Urine metabolomic profile in neonates with hypoxic-ischemic encephalopathy

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Abstract

Background: Metabolomics could provide valuable insights into hypoxemic-ischemic encephalopathy (HIE) revealing new disease-associated biochemical derangements. The study aimed to investigate urine metabolic changes in neonates with HIE compared to healthy controls, using targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS).
Patients and Methods: In this prospective, single-center study we enrolled neonates born at ≥ 36 weeks gestation with HIE (HIE group) and healthy controls (control group). We collected urine samples for metabolomic analysis on days one, three, and nine of life.
Results: Twenty-one full-term newborns were studied, 13 in the HIE group and eight in the control group. Six of the affected neonates had moderate/severe HIE and seven mild HIE. Therapeutic hypothermia was applied only in four neonates with moderate/severe HIE. Multivariate and univariate statistical analysis showed a clear separation between the HIE and the control groups. Discriminant metabolites involved pyruvic acid, amino acids, acylcarnitines, inositol, kynurenine, hippuric acid, and vitamins.
Conclusions: We have identified a specific metabolic profile in neonates with HIE, adding to the existing knowledge on the disease biochemistry that may potentially help in biomarker development. HIPPOKRATIA 2017, 21(2): 80-84.

Keywords: encephalopathy, neonate, perinatal asphyxia, brain injury, metabolomics

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Introduction

Perinatal asphyxia is one of the most dramatic situations in obstetrics and neonatology attributed to intrapartum events leading to fetal hypoxia and energy deprivation. Depending on the degree of cardiovascular compromise, the fetus may succumb in the womb[1] or be born depressed requiring immediate resuscitation at birth[2]. Of the asphyxiated newborns, some will eventually develop hypoxic-ischemic encephalopathy (HIE). The incidence of HIE in the western world has been calculated at 1.60 (range: 0.68-3.75) per 1,000 live births[3]. Unfortunately, most severely affected infants will die postnatally while survivors are at risk for long-term neurological handicaps[4].

Perinatal asphyxia is a large-extent event with a global effect on the infant. Clinically, this is manifested by the concomitant injury beyond the brain of almost all organs and systems[5]. Research performed heretofore clearly documented major biological alternations in HIE, but, in most studies, individual metabolites-molecules were evaluated[6]. This fact, however, hinders investigators from obtaining an overall image of the biochemical derangements that occur in asphyxia/HIE. To overcome this restriction, a relatively new scientific approach such as metabolomics has been applied. Metabolomics is a bio-analytical method that aims at the comprehensive profiling of small molecules in various biological fluids and tissues. Virtually, with this cutting-edge technological method, one may obtain a "snapshot" of the low molecular weight biochemical profile, thus enabling a better understanding of the complex metabolic pathways in normal and disease states. Moreover, metabolites that contribute to the separation of healthy from sick subjects may potentially serve as disease biomarker(s). Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are the most commonly applied analytical methods[7].

Nevertheless, despite the increasing scientific interest in the use of metabolomics in medicine, studies in neonates are sparse, particularly with respect to asphyxia/HIE. These are mainly experimental and investigate the metabolic profile in animal models of hypoxia-induced asphyxia alone[8] or in association with resuscitation protocols[9-12] and therapeutic hypothermia[13]. The latter is nowadays considered the standard care for neonates suffering moderate/severe HIE, and metabolomics may play a significant role in unraveling neuroprotective mechanisms

behind novel treatments. Surprisingly, only a small number of studies include clinical reports on neonates with HIE subjected[14,15] or not[16,17] to therapeutic hypothermia.

The aim of the present study was to investigate the urine metabolic profile of neonates born at ≥ 36 weeks gestation with HIE versus healthy neonates of comparable gestational age, using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Patients and Methods
Patients
This case-control study is part of a larger investigation on brain injury biomarkers in preterm and term neonates that was prospectively conducted in our center from April 2011 to November 2015. According to the protocol of the investigation, blood and urine samples were obtained and stored at -80ºC for later analysis. Results on circulating progenitor cells and their relation to biomarkers of brain injury have already been published[18]. From the database of this project, we selected i) neonates born at ≥ 36 weeks gestation who had HIE following an acute perinatal event (HIE group) and ii) healthy newborns of comparable gestational age (control group). In order to be eligible for the study, each of the selected neonates should have at least one urine sample available for metabolomic analysis. Diagnosis and classification of HIE were made using the modified Sarnat and Sarnat score and the findings of the amplitude-integrated electroencephalography (EEG)[19,20]. Neonates with major congenital abnormalities, known metabolic disorders, and those considered moribund were excluded from the study. Perinatal and neonatal demographic-clinical characteristics were recorded. The study was approved by the Ethical Committee (Scientific Council) of the Hippokrateion General Hospital (No 58/10.3.2011), and a written informed consent was obtained from parents.

