Improvement in the Prediction of Neonatal Hypoxic-Ischemic Encephalopathy with the Integration of Umbilical Cord Metabolites and Current Clinical Makers

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Objective To validate our previously identified candidate metabolites, and to assess the ability of these metabolites to predict hypoxic-ischemic encephalopathy (HIE) both individually and combined with clinical data.

Study design Term neonates with signs of perinatal asphyxia, with and without HIE, and matched controls were recruited prospectively at birth from 2 large maternity units. Umbilical cord blood was collected for later batch metabolomic analysis by mass spectroscopy along with clinical details. The optimum selection of clinical and metabolites features with the ability to predict the development of HIE was determined using logistic regression modelling and machine learning techniques. Outcome of HIE was determined by clinical Sarnat grading and confirmed by electroencephalogram grade at 24 hours.

Results Fifteen of 27 candidate metabolites showed significant alteration in infants with perinatal asphyxia or HIE when compared with matched controls. Metabolomic data predicted the development of HIE with an area under the curve of 0.67 (95% CI, 0.62-0.71). Lactic acid and alanine were the primary metabolite predictors for the development of HIE, and when combined with clinical data, gave an area under the curve of 0.96 (95% CI, 0.92-0.95).

Conclusions By combining clinical and metabolic data, accurate identification of infants who will develop HIE is possible shortly after birth, allowing early initiation of therapeutic hypothermia. *(J Pediatr 2021;229:175-81)*.

Hypoxic-ischemic encephalopathy (HIE) remains a major cause of neurologic disabilities in full term newborn infants. Intrapartum asphyxia is responsible for 23% of the annual global neonatal deaths, and results in long-term disability in more than 400 000 children.[1] The only treatment widely used to improve outcome in HIE is therapeutic hypothermia. Therapeutic hypothermia decrease cerebral injury and improves neurologic outcomes.[2] To be effective, therapeutic hypothermia must be commenced within 6 hours of birth, during a time in which the clinical condition of the infant may be changing rapidly. No robust, quantifiable measure of hypoxic brain injury is currently validated to help clinicians to make individual decisions on offering therapeutic hypothermia.[3]

In high-income countries, despite advanced obstetric care, approximately 2% of all deliveries will have clinical or biochemical evidence of perinatal asphyxia, but only approximately 20% of these infants will have significant hypoxic ischemic injury and will progress to develop clinical HIE.[4] A number of different potential biochemical markers have been proposed, but few are measurable and none are validated within the 6-hour therapeutic window. Over the last decade, we have studied the metabolic profiles of umbilical cord serum from infants after perinatal asphyxia and have reported metabolite alterations that can predict the development and grade of encephalopathy, and the neurologic outcome at 3 years.[5-8] This exploratory work was carried out using targeted, semitargeted, and untargeted methods, allowing us to develop a short list of candidate metabolites showing significant alteration in infants with HIE and perinatal asphyxia that may be useful in the prediction of outcome. In this study our aim was to validate these metabolites in a second independent cohort and assess their ability to predict HIE in a clinical context.

Methods

The BiHiVE2 cohort (Validation of Biomarkers in Hypoxic Ischaemic Encephalopathy) is a prospective observational cohort recruited between March 2013 and August 2015 (www.medscinet.net/BIHIVE, NCT02019147), using identical criteria to the discovery cohort.[8,9] Infants were recruited across 2 maternity

AUC	Area under the curve
HIE	Hypoxic-ischemic encephalopathy
HILIC	Hydrophilic interaction liquid chromatography
MS	Mass spectrometer
RF	Random Forest

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services: Cork University Hospital and Karolinska University Hospital, with 8000 and 4500 annual deliveries, respectively. Our aim was to recruit all infants who were at risk of HIE. Full term infants (>36 weeks of gestation) were recruited to the study if they had 1 or more of the following: a cord pH of less than 7.1, an Apgar score of less than 6 at 5 minutes of life, or the requirement for intubation or cardiopulmonary resuscitation at the time of delivery. Infants were followed throughout their neonatal course and the development of clinical encephalopathy was documented. Grade of HIE was assigned using a modified Sarnat score and was later confirmed by analysis of the electroencephalogram recordings by a neurophysiologist and expert in neonatal electroencephalograms. The BiHiVE2 study also included recruitment of healthy control infants who had umbilical cord blood biobanked at birth using identical collection, processing, and storage procedures. These contemporaneous control infants were recruited before birth, and enrolled if they had uneventful deliveries, Apgar scores of 8-10 at 1 and 5 minutes, and a normal newborn hospital stay. At discharge, infants were grouped as having perinatal asphyxia without the development of a clinical encephalopathy, having HIE, or controls. Infants with confirmed sepsis, suspected inborn errors of metabolism, or coexisting congenital abnormalities were excluded from analysis. The local hospital ethics committees approved this study's recruitment, consent, and sample processing procedures.

Mixed arterial/venous umbilical cord blood was drawn for all infants, immediately after delivery, immediately placed on ice and centrifuged to retain serum, which was aliquoted and at stored −80°C. A strict standard operating procedure was used to ensure that all serum was drawn, processed, aliquoted, tracked, and stored within 3 hours of delivery. The detailed methodology has been previously described. Samples were transported at constant at −80°C to the Phenome Centre at the University of Birmingham for metabolite quantification.

