| 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. The incidence of moderate to severe HIE is 1–3 per 1,000 live births in high-income countries. Hypoxic-ischaemic brain damage is a complex process that represents an evolving cascade of harmful events. The...
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. 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.

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. 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 injuries. They have also been used for protein profiling of cerebrospinal fluid (CSF) from preterm infants. The present study used antibody suspension bead array technology to assess the levels of brain enriched proteins and known inflammatory mediators 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.

### 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.

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 lumbar 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, 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. 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.
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) (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. 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 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). 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.
demonstrated a distinct inflammatory profile compared to the non-
neuroinflammatory pathways in CSF following asphyxia, which
proteins correlated with unfavourable outcomes (p value
CSF following perinatal asphyxia. This found that 51 unique CSF
proteins were altered in CSF compared with controls (Figure 1A). A
differential analysis was performed to compare the outcome groups
between both HIE grades and outcomes. 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 pro-
tein 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 dif-
fferences 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 con-
trols (data not shown). The four proteins that differed most between
outcome groups are show in Figure 3A-D.

2.5 | Ethics

This study was performed in accordance with European Community
guidelines and the Declaration of Helsinki. It was approved by the re-
ditional 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 clas-
sification of clinical signs. 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 dis-
tinct 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 be-
tween 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 re-
vealed almost identical paths and these created similar gradients from
favourable to unfavourable HIE grades and outcomes, respectively.

4 | DISCUSSION

We used an antibody array to analyse 178 proteins related to the
central nervous system and inflammation in CSF samples from in-
fants 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 biochemi-
cal changes in the CSF, which is in direct contact with the extracel-
lular matrix of the brain, as opposed to blood analyses, which may
not reflect events in the central nervous system. Proteins that indi-
cated 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 and has been correlated
with the risk of death or severe neurological impairment in HIE. It
is a commonly used biomarker for traumatic brain injuries and is used
in guidelines for managing cerebral anoxia following cardiopulmonary
resuscitation in adults, where increasing levels in serum predict an
unfavourable outcome. Secondary ischaemic injuries are common following severe traumatic brain injuries. 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. 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. 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. 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. 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
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. They also include beta-synuclein, which plays a detrimental role in Alzheimer’s disease and Parkinson’s disease. Nevertheless, agents that have the potential to reduce excitotoxicity are currently under investigation as promising HIE therapies.

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. A correlation has been indicated between increased myelin basic protein levels in serum and CSF and adverse outcomes in paediatric traumatic brain injuries and hypoxic-ischaemic brain injury models. The same correlation has been seen in traumatic brain injuries and hypoxic-ischaemic brain injury models. 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. 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 and in paediatric traumatic brain injuries. Furthermore, spectrin breakdown products have been shown to exist as exosomes in CSF when adults sustain traumatic...
| Analyte | Protein description | Function | Control Median | Control IQR | Patient Median | Patient IQR | ∆MFI | Log Fold Change | p Value |
|---------|---------------------|----------|----------------|-------------|----------------|-------------|------|----------------|---------|
| SNCB    | Beta-synuclein      |          | 425            | 1,480       | 1,029          | 972         | 721  | 762–1,224       | 0.5727  |
| ATP6V1G2| V-type proton ATPase subunit C2 | | 1,954 | 1,029–2,057 | 1,024–1,420 | 1,420–1,783 | 1,320–1,783 | 7.21E−05 |
| NSE     | Neuron-specific enolase | | 1,622 | 625–1,318 | 526–1,318 | 526–1,318 | 526–1,318 | 0.4211 |
| ALDOC   | Aldolase C          |          | 1,622 | 760 | 772 | 772 | 772 | 1.21E−04 |
| SPAN1   | Spectrin alpha chain 1&2 | | 1,622 | 1,622–3,928 | 1,622–3,928 | 1,622–3,928 | 1,622–3,928 | 1.21E−04 |
| PRRT2   | Proline-rich transmembrane protein 2 | | 1,622 | 577 | 577 | 577 | 577 | 1.49E−04 |
| SLC12A5 | Solute carrier family 12 member A5 | | 1,622 | 1,003 | 972 | 972 | 972 | 1.49E−04 |
| RTN1    | Reticulon−1 4&5 | | 1,622 | 1,622–3,928 | 1,622–3,928 | 1,622–3,928 | 1,622–3,928 | 1.21E−04 |
| ARPP32  | cyclic AMP-regulated phosphoprotein | | 1,622 | 1,003 | 972 | 972 | 972 | 1.49E−04 |
| APPB1   | Amyloid precursor protein | | 1,622 | 1,003 | 972 | 972 | 972 | 1.49E−04 |
| DSCAM   | Down syndrome cell adhesion molecule | | 1,622 | 1,003 | 972 | 972 | 972 | 1.49E−04 |
| NPTX1   | Neuronal pentraxin−1 | | 1,622 | 1,003 | 972 | 972 | 972 | 1.49E−04 |

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 (α = 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.
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. 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 utmost 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.

ACKNOWLEDGEMENTS

We would like to thank Dr David Just for performing the initial proteomic analysis, Naify Ramadan for providing technical assistance, Professor Ásgeir Haraldsson for reviewing the manuscript and for providing valuable advice and Dr Louise Steinhoff for English proofreading.

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 proofreading.

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

How to cite this article: Leifsdottir K, Thelin EP, Lassarén P, et al. Proteomic profiles in cerebrospinal fluid predicted death and disability in term infants with perinatal asphyxia: A pilot study. Acta Paediatr. 2022;111:961-970. doi:10.1111/apa.16277