Urine sample collection and preparation
Urine samples were collected from newborns, using sterile urine bags during the first 12 hours after birth (hereafter referred to as day one) and thereafter on days three (while on therapeutic hypothermia if applied) and nine of life. In healthy neonates, urine samples were similarly collected during their three-day hospital stay after birth. Moreover, parents were encouraged to bring samples corresponding to day nine of life from home to the hospital immediately after the urine collection. As aforesaid, all samples were frozen and stored until metabolomic analysis. Further details on the sample processing are provided in the supplementary material (Appendix I, available online, link in the Acknowledgement).

LC-MS/MS
All samples were subjected to a targeted metabolomics analysis by an in-house HILIC-MS/MS as previously described[21]. This method provides quantitative analysis of 110 small molecules. The method has been used by our group for a number of studies[22] and has been validated extensively in the analysis of biological samples. More details of the method are given in the supplementary material (Appendix I, available online, link in the Acknowledgement).

Data Analysis
The MetSizeR R-package was used for the calculation of the sample size needed to find significant changes in 10 % out of a total of 100 metabolites studied. The target false discovery rate (FDR) was set at 0.05. A sample size of four subjects per group was found adequate for the goals of our study.

Statistical analysis of the demographic and perinatal-clinical characteristics was performed by using the IBM SPSS Statistics for Windows software (IBM SPSS, IBM Corp., Armonk, NY, USA), version 19.0. Numerical data are expressed as means ± standard deviation, and differences between two groups were assessed using the t-test. The Fisher's exact test was used for the comparison of categorical variables.

Tools for data processing of LC-MS/MS, multivariate/univariate analysis methods, and visualization are described in detail in the supplementary material (Appendix I, available online, link in the Acknowledgement).

Results
Study population
Twenty-one full-term newborns were included in the study, 13 in the HIE group and eight in the control group. Six of the affected neonates had moderate or severe HIE and seven mild HIE. The HIE and control groups were comparable regarding gestational age (38.1 ± 1.5 vs 37.5 ± 1.2 weeks gestation, p =0.490), birth weight (3,139 ± 528 vs 2,873 ± 249 g, p =0.137), sex (8/13 vs 4/8 male neonates, p =0.605), and mode of delivery (7/13 vs 6/8 caesarian section, p =0.4). Eight and six neonates from the HIE and control groups, respectively, were inborn (p =0.656). As expected, neonates with HIE vs controls had significantly lower Apgar scores at one (2.3 ± 2.4 vs 8.2 ± 0.5, p <0.001) and five (5.5 ± 2.1 vs 8.9 ± 0.4, p <0.001) minutes. Therapeutic hypothermia was applied only in four neonates with moderate/severe HIE as two had already passed the therapeutic window of the first six hours after birth upon admission in our neonatal intensive care unit (NICU). Death before the hospital discharge occurred in two neonates with moderate/severe HIE.

LC-MS/MS data
In total, 43 urine samples were analyzed: 25 in the HIE group (nine, eight, and eight samples for days one, three, and nine, respectively) and 18 in the control group (eight, six, and four samples for days one, three, and nine, respectively).

Approximately 40 metabolites were detected and semi-quantified in the urine samples. These included organic acids, amino acids and their derivatives, vitamins, nucleosides, sugars, and other metabolites.

A clear discrimination between HIE and control

groups was seen on days one and three in Partial least squares-Discriminant Analysis (PLS-DA) models (Figure 1). Permutation plots and p-values from CV-ANOVA confirmed the statistical strength of the differentiation. In addition, univariate analysis showed significant alterations for 15 metabolites (Table 1). Differentiation between the two groups at day nine by PLS-DA (Figure 1) was not proven valid as shown in the permutation plot (Figure 1, inset for day nine) and CV-ANOVA tests. Metabolites that were highlighted by both multivariate and univariate statistical analysis as differentiating between the two groups on day one and three listed in descending order of VIP (variable importance in projection) value are shown in Table 2. PLS-DA could not differentiate neonates with moderate/severe HIE from those with mild HIE.