Metabolite Quantification

Metabolite biomarkers for validation were identified from our biomarker discovery work analysis in the BiHiVE discovery cohort recruited using identical recruitment and laboratory methods from 2009 to 2011.[8] Quantification of metabolites was performed applying 2 complementary assays (hydrophilic interaction liquid chromatography [HILIC] and C_{18} reversed phase) using an electrospray TSQ Quantiva triple quadrupole mass spectrometer (MS) coupled to an Ultimate 3000 UHPLC (both made by Thermo Scientific, Waltham, Massachusetts). Full details of the methods applied are detailed in the **Appendix** (available at www.jpeds.com). Of the 33 metabolites of interest, 27 were accurately quantified using the UHPLC-MS/MS assay. Tetradecadiencarnitine was excluded as a standard was not commercially available. PCae (38:0), acetone, 3-hydroxybutyrate, glycerol, and erythrose-4-phosphate were excluded because they could not be assayed using the assays outlined with a suitable validation of the method to demonstrate accuracy and precision of quantification (calibration accuracy, calibration results, and chromatograms available in the **Appendix**).

Statistical Analyses and Machine Learning

Nonparametric univariate hypothesis testing (Kruskal-Wallis) was performed to determine candidate metabolites that displayed a significant difference across the 3 outcome groups (HIE vs perinatal asphyxia vs control), with a post hoc Dunn test to examine between-group differences. Statistical significance was set at a P value of less than .05 after correction for multiple comparisons using the Holm-Bonferroni method.[10] The linear correlation between all measured metabolites, and clinical factors (Apgar scores at 1 and 5 minutes), was calculated using the nonparametric pairwise Spearman correlation coefficient. The resulting correlation matrix is graphically presented in the form of a undirected correlation graph, or "spring" graph.[11-13] Here, a network of "nodes" and "arcs" is created such that each node represents a measured variable (size of node is proportional to each variables' correlation with HIE classification) and each arc represents a spring (spring strength is proportional to the correlation between 2 connected nodes). Edges are only included in the network if the correlation coefficient is positive, and significant at a critical P value of .001. Once the network is constructed it is allowed to "relax." The spring plot can be viewed as a simple multivariate cluster analysis, where nodes that cluster close to each other can be considered to be highly correlated in a multivariate sense. Networks were constructed using the graph visualization software Graphviz (AT&T Labs Research, Florham Park, New Jersey) using the "neato" virtual physics model.[14]

Logistic regression combined with Least Absolute Shrinkage and Selection Operator (LASSO) feature selection[15] was used to determine the subset of metabolites needed to create an effective multivariable model for the prediction of HIE. LASSO is a continuous subset selection algorithm which can lessen, and in some cases remove, the effect of unimportant predictors. LASSO operates by limiting the overall magnitude of the coefficients of the model so that important predictors are included and less important predictors shrink, potentially to zero, with the aim of creating the most accurate model possible using the least amount of predictor variables.

To further validate the results from the logistic regression, complementary classification models were created using the random Forest (RF) Algorithm.[16] RF is a machine learning method for classification that operate by constructing a multitude of decision trees during the training phase and outputting the class that is the mode of the classes of the individual trees. Input variables were the same as those used for the logistic regression models. To avoid overfitting and ensure that the resulting models were robust and generalizable, all model optimization was subjected to 10-fold cross-validation (averaged over with 10 Monte Carlo repetitions). The resulting optimal classifier models were assessed using receiver operator characteristic curve analyses.

Finally, the feature selection methods described above were compared with, and integrated with, clinical markers previously found to be helpful in outcome prediction.[17]

Results

Over the recruitment period, 30 208 deliveries were screened. Of these 287 (0.95%) were inborn, met case recruitment criteria at birth, and were enrolled in the study with informed parental consent. We also recruited 224 control infants. Of the cases, 48 progressed to clinical and electrophysiologic HIE, 10 had alternative diagnoses (confirmed or suspected sepsis and neonatal stroke), and 229 did not develop HIE and were classed as perinatal asphyxia (perinatal asphyxia group). Of the 48 infants with confirmed HIE, 41 had available serum suitable for analysis. These 41 infants with HIE were matched for sex, gestational age, and birth weight with 41 infants from each of the perinatal asphyxia and control groups (total n = 123). Of the infants with HIE, 1 had severe HIE, 12 had moderate HIE, and 28 had mild HIE based on Sarnat grading at 24 hours. Demographic data for each group are provided in **Table I** (available at www.jpeds.com). After metabolite quantification, 2 infants were excluded owing to failure of accurate quantification in the processed samples. One infant was excluded from the analysis owing to incomplete clinical data. Data from the remaining 120 infants (41 HIE, 39 perinatal asphyxia, and 40 controls) were analyzed.

In the univariate analysis, of the 27 quantifiable metabolites, 15 were validated as being significantly altered across the 3 outcome groups (HIE vs perinatal asphyxia vs control). These metabolites together with associated statistics are displayed in **Table II**. The metabolites most significantly altered (based on corrected *P* values) in both perinatal asphyxia and HIE were palmitoyl, lactic acid, succinic acid, and uridine (all $P = .0001$). The metabolites that were not significantly altered in perinatal asphyxia, but were altered in HIE were oleic acid, tryptophan, and linoleic acid. No metabolite was significantly increased in the HIE group compared with the perinatal asphyxia group alone.

