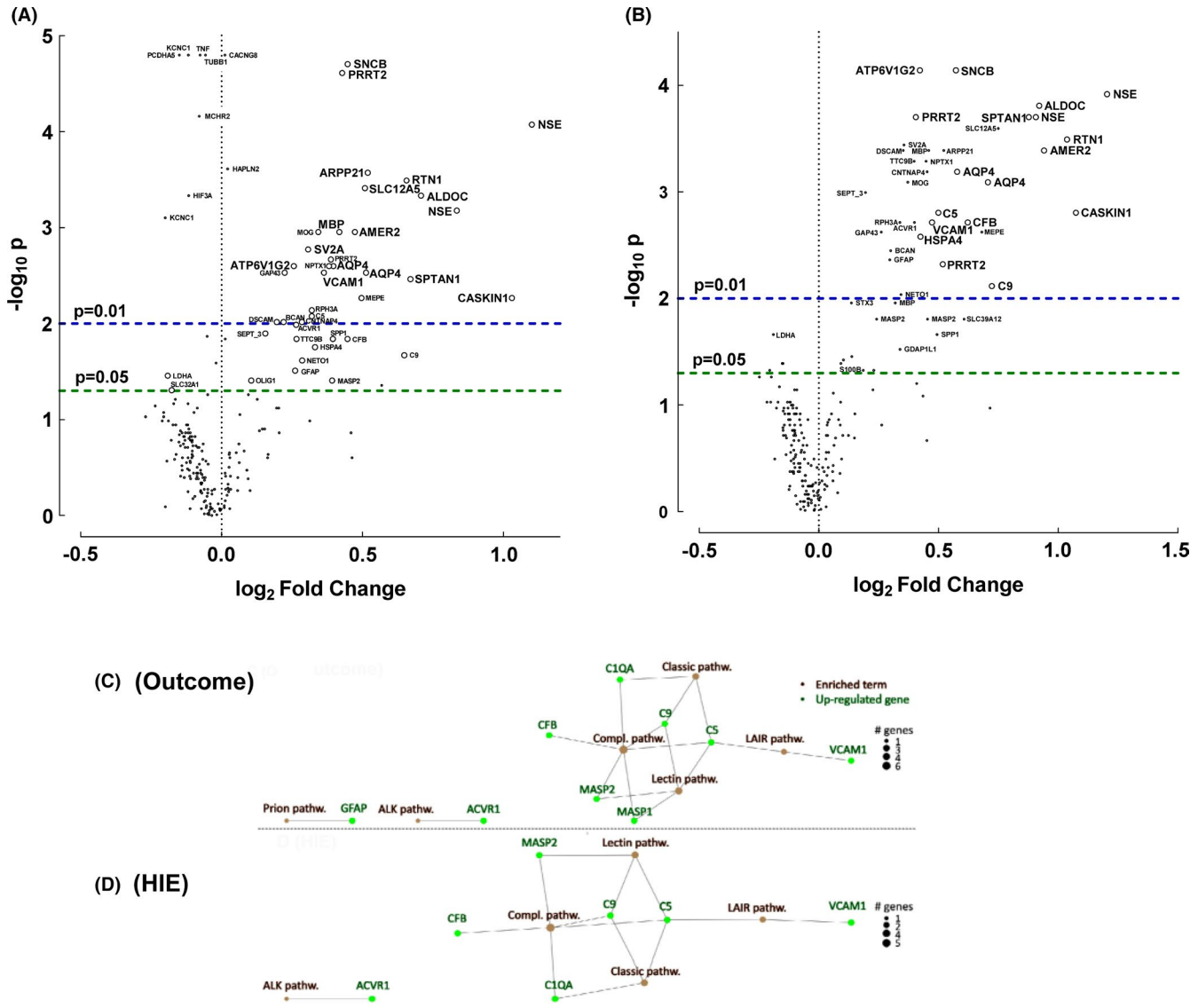

FIGURE 1 Volcano plots describe the relative abundances of CSF proteins in infants with hypoxic-ischaemic encephalopathy (HIE) and controls. The Mann–Whitney U test was used to calculate the differences in median fluorescent intensity (MFI) for each analyte, transform them into $\log_2$ fold changes, which are above zero when increased in HIE, and plot them against $-\log_{10}$ p-values. Figure 1A describes the differences between the 18 HIE patients and 10 controls. Figure 1B describes the differences between HIE patients with adverse outcome and the controls. Pathway analysis shows the upregulation of neuroinflammatory proteins in CSF following perinatal asphyxia, reflected in lectin, complement and classical pathways. The complement pathway was the most important pathway in both outcomes (C) and HIE grades (D)

unfavourable outcome.[21] Secondary ischaemic injuries are common following severe traumatic brain injuries.[22] These are probably due to the deranged cerebral metabolism caused by a regional cerebral mismatch between perfusion and metabolic demand. This is a pathology shared with anoxic injuries and presumably with HIE as well. Aldolase C, a primarily astrocytic protein, is released when there is an astrocyte injury. It has been indicated as a marker of brain damage following traumatic brain injuries and hypoxic ischaemia in animal models.[6,23] A proteomic screening of human adult CSF following traumatic brain injury identified Aldolase C as one of the most promising biomarkers of cell death and functional outcome.[24] This could have a clinical use in HIE. ATPase H^+ transporting V1 subunit G2, which is involved in cell metabolic turnover, has been associated with chronic and progressive traumatic brain injuries with a delayed onset of symptoms.[25] These proteins might indicate the metabolic derangement preceding the secondary phase of a hypoxic-ischaemic brain injury, which leads to mitochondrial impairment and eventually apoptotic neuronal death. This might be of value in clinical decision making, because this time point in the pathological process has been referred to as the window of opportunity for therapeutic interventions.[26] Metabolic derangements during hypoxic ischaemia may lead to disrupted synaptic function, which can induce excitotoxicity and exacerbate brain damage.

4.2 | Synaptic proteins

Several synaptic associated regulatory proteins were increased in our study and correlated with adverse outcomes. None of these