Discussion

In the present study, we evaluated the urine metabolic profile of neonates born at ≥ 36 weeks gestation with HIE compared to healthy controls using a targeted metabolic
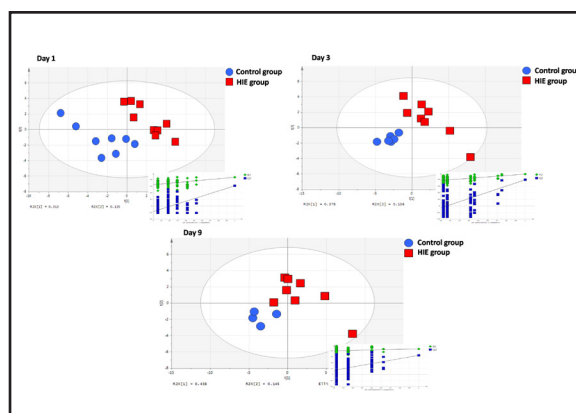

Figure 1: Partial least squares-Discriminant Analysis (PLS-DA) scores plots in the first two components [t1] and [t2] on days 1, 3 and 9 with the permutation tests performed to validate the grouping in corresponding insets (bottom right of each plot). Hypoxic-ischemic encephalopathy (HIE) samples are differentiated from controls in days 1 and 3, but group separation is not valid on day 9 (permutation fails).

Table 1: Metabolites contributing to the differentiation between neonates with hypoxic-ischemic encephalopathy (HIE) and healthy controls on days one, three, and nine. The arrows ↑ and ↓ indicate increased and decreased metabolite levels, respectively, in the HIE vs the control group whereas dash (-) comparable levels between the two groups. Significant p values are shown in bold font.

	Neonates with HIE vs controls								
	Day 1			Day 3			Day 9		
Metabolite	Trend	Fold change	p value	Trend	Fold change	p value	Trend	Fold change	p value
Pyruvic acid	-	-	0.831	↑	**2.88**	**0.034**	-	-	0.179
Leucine-isoleucine	↓	**0.45**	**0.014**	↑	**1.65**	**0.017**	-	-	0.271
Norvaline-valine	-	-	0.064	↑	**1.27**	**0.003**	-	-	0.063
Tyrosine	↓	**0.51**	**0.039**	-	-	0.107	-	-	0.115
Tryptophan	↓	**0.55**	**0.042**	-	-	0.071	-	-	0.127
Phenylalanine	↓	**0.5**	**0.011**	↑	1.77	**0.05**	-	-	0.182
Threonine	↓	**0.41**	**0.029**	-	-	0.206	-	-	0.057
Serine	-	-	0.127	-	-	0.101	↑	**1.32**	**0.046**
N-Acetyl-aspartate	↓	**0.51**	**0.002**	-	-	0.406	-	-	0.312
Inositol	-	-	0.121	↑	**1.34**	**0.007**	↓	**0.59**	**0.008**
Acetylcarnitines	-	-	0.498	↑	**3.97**	**0.032**	-	-	0.154
Kynurenate	↓	**0.47**	**0.013**	-	-	0.087	-	-	0.095
Hippuric acid	↓	**0.75**	**0.021**	-	-	0.603	-	-	0.111
Pyridoxine	-	-	0.406	↑	**1.64**	**0.047**	-	-	0.103
Thiamine	-	-	0.221	-	-	0.103	↑	**4**	**0.014**

Table 2: VIP (variable importance in projection) values of significant metabolites found by both multivariate and univariate statistical analysis on days one and three.

	Metabolite	VIP value
Day 1	N-acetyl-aspartate	1.50
	Phenylalanine	1.30
	Kynurenate	1.29
Day 3	Pyridoxine	1.50
	Norvaline-valine	1.34
	Pyruvic acid	1.18

approach (LC-MS/MS). Results showed significant differences in several metabolites between neonates suffering HIE and healthy ones, which could potentially serve as disease biomarkers.

Hypoxia-ischemia is characterized by a shift towards anaerobic metabolism. This is well-documented in various preclinical and clinical studies of neonatal asphyxia/HIE that show accumulation of lactate[8,10,16,23] and Kreb's cycle intermediates (citrate, alpha keto-glutarate, succinate, fumarate)[8,23-25] owing to diminished high energy stores (adenosine triphosphate). We were not able to document similar metabolic alternations during the first day

after the acute perinatal event. Nevertheless, we noted significantly higher pyruvic acid in neonates with HIE at day three, a finding which suggests an ongoing dysfunctional aerobic metabolism.