A spring correlation graph of the 27 metabolites and the select clinical variables (**Figure 1**) showed that the clinical measures (Apgar scores) were the most important for the prediction of HIE vs non-HIE groups (perinatal asphyxia and controls combined) and they were not highly correlated with the metabolome. Lactic acid was the most highly correlated with the occurrence of HIE and was highly correlated with succinic acid and uridine. Palmitoyl-L-carnitine, decanoly-L-carnitine, oleic acid, tryptophan, and alanine were also highly correlated with the occurrence of HIE. Tryptophan and alanine showed less correlation to the other metabolites, allowing them to provide additional weight to the model.

Table III shows predictive values using metabolite data, clinical data and combined data. Using LASSO logistic regression, the best prediction of HIE using metabolite data alone was achieved with a combination of lactic acid, acetyl-L-carnitine, kynurenine, tryptophan, and oleic acid (**Table III**).

Table II. Metabolites showing altered expression between controls and infants with perinatal asphyxia and HIE

				Control vs perinatal asphyxia	Control vs HIE
Metabolites	**Control**	**Perinatal asphyxia**	**HIE**	***P* value**	***P* value**
Palmitoyl L carnitine	**0.04** (0.02)	**0.08** (0.07)	**0.07** (0.04)	**.0003**	**.0004**
Phenylalanine	**14.57** (2.43)	**16.06** (4.42)	**14.98** (2.59)	**.0406**	.3970
Leucine	**16.4** (4.11)	**19.28** (6.51)	**18.91** (6.66)	**.0493**	.0668
Creatine	**3.95** (1.73)	**4.61** (1.16)	**4.82** (0.0012)	**.0264**	**.0184**
Butyryl L Carnitine	**0.01** (0.01)	**0.02** (0.01)	**0.02** (0.01)	**.0031**	**.0176**
lactic acid	**421.63** (209.75)	**634.21** (185.72)	**725.66** (372.67)	**<.0001**	**<.0001**
Oleic acid	**110** (64.20)	**156.13** (109.5)	**160.57** (111.06)	.1354	**.0101**
Succinic acid	**0.56** (0.34)	**1.15** (0.84)	**1.02** (0.73)	**<.0001**	**<.0001**
Uridine	**0.7** (0.51)	**1.12** (0.69)	**1.24** (0.68)	**<.0001**	**<.0001**
Alanine	**39.52** (14.56)	**44.57** (21.51)	**46.45** (14.62)	**.03845**	**.0095**
Taurine	**22.31** (8.82)	**26.34** (9.74)	**26.10** (8.79)	**.0055**	**.0058**
Decanoyl L carnitine	**0.008** (0.0056)	**0.02** (0.01)	**0.02** (0.01)	.0619	**<.0001**
Tryptophan	**14.72** (3.61)	**14.41** (4.04)	**13.21** (2.61)	.6149	**.03328**
Acetyl L carnitine	**0.73** (0.34)	**1.09** (0.73)	**1.08** (0.52)	**.0011**	**.0019**
Linoleic acid	**48.81** (25.12)	**62.78** (42.72)	**60.66** (37.28)	.0968	**.0428**
DL Indole 3 lactate	**0.48** (0.17)	**0.53** (0.23)	**0.53** (0.20)	.0756	.2164
Glutamine	**1.50** (0.67)	**1.63** (0.66)	**1.63** (1.01)	.7040	.8328
Isoleucine	**12.71** (10.65)	**12.69** (9.00)	**14.04** (7.64)	.8406	1
Kynurenine	**2.29** (0.76)	**2.22** (0.87)	**2.11** (0.77)	.9345	.4480
Methionine	**5.04** (0.97)	**5.32** (1.67)	**5.17** (1.01)	.7899	.7177
Proline	**16.86** (3.56)	**16.55** (5.73)	**17.48** (2.83)	.5346	.2905
Tyrosine	**42.45** (10.01)	**43.58** (16.32)	**47.24** (11.62)	.5384	.4405
Valine	**42.65** (8.06)	**43.85** (10.52)	**41.62** (13.09)	.8670	1
Phosphocholine	**2.17** (0.81)	**2.09** (1.12)	**2.00** (1.10)	.9187	1
Linolenic acid	**3.40** (2.39)	**4.82** (6.25)	**4.36** (4.56)	.2304	.2310

Median values (IQR) are listed for each metabolite in milligrams per milliliter. All *P* values are corrected for multiple comparisons. Bold indicates values significantly altered ($P < .05$) between groups.