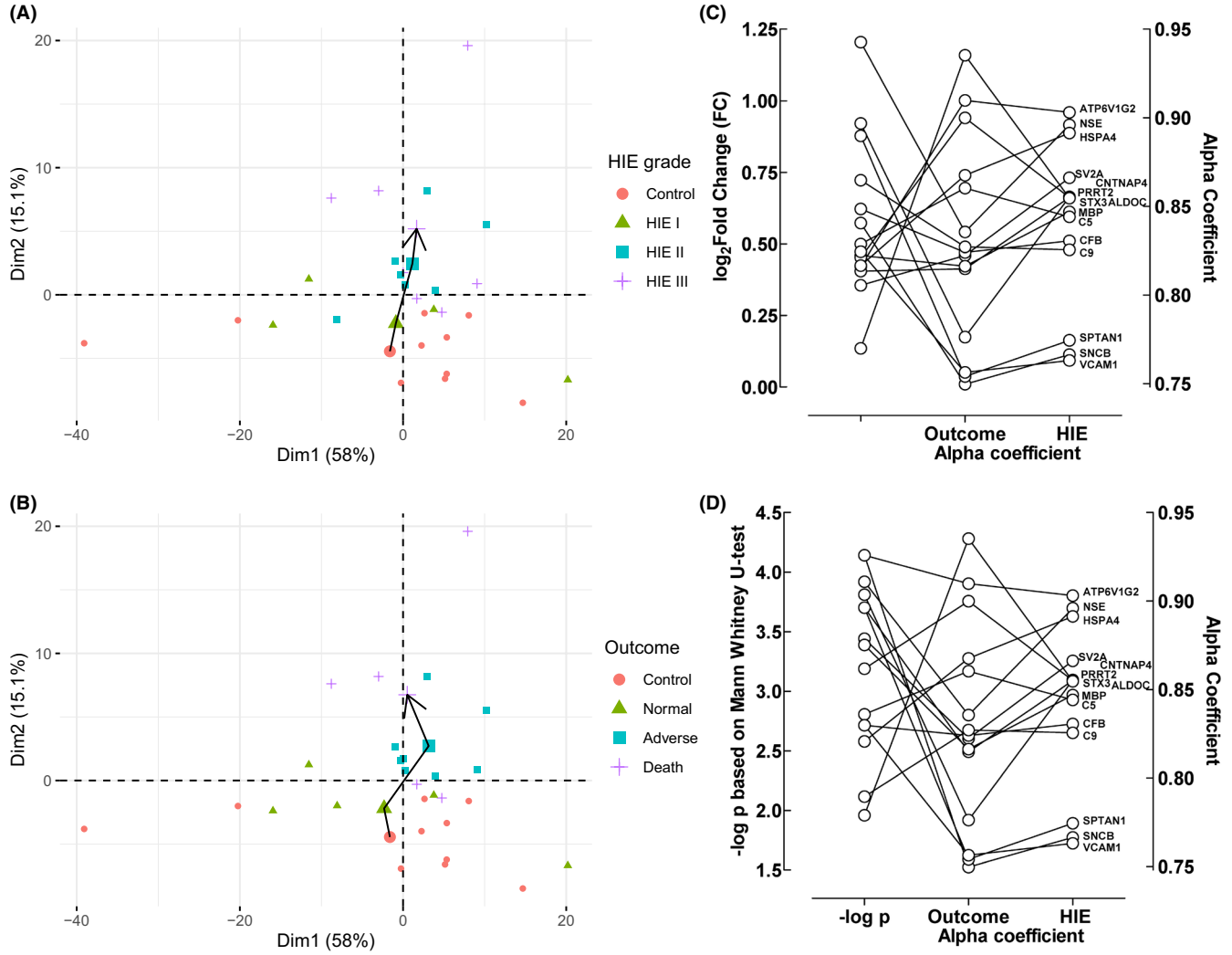

FIGURE 2 The principal component analysis (PCA) score plot of all 178 proteins in the CSF of patients with hypoxic-ischaemic encephalopathy (HIE) and controls. Patients were grouped by either HIE grades (A) or outcomes (B). Centroids are depicted as enlarged symbols and connected in paths from most to least favourable. The contribution of each protein to the separation of the data is expressed as alpha coefficients. Dim1 and Dim2 are the first two principal components. Relationships are described between the alpha coefficients on PCA and fold changes (FC) and expressed on volcano plots (C) and -$\log_{10}$ p-values (D). Fold changes describe the $\log_2$ transformed median fluorescent intensity (MFI) differences between HIE patients and controls. Values are based on Mann–Whitney U test. FC; Log_2Fold Change

proteins have previously been investigated in relation to HIE. These include synaptic vesicle glycoprotein 2A, a regulator of neurotransmitter release, and reticulon-1, which takes part in excitotoxic neurotransmitter release and may mediate brain damage in hypoxia ischaemia through apoptosis.[27] They also include beta-synuclein, which plays a detrimental role in Alzheimer's disease and Parkinson's disease.[28] Nevertheless, agents that have the potential to reduce excitotoxicity are currently under investigation as promising HIE therapies.[29]

4.3 | Cytoskeletal proteins

Cytoskeletal proteins are released when there is cellular damage or death, and this means that they may serve as markers of brain damage. The myelin basic protein and the alpha II-spectrin protein both increased following perinatal asphyxia and were correlated with unfavourable outcomes. Myelin basic protein is an essential component of the myelin sheath and myelin damage has been correlated with white matter injuries and epilepsy.[30] A correlation has been indicated between increased myelin basic protein levels in serum and CSF and adverse outcomes in paediatric traumatic brain injuries and HIE.[31,32] The same correlation has been seen in traumatic brain injuries and hypoxic-ischaemic brain injury models,.[33,34] Alpha II-spectrin is a protein that is essential for maintaining the integrity of brain cells, as it provides a link between the cytoskeleton and the plasma membrane.[35] It is a novel biomarker for neonatal HIE. It is notable that the present data are in line with suggestions that alpha II-spectrin might be a promising biomarker of brain injuries in infants following cardiac operations[36] and in paediatric traumatic brain injuries.[37] Furthermore, spectrin breakdown products have been shown to exist as exosomes in CSF when adults sustain traumatic