We found several categories of amino acid decreased in the urine compared to controls during the first day of life. These included branched-chain amino acids [(BCAAs), leucine-isoleucine] as well as aromatic (phenylalanine, tyrosine, tryptophan), neutral (threonine), and basic acids (aspartate). BCAAs and aromatic acids are biochemical precursors of important neurotransmitters including dopamine, serotonin and melatonin[26]. Others, like aspartate, serve *per se* as excitatory amino acids or are closely related to amino acids with a similar action such as glutamate[27]. Perturbations of the amino acids in HIE have already been reported in previous investigations. Nevertheless, in some of these studies performed in animals[8,28] and humans[14,17,25], a significant increase of several amino acids was noted following hypoxia and asphyxia/HIE, whereas others in humans showed elevated or reduced amino acid levels[29]. In these studies, however, amino acids were evaluated in cord blood, that is, immediately after the induction of acute hypoxia/ischemia or the acute perinatal event in humans, whereas in our study they were measured considerably later. A dynamic change of the urine metabolome has been documented to occur over time in hypoxic animals (up to four hours)[23] and in neonates with HIE up to one month after birth[15]. Interestingly, we found that most of the amino acids normalized or even increased compared to healthy controls after the third day of life. This could be attributed to a relative amelioration of the catabolic state and parenteral nutritional support of the sick neonates.

Inositol showed a marked elevation in our neonates with HIE on day three but decreased, thereafter**.** Inositol is widely distributed in human tissues and cells, and it is a precursor for phosphorylated compounds that are involved in signal transduction[30]. Its most widely occurring stereoisomer, myo-inositol, has been documented to increase in the blood following perinatal asphyxia or HIE in animals[24] and humans[25]. A similar elevation of myo-inositol has been observed in the cerebrospinal fluid of fetal sheep suffering from hypoxia. In this case, brain injury was attributed to osmolytic cell changes causing cell edema[31].

As with previous studies, we observed elevated urine acylcarnitines levels in the HIE group as compared to controls. Carnitine is the transporter of fatty acids across the inner mitochondrial membrane for β-oxidation. Metabolomic studies involving animal models of asphyxia-resuscitation or hypoxia showed lower plasma free carnitine and higher long-chain acylcarnitines after hypoxia as a consequence of incomplete β-oxidation[8,11]. Similar results were recently reported with the untargeted metabolic analysis of cord blood in infants with asphyxia and HIE[17]. Moreover, therapeutic hypothermia was reported to achieve its neuroprotective action in neonatal brain injury via a decrease in acetylcholine with a concurrent increase in carnitine[13].

This study detected two other metabolites to be significantly decreased in the urine of neonates with HIE: kynurenine and hippuric acid (hippurate). Kynurenine is the initial product of tryptophan metabolism and may be converted to kynurenic acid, a neuroprotective molecule that antagonizes glutamate receptors induced neurotoxicity[32]. Denihan et al found a significant reduction of kynurenine in the cord blood of infants developing HIE as well. In the latter study, as in our case, a significant decrease in tryptophan (the precursor of kynurenine) was observed in neonates with HIE[17]. Regarding decreased hippuric acid, our results are in line with previous reports in animal models of neonatal hypoxia[10,23]. Lower levels of hippurate were also observed in the urine of stroke patients, although this could be related to folic acid deficiency, which is a known risk factor for stroke[33]. Lastly, we observed significantly elevated pyridoxine (vitamin B6) and thiamine (vitamin B1) levels in cases compared to controls at days three and nine, respectively. These vitamins are coenzymes involved in several biochemical pathways essential for every aspect of brain function[34]. As far as we know, there are no reports on these vitamins in relation to HIE.

Overall, our study extends existing knowledge on the underlying pathophysiological mechanisms in HIE. Moreover, in the present study, neonates were serially evaluated over time corresponding to the primary and secondary energy failure. Nevertheless, our study has limitations as well. We did not evaluate depressed at birth neonates who did not develop HIE. Worth noting that cases with mild HIE could not be differentiated from those with moderate/severe HIE, at least with the analytical method we used in this study. The clinical distinction between mild and moderate HIE is not always an easy task while at the same time categorizing is critical for the immediate management (e.g., initiation of therapeutic hypothermia) and long-term prognosis. Additionally, consequently to the small number of studied infants, we could not investigate the effect of treatment (hypothermia vs supportive care only) or outcome (death) on the metabolic profiling. Apparently, a single-center study, a priori limits the number of HIE cases that could have been evaluated.

In conclusion, through a targeted metabolomic analysis, we identified a specific metabolic profile in neonates with HIE. Such a biochemical fingerprint could potentially help in the development of biomarkers for the early identification of neonates at high-risk for adverse neurological outcome as well as of therapeutic strategies that would improve the outcome in the affected neonates.

Conflict of interest
The authors declare no conflict of interest.

The supplementary material regarding the Methods and Data processing of this study are available online in the electronic version: Appendix I.

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