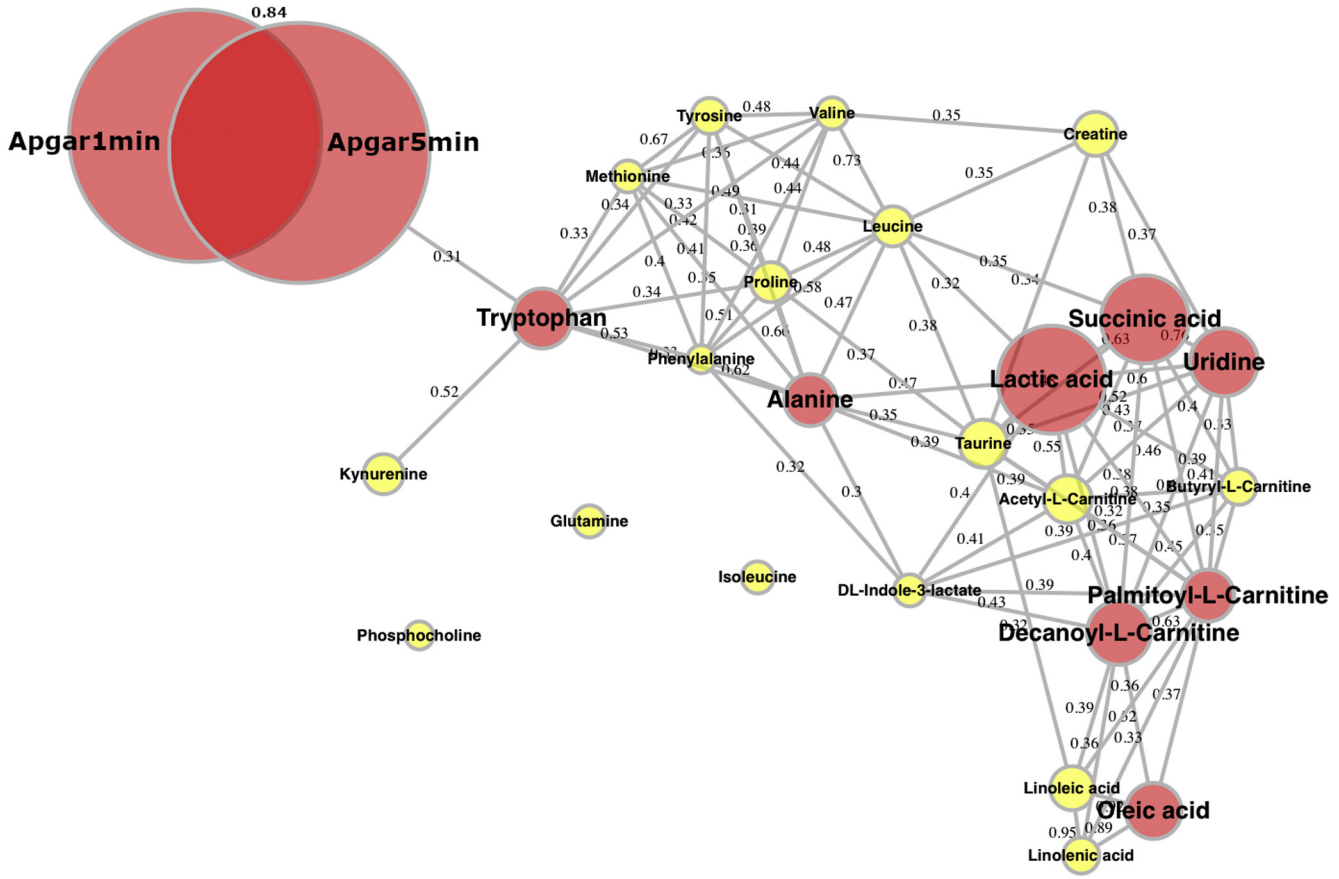

Figure 1. Spring-embedded correlation plot as a simple multivariable cluster analysis. Node size is proportional to the Spearman correlation with HIE score (*red* = $P < .05$) and arc being proportional to the Spearman correlation between nodes (with $P < .001$). Position of uncorrelated nodes is arbitrary.

Based on previous findings in a larger cohort, a clinical model to predict the onset of HIE was built using Apgar scores at 1 minute, Apgar score at 5 minutes, and lactic acid.[16] The predictive accuracy of this model (**Table III**) was similar to the previously reported cohort. The best prediction using both clinical and metabolite data was provided using lactic acid, alanine, and Apgar scores at 1 and 5 minutes (**Table III** and **Figure 2** [available at www.jpeds.com]). The optimum model for distinguishing between perinatal asphyxia and patients with HIE (excluding control infants) was obtained using lactic acid, alanine, Apgar scores at 1 and 5 minutes, the most intensive resuscitation required at birth, and the need for assisted ventilation at 10 minutes of age. This gave an

Table III. Summary statistics for logistic regression and RF models for the classification of HIE vs the non-HIE groups (control and perinatal asphyxia) using metabolite measurements, clinical data, and combined metabolite and clinical data

Models	Metabolite data	Clinical data	Combined data
1. Logistic regression			
Accuracy (95% CI)	73.7 (60%-87%)	86.8 (72%-96%)	91.9 (78%-98%)
Sensitivity	80%	80%	92.31%
Specificity	62%	100%	90.91%
AUC (95% CI)	0.74 (0.67-0.80)	0.92 (0.87-0.96)	0.92 (0.87-0.95)
2: RF			
Accuracy (95% CI)	68.4 (51%-83%)	89.5 (75%-97%)	97.3 (86-99%)
Sensitivity	80%	84%	96.15%
Specificity	46%	100%	100%
AUC (95% CI)	0.67 (0.62-0.71)	0.92 (0.89-0.95)	0.96 (0.92-0.95)

overall accuracy of 86%, area under the curve (AUC) of 0.90 (95% CI, 0.84-0.94); positive predictive value, 80%; negative predictive value, 92%; sensitivity, 92%; and specificity, 80%.

