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REVIEW ARTICLE

Understanding neonatal hypoxic-ischemic encephalopathy with metabolomics

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Abstract

Background: Hypoxic-ischemic encephalopathy (HIE), a serious complication of perinatal asphyxia, is commonly associated with an unfavorable outcome. In-depth research is important not only for the interpretation of the underlying biological alternations but may also provide the basis for the development of novel diagnostic and therapeutic tools. The application of metabolomics in perinatal asphyxia/HIE is a relatively new approach.

Methods: We performed a narrative, non-systematic review in the literature of metabolomic studies involving newborn animals and humans exposed to hypoxia-ischemia or developing perinatal asphyxia/HIE.

Results: Fifteen animal studies, nine studies in human neonates, and two review articles were evaluated. Changes in the metabolomic profile of newborn animals exposed to hypoxia-ischemia and of asphyxiated neonates with HIE are presented in relation to the underlying pathophysiology. The clinical relevance of these findings is further discussed in a comprehensible to the bedside clinician manner.

Conclusions: Metabolomics may provide an explanation for the various metabolic alternations occurring in perinatal asphyxia/HIE, elucidate the biological background of the applied therapeutic interventions and promote the development of novel diagnostic-prognostic biomarkers of the disease. HIPPOKRATIA 2017, 21(3): 115-123.

Keywords: Neonatal care, perinatal asphyxia, metabolic pathways, therapeutic hypothermia, biomarker

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Introduction

Hypoxic-ischemic encephalopathy

Hypoxic-ischemic encephalopathy (HIE) following perinatal asphyxia remains an issue of great clinical importance. The majority of the nearly one million deaths that occur every year consequent to perinatal asphyxia worldwide happen in low- and middle-resource countries[1]. Nevertheless, 1-5 term neonates per 1,000 live births will develop HIE even in countries with advanced medical systems[2,3]. Moreover, despite progress in perinatal and neonatal care, depending on the severity of the brain injury, a significant number of the affected neonates will die or suffer neurological sequels[4,5].

HIE is mainly caused by energy failure of the brain due to an acute perinatal event. However, experimental and clinical observations have demonstrated that the development of HIE is rather an evolving process. The initial hypoxic and/or ischemic insult switches metabolism to anaerobic energy production, and with prolonged oxygen deprivation "primary energy failure" is established characterized by depletion of high-energy metabolites [adenosine triphosphate (ATP)]. This leads to depolarization of the mitochondrial membranes, intracellular calcium accumulation, cytotoxic edema, extracellular accumulation of excitatory amino acids, and finally death of some neuronal cells[6,7]. After resuscitation, oxidative metabolism returns gradually (in 30-60 min) to baseline whereas many neurons recover, at least partially. However, after a latent period of around six hours, high-energy metabolites decrease again, owing to the deterioration of the mitochondrial function, and give rise to the so-called "secondary energy failure". Oxidative stress, accumulation of toxic metabolites and delayed apoptosis are additional features of this phase. There is a "tertiary phase" of repair and reorganization that may last for months, but the precise mechanisms involved in this phase are not clear[6,7].

Therapeutic hypothermia (TH) initiating during the first six hours is nowadays considered the standard of care for the management of asphyxiated neonates with moderate-severe HIE, as it significantly alters prognosis, improving survival and neurodevelopmental outcome[4]. On the other hand, TH is not a panacea and has endogenous limitations, as, for example, its applicability only during the first six hours after birth[7]. This fact renders the need for more research mandatory in order to elucidate underlying biological mechanisms of perinatal

asphyxia/HIE. Potentially, this research could aid in the development of future pharmaceutical interventions for neuroprotection and also of reliable disease biomarkers. Metabolomics, the younger "-omics" discipline, could be the scientific answer to these issues.

Metabolomics

Metabolomics involves the development and application of unbiased/global analysis of biological samples (e.g., animal/human biofluids, tissue or cell extracts) with the aim to discover biomarkers related to a certain condition such as disease, stress or environmental factors. Analytical methods utilized in metabolomics research aim at the characterization of the entire metabolic content of the samples under study and then at the relation of their concentration patterns to properties of the samples[8]. Therefore, metabolomics involves multidisciplinary research driven by scientists with different expertise, for example, life and medical sciences, analytical chemistry, statistics, biochemistry, nutrition, and agricultural or environmental sciences.