TABLE 2 Protein alterations predicting outcome following perinatal asphyxia

			HIE patients with adverse outcome		Control				
Analyte	Protein description	Function	Median	IQR	Median	IQR	ΔMFI	Log_2 Fold Change	p MWu-test
SNCB	Beta-synuclein	4&5	2553	1420–1783	1717	1420–1783	837	0.5727	7.21E−05
ATP6V1G2	V-type proton ATPase subunit G2	2	1194	1022–1338	891	837–915	303	0.4221	7.21E−05
NSE	Neuron-specific enolase	1&2	2231	1900–3764	968	887–1070	1263	1.2046	1.21E−04
ALDOC	Aldolase C	1&2	1578	1275–1951	834	758–984	745	0.9208	1.55E−04
SPTAN1	Spectrin alpha chain	1&3	760	525–1138	414	355–458	346	0.8772	1.98E−04
PRRT2	Proline-rich transmembrane protein 2	5	772	1240–1757	583	832–1299	189	0.4051	1.98E−04
SLC12A5	Solute carrier family 12 member A5	5	1610	1226–2209	957	783–1061	653	0.7501	2.53E−04
RTN1	Reticulon−1	4&5	1480	963–2357	721	649–768	759	1.0370	3.22E−04
SV2A	Synaptic vesicle glycoprotein 2A	5	1029	909–1298	804	705–899	225	0.3555	3.62E−04
ARPP21	cyclic AMP-regulated phosphoprotein 21	2&5	1147	1057–2248	799	686–919	348	0.9408	4.08E−04
DSCAM	Down syndrome cell adhesion molecule	7&8	582	492–867	456	371–474	126	0.5216	4.08E−04
AMER2	APC membrane recruitment protein 2	7	1003	598–2183	523	476–555	481	0.4594	4.08E−04
MBP	Myelin basic protein	1&3	792	728–1004	576	474–606	216	0.3528	4.08E−04
TTC9B	Tetratricopeptide repeat domain 9B	8	732	610–1078	556	507–600	177	0.4483	5.15E−04
NPTX1	Neuronal pentraxin−1	4	2078	1884–2377	1523	1437–1786	555	0.3981	5.15E−04
AQP4	Aquaporin−4	6&7	5592	4707–7303	3747	2663–4056	1845	0.5775	6.47E−04
CNTNAP4	Contactin 4	5&7	718	618–854	525	409–572	193	0.4520	6.47E−04
MOG	Myelin oligodentrocyte glycoprotein	8	1486	1355–1678	1149	1019–1270	337	0.3714	8.11E−04
CASKIN1	CASK-interacting protein 1	5	2153	1864–3528	1023	845–1960	1131	1.0742	1.56E−03
C5	Complement factor 5	7	970	813–1124	686	603–812	284	0.4993	1.56E−03
CFB	Complement factor B	7	2773	2232–3377	1802	1711–2501	971	0.6220	1.93E−03
VCAM1	Vascular adhesion molecule	7	1609	1260–1733	1159	768–1447	450	0.4730	1.93E−03
ACVR1	Activin A receptor type 1	8	1152	1003–1322	873	802–1033	279	0.3995	1.93E−03
RPH3A	Rabphilin 3A	5	693	626–919	548	475–637	145	0.3387	1.93E−03
GAP43	Growth associated protein 43	8	7728	7277–9030	6451	4534–6745	1277	0.2606	2.37E−03
MEPE	Matrix extracellular phophoglycoprotein	3	1074	791–1777	670	548–876	404	0.6808	2.37E−03
HSPA4	Heat shock protein family A member 4	7	551	473–756	411	328–428	141	0.4247	2.63E−03