Using machine learning, we examined the ability of RF plots to improve the prediction of HIE. The use of RF plots improved the outcome prediction across all models. Feature selection was repeated. RF selection confirmed the same features as identified through logistic regression allowing us to include the same variables/features in the models, with improved predictive accuracy (**Table III**).

When using the metabolite data alone, the optimum model for the prediction of HIE was created using lactic acid, tryptophan, kynurenine, acetyl-L-carnitine, and oleic acid. When using clinically available data alone, the optimum model for predicting HIE was created using Apgar scores at 1 and 5 minutes and lactate levels at birth. Overall, the best prediction of HIE was found using RF analysis of a combined model of Apgar scores at 1 and 5 minutes, lactic acid, and alanine levels measured at birth (**Table III**).

The RF combined model was tested on its ability to specifically differentiate between perinatal asphyxia and HIE. Control infants were excluded from this analysis and the predictive value of the model was reassessed. The accuracy and predictive values remained high, with a sensitivity of 86%, a specificity of 75%, a positive predictive value of 86%, a negative predictive value of 75% and an AUC of 0.93 (95% CI, 0.91-0.95).

Discussion

We have shown that the measurement of additional metabolites at birth can be used, in conjunction with clinically available data, to improve our ability to predict the development of HIE. The infant's condition at birth, combined with both lactic acid and alanine measurements gave an AUC of 0.92 (95% CI, 0.87-0.95). This predictive ability was improved further with the use of machine learning techniques giving an optimum AUC of 0.96 (95% CI, 0.92-0.95). Importantly we have shown that we can accurately differentiate infants who will progress to HIE from those with perinatal asphyxia who will recover quickly and have a normal neonatal outcome.[4]

The earlier that therapeutic hypothermia can be started, the better the outcome.[18] In the initial hours after birth, neurologic signs evolve and fluctuate, making prediction of HIE and assessment of HIE grade difficult.[19,20] We know that using current eligibility criteria, even in expert centers, we will miss approximately 20% of infants who might benefit from cooling.[21,22] Having some quantitative measure of the degree of injury would be useful. The outcome following HIE varies considerably with the grade of encephalopathy.[23] A rapid quantitative test that uses the infant's condition at birth as well as metabolite quantification might aid these urgent therapeutic decisions.

In an effort to improve prediction, we studied metabolic phenotyping. Advances in mass spectrometry and nuclear magnetic resonance spectroscopy can allow a snapshot of thousands of metabolites at 1-time point. Because neonatal HIE is a sudden, acute event associated with recent global ischemia, we would expect HIE to be associated with widespread cellular hypoxia and metabolic disturbance. A number of studies have examined this metabolic profile in HIE using targeted (ie, a small number of predefined metabolites), semitargeted (ie, a large number of metabolites), and untargeted techniques (ie, all mass/charge ratios analyzed, no predefined metabolites).[7,8,24,25] Studies have used urine, serum, plasma, or cerebrospinal fluid in both animals and humans.[26] Hence, the metabolites reported and degree of alteration has varied.

Despite this, a number of characteristic metabolic phenotypes have been identified. The accumulation and delayed recovery of Krebs cycle intermediates (eg, fumarate, succinate, malate, and alpha keto-glutarate) have been described in a number of animal models.[26-28] These energy metabolites are a product of the shift toward anaerobic conditions, illustrated by the accumulation of lactate and mitochondrial dysfunction leading to disturbance of the Krebs.

A small number of studies have examined metabolic alterations in human infants, showing patterns of metabolite alterations are similar to those found in animal studies. In the BiHiVE discovery cohort, using a quantitative direct injection-MS/MS and liquid chromatography MS/MS approach we have previously shown significant alterations in HIE and perinatal asphyxia in 29 serum metabolites from 3 distinct classes; amino acids, acylcarnitines, and glycerophospholipids.[9] Next, a 1D 1H-NMR spectroscopy method was used to specifically characterize primary energy metabolites.[8] In the same perinatal asphyxia cohort, a broader untargeted analysis of cord blood was performed using direct infusion Fourier-transform ion cyclotron resonance mass spectrometry.[24] Twenty-nine putatively annotated metabolic features were significantly different in perinatal asphyxia after false discovery correction ($q < 0.05$), with 8 of these also significantly altered in HIE. Pathway analysis revealed HIE was associated with significant perturbation of the tryptophan and pyrimidine pathways.

This work has allowed us to build a short list of putative metabolites, which we have now quantified in a separate validation cohort, the BiHiVE 2 cohort, using a targeted MS method. Of these, 15 of 27 candidate metabolites were confirmed to have altered expression, providing confidence that the alterations are likely to be reliable. Of these, lactic acid and alanine were the metabolites that aided the most to the differentiation of infants with HIE from the other groups.

Lactic acid is the primary end product produced by the fermentation of pyruvate to yield 2 adenosine triphosphate molecules during anaerobic metabolism. Studies have shown strong correlations between lactate and pH or base deficit in umbilical cord blood samples and postnatal infant samples.[29,30] We have shown that, although a number of Krebs cycle metabolites were altered, lactate was the most affected, and in predictive modeling at the time of birth additional Krebs cycle metabolites such as succinate and fumarate did

not add to the predictive ability of the model. For improvement of our predictive power, alanine provided the most additional information.