Holistic methods apply a hypothesis-free approach and aim at the identification and determination of non-anticipated markers. In this approach, data leads the analysis. The markers found should subsequently be validated and linked within biochemical pathways. Metabolomics approaches can be combined with data from other -omics fields such as genomics, proteomics, and transcriptomics in order to reach a systems biology perspective to integrate different systems to provide descriptive and predictive models.

Thus far, biomarker discovery for disease and drug efficacy represent the major fields of metabolomics research and development[9]. In particular, the search for early biomarkers of disease (diagnostic markers)[10], biomarkers of drug efficacy (pharmacometabonomics)[11], biomarkers of disease progression (prognostic markers) or drug toxicity (safety assessment)[12] have attracted major efforts from the research community.

With regard to instrumental methods, holistic analysis necessitates the use of information-rich spectroscopy techniques such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS)[13,14]. Liquid phase separations, in particular, High-Performance Liquid Chromatography (HPLC) and Ultra High-Performance Liquid Chromatography [U(H)PLC], are the key analytical platforms. These are used in the pharmaceutical industry for the determination of small molecules in an extensive range of applications. When combined with mass spectrometry (MS), they provide the essential technology in the quest for wide metabolome coverage for biomarker discovery[13,14]. Due to the versatility offered, HPLC/U(H)PLC is the most powerful tool for the analysis of a multitude of molecules from different groups, molecules that have different molecular properties and exist in the same sample in different concentration ranges. As a result, LC-MS dominates the field of metabolomics analysis.

Methods

Given the dramatic consequences of perinatal asphyxia/HIE and the potential of metabolomics in the exploration of diseases and biomarker discovery, this review aims to make metabolomics comprehensible to the bedside clinician presenting the main results of the pertinent metabolomic studies regarding pathophysiology and current clinical practice. Therefore, we searched the Medline (PubMed) for metabolomic articles in English related to perinatal asphyxia/HIE, hypoxia-ischemia, and neonatal resuscitation published from 2000 to 2017. The following keywords were used in advanced search: (metabolom*) AND (neonat* OR perinatal OR newborn) AND (asphy* OR hypox* OR ischem* OR ischaem* OR anoxia OR encephalopathy OR brain injury). Moreover, our search was expanded to most recent neonatal resuscitation guidelines and therapeutic hypothermia (TH) in neonates with HIE to support our statements.

We proceeded in discussing metabolic derangements and their biological interpretation in perinatal asphyxia/HIE, and translate metabolomic investigations to clinical practice focusing on oxygen concentration during neonatal resuscitation, therapeutic hypothermia, as well as outcome prognosis. Finally, we investigated the role of metabolomics in the discovery of novel HIE biomarkers. To answer these topics, a narrative but comprehensive review is presented.

Results

From the 102 articles retrieved, only a number of them, either experimental or human (summarized in Table 1 and Table 2, respectively), were specifically referred to metabolomics in relation to perinatal asphyxia/HIE, neonatal hypoxia-ischemia, and resuscitation protocols. We also retrieved two review articles explicitly focusing on metabolomics and asphyxia/HIE[15,16] and the most recent guideline article on neonatal resuscitation from the International Liaison Committee on Resuscitation (ILCOR)[17]. Duplicate and irrelevant articles were not included. However, evaluations of methodological quality were not used to exclude studies from this review. The flow chart of the recovered and analyzed studies from the PubMed is shown in Figure 1.

Discussion

A. Metabolomics in perinatal asphyxia-HIE: possible clinical relevance

Metabolomics, as a holistic approach, has significant advantages over the traditional measurement of individual metabolites, allowing the simultaneous evaluation of various metabolic pathways in large-scale events such as hypoxia-ischemia following perinatal asphyxia. Metabolomics may not only markedly improve our understanding of the pathophysiological-biochemical alternations occurring in perinatal asphyxia-HIE but also provide evidence for important clinical questions.

Table 1: Summary of metabolomic studies in animal models of hypoxia/asphyxia and/or resuscitation protocols.