Note: Proteins in CSF that exhibited statistically significant differences in median fluorescent intensity (MFI) levels between patients with adverse outcome and controls at threshold of $p < 0.005$, established by Mann–Whitney (MW) u-test and the sequentially rejective Bonferroni test ($\alpha = 0.005$). 1 = Marker of brain cellular damage; 2 = Energy metabolism; 3 = Brain cell structure; 4 = Apoptotic properties; 5 = Synaptic regulation; 6 = Brain vascular regulation; 7 = Neuroinflammation; 8 = Neurotrophic properties.

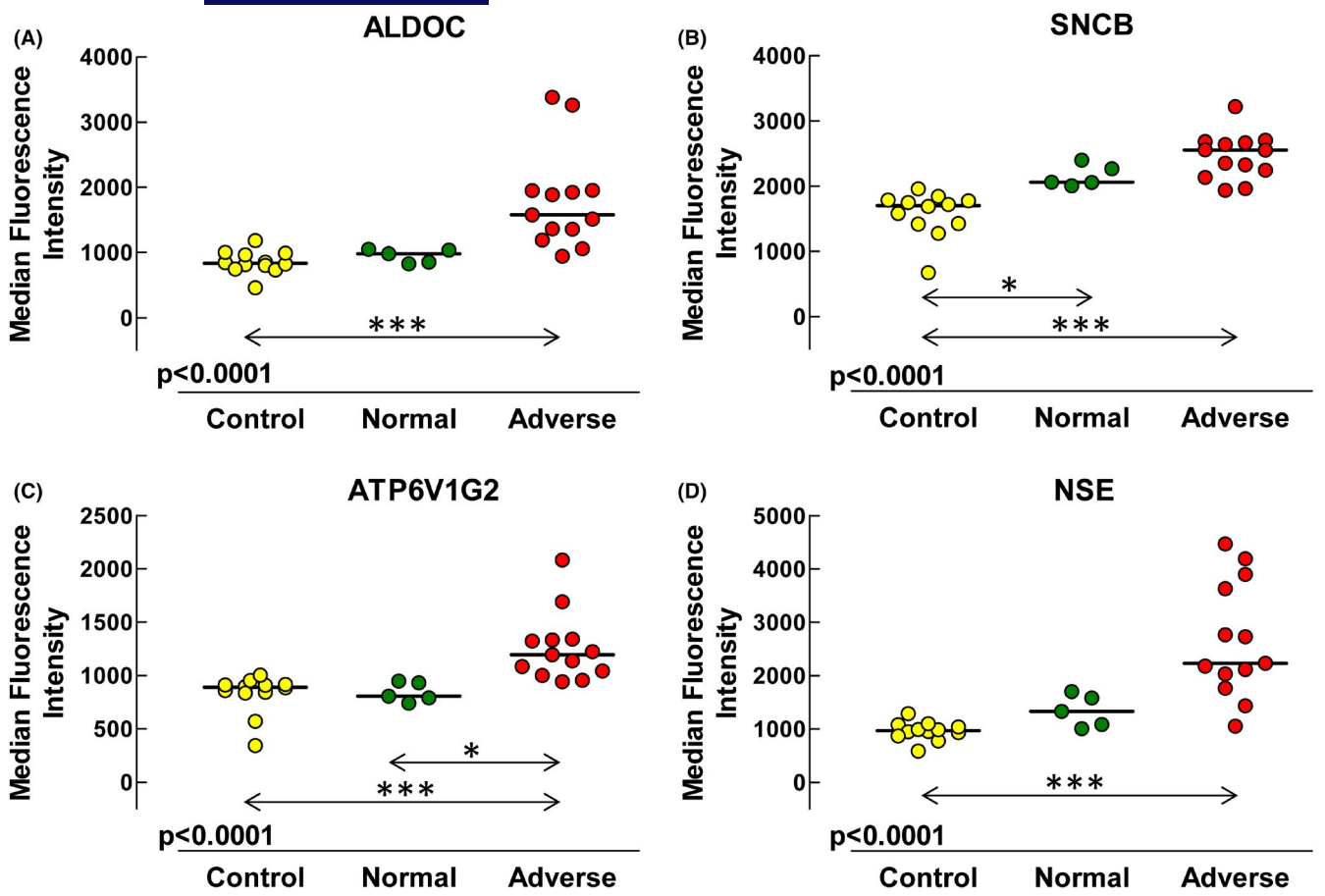

FIGURE 3 Scatter plot of the proteins that exhibited the largest differences between the outcome groups: aldolase C (ALDOC) (A), Beta-synuclein (SNCB) (B), ATPase H^+ transporting V1 subunit G2 (ATP6V1G2) (C) and neuron-specific enolase (NSE) (D). Adverse outcomes are defined as death or abnormal neurological outcomes at 18 months. Data were analysed with the Kruskal-Wallis test and Dunn's Multiple Comparison Test ($p < 0.0001$)

brain injuries. They function as cell death signalling molecules in the pathological pathways, leading to neurodegeneration.[38]

4.4 | Neuroinflammatory pathway proteins

The key cellular pathways of hypoxic-ischaemic brain injuries include the upregulation of the innate immune system. Inflammatory mediators may be produced within minutes of a brain insult and continue to expand for weeks and even months. They are the main contributors to the chronic prolonged phase of the injury, when the regeneration and repair of neurons may be prevented and neurodevelopment altered.[4] Clinical and experimental data that underline the importance of inflammatory mediators in perinatal brain injuries continue to emerge.[12,39,40] The present study found that increased levels of several inflammatory biomarkers correlated with unfavourable outcomes in patients. Furthermore, pathway analysis confirmed the importance of the complement pathway. It is of upmost importance to recognise the neuroinflammatory reaction in hypoxic-ischaemic brain injuries, as this may open up new possibilities for therapeutic interventions.

4.5 | Strengths and limitations

The study´s main strength was that we used a protein array, which is an emerging technique in hypoxic-ischaemic brain injuries. Doing this enabled us to provide novel insights into the underlying pathophysiology of brain disease. This technique also enabled us to simultaneously quantify 178 proteins in small CSF samples.