Alanine is a nonessential amino acid and plays a key role in gluconeogenesis in mammals. The breakdown of muscle with release of adenosine triphosphate and glucose produces pyruvate, which is transaminated to alanine to enable transport to the liver for further gluconeogenesis.[31] In addition, during anaerobic metabolism, lactate is converted to pyruvate, which is again transaminated to alanine for transport to the liver. This catabolic mechanism, the glucose-alanine cycle between tissues and liver improves the efficiency of adenosine triphosphate production from amino acids and pyruvate. Thus, during anaerobic metabolism it is not surprising that we see a rise in circulating alanine.

An additional source of alanine is the breakdown of tryptophan. Tryptophan is an essential amino acid that is degraded to produce alanine by the kynurenine pathway.[31] Alanine is converted to pyruvate, which in low energy states will convert to acetyl-coenzyme A and feedback into the Krebs cycle to support energy production. There is increasing interest in the role of kynurenine pathway dysregulation in the development of neurologic disorders owing to the neurotoxic effect of the downstream product quinolinic acid.[32,33] The regulation of this pathway is very different in the developing brain and levels of kynurenine are much higher in the fetus and newborn.[34,35] Alterations have been reported in a rat model of perinatal asphyxia.[36] Of the 5 metabolites adding most to the predictive model, 3—tryptophan, kynurenine, and alanine—lie on this pathway. Thus, the kynurenine pathway seems to be giving us additional information regarding the state of tissue energy production beyond disruption of the Krebs cycle.

Differences between our discovery and validation cohort findings do exist; a number of the metabolites from our discovery cohort did not show significant alterations in the validation cohort. This finding may be due to differences in the assay technique, biological variability between individual infants, or difference in the severity profile of the HIE cohort. In the original discovery cohort, a greater proportion of infants had severe HIE, whereas in our validation cohort, only 1 infant was graded as having severe HIE. We have previously shown that the profile in infants with severe HIE is different, with more severe perturbations.5 This result may explain why not all 27 metabolites were altered in this validation cohort. It is likely that, in a more severely affected cohort, the alterations seen would be more significant.

There are limitations to our study. Although we maintained a strict standard operating procedure for the collection and processing of blood, umbilical cord blood can be difficult to collect. Not all infants with perinatal asphyxia were recruited to the study. However we did recruit 48 infants with HIE from an inborn population of 30 000 infants, close to our expected rate of 2 per 1000 deliveries. We chose serum for this study because our previously reported metabolite alterations were also in serum, and we wished to attempt to replicate this work. Further study is required to see if plasma sample patterns will be similar. Although umbilical cord blood can suffer from hemolysis, we have examined this in our samples, collected using the same standard operating procedures, and shown that, although some Krebs cycle metabolites are altered by hemolysis, alanine and lactate were not.[37]

Through a process of biomarker discovery and validation, and building on supportive preclinical data, we have shown that the most predictive metabolites for the development of HIE were lactic acid, acetyl-L-carnitine, kynurenine, tryptophan, and oleic acid. Activation of tryptophan breakdown through the kynurenine pathway to alanine seems to occur predominantly in those infants who progress to HIE. Metabolites in umbilical cord blood were not sufficiently predictive on their own, but when combined with clinical data could accurately predict the development of HIE. The metabolites that added most to the combined model were alanine and lactic acid. Developing this knowledge into a rapid bedside test would have the potential to quickly identify those infants who will progress to HIE and allow timely intervention. ■

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Data Statement

Data sharing statement available at www.jpeds.com.

References

1. Lee AC, Kozuki N, Blencowe H, Vos T, Bahalim A, Darmstadt GL, et al. Intrapartum-related neonatal encephalopathy incidence and impairment at regional and global levels for 2010 with trends from 1990. Pediatr Res 2013;74(Suppl 1):50-72.
2. Shankaran S. Neonatal encephalopathy: treatment with hypothermia. J Neurotrauma 2009;26:437-43.
3. Douglas-Escobar M, Weiss MD. Biomarkers of hypoxic-ischemic encephalopathy in newborns. Front Neurol 2012;3:144.
4. Finder M, Boylan GB, Twomey D, Ahearne C, Murray DM, Hallberg B. Two-year neurodevelopmental outcomes after mild hypoxic ischemic encephalopathy in the era of therapeutic ypothermia. JAMA Pediatr 2020;174:48-55.
5. Ahearne CE, Denihan NM, Walsh BH, Reinke SN, Kenny LC, Boylan GB, et al. Early cord metabolite index and outcome in perinatal asphyxia and hypoxic-ischaemic encephalopathy. Neonatology 2016;110:296-302.
6. Denihan NM, Boylan GB, Murray DM. Metabolomic profiling in perinatal asphyxia: a promising new field. Biomed Res Int 2015;2015:254076.
7. Reinke SN, Walsh BH, Boylan GB, Sykes BD, Kenny LC, Murray DM, et al. 1H NMR derived metabolomic profile of neonatal asphyxia in umbilical cord serum: implications for hypoxic ischemic encephalopathy. J Proteome Res 2013;12:4230-9.
8. Walsh BH, Broadhurst DI, Mandal R, Wishart DS, Boylan GB, Kenny LC, et al. The metabolomic profile of umbilical cord blood in neonatal hypoxic ischaemic encephalopathy. PLoS One 2012;7:e50520.
9. O'Sullivan MP, Looney AM, Moloney GM, Finder M, Hallberg B, Clarke G, et al. Validation of altered umbilical cord blood MicroRNA expression in neonatal hypoxic-ischemic encephalopathy. JAMA Neurol 2019;76:333-41.
10. Holm S. A Simple sequentially rejective multiple test procedure. Scand J Stat 1979;6:65-70.

11. Broadhurst DI Kell DB. Statistical strategies for avoiding false discoveries in metabolomics and related experiments. Metabolomics 2006;2:171-96.
12. Landi A, Broadhurst D, Vernon SD, Tyrrell DL, Houghton M. Reductions in circulating levels of IL-16, IL-7 and VEGF-A in myalgic encephalomyelitis/chronic fatigue syndrome. Cytokine 2016;78:27-36.
13. Hollywood KA, Winder CL, Dunn WB, Xu Y, Broadhurst D, Griffiths CE, et al. Exploring the mode of action of dithranol therapy for psoriasis: a metabolomic analysis using HaCaT cells. Mol Biosyst 2015;11:2198-209.
14. Ellson J, Gansner ER, Koutsofios E, North SC, Woodhull G. Graphviz and dynagraph—static and dynamic graph drawing tools. Graph drawing software. Springer, Berlin, Heidelberg; 2004. p. 127-48.
15. Tibshirani R. Regression shrinkage and selection via the lasso. J R Stat Soc Series B Stat Methodol 1996;58:267-88.
16. Breiman L. Random forests. Mach Learn 2001;45:5-32.
17. O'Boyle D, Mooney C, Finder M, Hallberg B, Boylan GB, Murray DM. A bioinformatics approach to predicting HIE following perinatal asphyxia. Poster presented at The 7th Congress of the European Academy of Paediatric Societies. October 30, 2018 – November 2, 2018; Paris, France.
18. Thoresen M, Tooley J, Liu X, Jary S, Fleming P, Luyt K, et al. Time is brain: starting therapeutic hypothermia within three hours after birth improves motor outcome in asphyxiated newborns. Neonatology 2013;104:228-33.
19. Biselele T, Naulaers G, Bunga Muntu P, Nkidiaka E, Kapepela M, Mavinga L, et al. A descriptive study of perinatal asphyxia at the University Hospital of Kinshasa (Democratic Republic of Congo). J Trop Pediatr 2013;59:274-9.
20. Natarajan G, Pappas A, Shankaran S. Outcomes in childhood following therapeutic hypothermia for neonatal hypoxic-ischemic encephalopathy (HIE). Semin Perinatol 2016;40:549-55.
21. DuPont TL, Chalak LF, Morriss MC, Burchfield PJ, Christie L, Sánchez PJ. Short-term outcomes of newborns with perinatal acidemia who are not eligible for systemic hypothermia therapy. J Pediatr 2013;162:35-41.
22. Gagne-Loranger M, Sheppard M, Ali N, Saint-Martin C, Wintermark P. Newborns referred for therapeutic hypothermia: association between initial degree of encephalopathy and severity of brain injury (What about the newborns with mild encephalopathy on admission?). Am J Perinatol 2016;33:195-202.
23. Sarnat HB, Sarnat MS. Neonatal encephalopathy following fetal distress. A clinical and electroencephalographic study. Arch Neurol 1976;33:696-705.
24. Denihan NM, Kirwan JA, Walsh BH, Dunn WB, Broadhurst DI, Boylan GB, et al. Untargeted metabolomic analysis and pathway discovery in perinatal asphyxia and hypoxic-ischaemic encephalopathy. J Cereb Blood Flow Metab 2019;39:147-62.
25. Sánchez-Illana Á, Núñez-Ramiro A, Cernada M, Parra-Llorca A, Valverde E, Blanco D, et al. Evolution of energy related metabolites in plasma from newborns with hypoxic-ischemic encephalopathy during hypothermia treatment. Sci Rep 2017;7:17039.
26. Beckstrom AC, Humston EM, Snyder LR, Synovec RE, Juul SE. Application of comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry method to identify potential biomarkers of perinatal asphyxia in a non-human primate model. J Chromatogr A 2011;1218:1899-906.
27. Solberg R, Enot D, Deigner HP, Koal T, Scholl-Bürgi S, Saugstad OD, et al. Metabolomic analyses of plasma reveals new insights into asphyxia and resuscitation in pigs. PLoS One 2010;5:e9606.
28. Atzori L, Xanthos T, Barberini L, Antonucci R, Murgia F, Lussu M, et al. A metabolomic approach in an experimental model of hypoxia-reoxygenation in newborn piglets: urine predicts outcome. J Matern Fetal Neonatal Med 2010;23(Suppl 3):134-7.
29. Westgren M, Divon M, Horal M, Ingemarsson I, Kublickas M, Shimojo N, et al. Routine measurements of umbilical artery lactate levels in the prediction of perinatal outcome. Am J Obstet Gynecol 1995;173:1416-22.
30. Gjerris AC, Staer-Jensen J, Jørgensen JS, Bergholt T, Nickelsen C. Umbilical cord blood lactate: a valuable tool in the assessment of fetal metabolic acidosis. Eur J Obstet Gynecol Reprod Biol 2008;139:16-20.
31. Nelson DL, Cox MM. Principles of Biochemistry. 4th Edition. New York (NY): WH Freeman & Company; 2005. p. 684-5.
32. Lovelace MD, Varney B, Sundaram G, Lennon MJ, Lim CK, Jacobs K, et al. Recent evidence for an expanded role of the kynurenine pathway of tryptophan metabolism in neurological diseases. Neuropharmacology 2017;112(Pt B):373-88.
33. Tan L, Yu JT. The kynurenine pathway in neurodegenerative diseases: mechanistic and therapeutic considerations. J Neurol Sci 2012;323:1-8.
34. Savitz J, Drevets WC, Smith CM, Victor TA, Wurfel BE, Bellgowan PS, et al. Putative neuroprotective and neurotoxic kynurenine pathway metabolites are associated with hippocampal and amygdalar volumes in subjects with major depressive disorder. Neuropsychopharmacology 2015;40:463-71.
35. Notarangelo FM, Pocivavsek A. Elevated kynurenine pathway metabolism during neurodevelopment: Implications for brain and behavior. Neuropharmacology 2017;112(Pt B):275-85.
36. Ceresoli-Borroni G, Schwarcz R. Neonatal asphyxia in rats: acute effects on cerebral kynurenine metabolism. Pediatr Res 2001;50:231-5.
37. Denihan NM, Walsh BH, Reinke SN, Sykes BD, Mandal R, Wishart DS, et al. The effect of haemolysis on the metabolomic profile of umbilical cord blood. Clin Biochem 2015;48:534-7.