The study also had several limitations that must be acknowledged. It is important to point out that there was a time lapse between recruiting the patients and analysing the samples, as well as presenting the results. This means that it is possible that some of the frozen protein samples deteriorated over time. Also, hypothermia was not a standard treatment for HIE at the time of recruitment, so we did not have cooled infants in our patient group. On the other hand, this provide us with important information about brain pathology without the influence of therapeutic hypothermia. Another limitation was that the developmental evaluation, carried out with the Bayley Scales of Infant and Toddler Development, Second Edition, was only performed on infants with abnormal neurological symptoms at 18 months of age. Infants without symptoms did not undergo this test.

5 | CONCLUSION

This study has demonstrated an unprecedented array of CSF proteomic profiling alterations in protein levels following perinatal asphyxia and showed that these were associated with the severity of HIE and long-term outcomes. These can provide biomarkers for perinatal asphyxia. Several of these proteins are novel biomarkers for long-term outcomes after HIE and will require external validation in larger patient cohorts. Alterations in several novel biomarkers have previously been observed in biofluids in similar cerebral conditions, like traumatic brain injuries. This suggests a shared pathophysiology. Our study also characterised the pathological pathways involved in perinatal asphyxia, and this may open up new therapeutic options for reducing long-term morbidity and mortality.

As a result of our findings, we suggest that these markers should be used to monitor different pathophysiological processes following HIE. This could present tentative treatment options, but further research is warranted.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request in a format that adheres to current Swedish and European Union legislation regarding study participant anonymity.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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