Table I. Demographics details and comparisons of all 3 study groups

Characteristics	HIE (n = 41)	Perinatal asphyxia (n = 40)	Control (n = 40)	*P* value
Gestational age (wk)	40.0 ± 1.3	40.2 ± 1.1	40.2 ± 1.3	.9895
Sex (M/F)	23/18	22/19	23/18	.9721
Birth weight (g)	3624 ± 491	3635 ± 486	3649 ± 597	.9569
Mode of delivery				.0216
Unassisted vaginal	11 (27)	18 (44)	24 (58)	
Assisted (ventrose)	20 (48)	13 (31)	6 (15)	
Assisted (forceps)	4 (10)	4 (10)	5 (12)	
Emergency caesarean delivery in labor	6 (15)	6 (15)	6 (15)	
1-minute Apgar score	2 (1.0-3.5)	6 (5-8)	9 (8.5-9.0)	<.0001
5-minute Apgar score	5 (4-6)	9 (8-10)	10 (9-10)	<.0001
Maternal ethnicity				.1158
Caucasian	38	36	37	
African	1	3	1	
Asian	1	2	3	
Maternal age (y)	31.0 ± 5.1	31.0 ± 5.5	31.0 ± 5.1	.6386

Values are mean ± SD, median (IQR), or number (%).

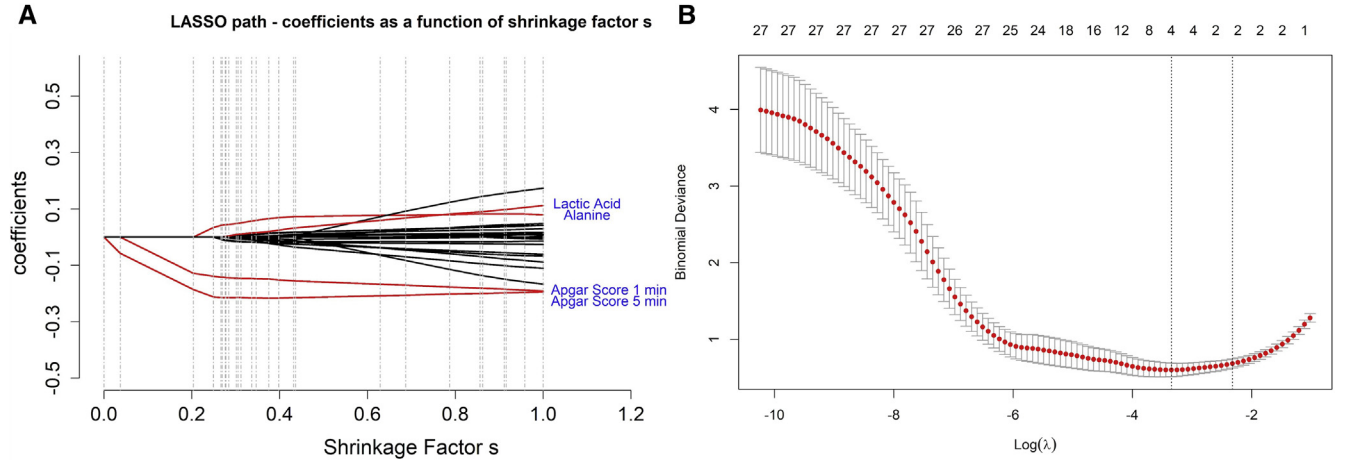

Figure 2. **A,** Plot showing the coefficients of the predictors included in the model as the shrinkage factor increases in the LASSO model to distinguish between HIE and non-HIE groups. At an optimum shrinkage factor of (X), 4 variables were included in the final model (shown in *red*) = Apgar 1, Apgar 2, lactate and alanine. **B,** Elbow plot showing the average levels of deviance for models built to classify between HIE and non-HIE groups using each possible value of the shrinkage factor (logLambda = *x*-axis). The number of included variables for each value of lambda is shown on the top axis. The lowest levels of deviance were observed in the model using 4 predictor variables; shrinkage factor = 3.2-3.7.