Author/year	Experimental model	Aim	Biological fluid	Analytical platform	Key findings
van Cappellen van Walsum et al/2001[41]	Fetal lambs	Investigate if mild hypoxia induces changes in cerebral metabolism vs. severe hypoxia	Cerebrospinal fluid	^{1}H NMR	• Increased choline in severe hypoxia • After 2 hours of mild hypoxia and in severe hypoxia, levels of lactic acid, alanine, phenylalanine, tyrosine, lysine, branched chain amino acids, and hypoxanthine were found increased
Atzori et al/2010[49]	Newborn piglets	Characterize the metabolic profiles of newborn undergoing hypoxia-reoxygenation	Urine	^{1}H NMR	• Metabolic variations were observed in the urine of piglets treated with different oxygen concentrations. Discriminant metabolites: urea, creatinine, malonate, methylguanidine and hydroxyisobutyric acid
Solberg et al/ 2010[19]	Newborn piglets	Detection of markers of hypoxia	Plasma	Flow injection analysis MS/MS and LC-MS/MS	• Ratios of alanine to branched chained amino acids and of glycine to BCAA were highly correlated with the duration of hypoxia • Reoxygenation with 100% oxygen delayed cellular metabolic recovery • Metabolites of the Krebs cycle (alpha keto-glutarate, succinate, fumarate) were significantly reduced at different rates depending on the resuscitation, showing a delay in recovery in the 100% reoxygenation groups. • Oxysterols and acylcarnitines showed different responses to reoxygenation
Beckstrom et al/2011[22]	Newborn non-human primate	Identify significant metabolites affected by birth asphyxia	Blood	GC×GC-TOFMS	• 10 metabolites increased after asphyxia • Lactate, creatinine, succinic acid, malate and arachidonic acid could help as potential biomarkers
Liu et al/ 2011[18]	Neonatal rats	Distinguish different insults, treatments and recovery stages after applying hypothermia	Brain slice	^{1}H/^{31}P NMR	• Metabolites differed in treatment and outcome groups, especially phosphocreatine, ATP and ADP • ATP levels severely decreased at normothermia, and restored equally by immediate and delayed hypothermia • Cell death was decreased by immediate hypothermia, but was equally substantially greater with normothermia and delayed hypothermia
Skappak et al/2013 [23]	Newborn piglets	Identify hypoxia using urinary metabolomic profiling	Urine	NMR	• 13 urinary metabolites differentiated hypoxic versus nonhypoxic animals (1-methylnicotinamide, 2-oxoglutarate, alanine, asparagine, betaine, citrate, creatine, fumarate, hippurate, lactate, N-acetylglycine, N-carbamoyl-β-alanine, and valine). • Using metabolomic profile, it was able to blindly identify hypoxic animals correctly 84% of the time compared to nonhypoxic controls • Metabolomic profiling of urine has potential for identifying neonates that have undergone episodes of hypoxia
Liu et al/ 2013[25]	Neonatal rats	Distinguish metabolic differences in glia and neurons	Brain slices	^{13}C NMR	• [2-C]Glutamine increased in the hypothermia group compared to delayed hypothermia and normothermia group • [3,4-C]glutamate, [2-C]taurine and phosphocreatine were mostly associated with adenosine triphosphate preservation
Liu et al/ 2013[51]	Neonatal mice	Identify biomarkers and distinguish differences applying hypothermia	Brain extracts	^{1}H NMR	• Hypothermia group was separated from non-hypothermia and controls
Fanos et al/2014[20]	Piglet model	Investigate metabolomic profiles according to oxygen concentration (18%, 21%, 40%, and 100%) administered at resuscitation	Urine	^{1}H NMR	• 21% of oxygen is the most “physiological” and appropriate concentration to be used for resuscitation
Takenouchi et al/2015[29]	Neonatal rats	Decipher the mechanisms through which hypothermia regulates metabolic dynamics in different brain regions	Brain tissue	MS/MS	• 107 metabolites were investigated • Hypothermia diminished the carbon biomass related to acetyl moieties, such as pyruvate and acetyl-CoA, and increased deacetylated metabolites (carnitine and choline) • Hypothermia diminished the acetylcholine contents in hippocampus and amygdala, where carnitine was increased

Chun et al/2015[39]	Non-human primate model	Identify indicators of brain injury, repair and prediction of neurodevelopmental outcome	Plasma	GC×GC-TOFMS	● 63 metabolites identified as potential biomarkers ● 8 metabolites (arachidonic acid, butanoic acid, citric acid, fumaric acid, lactate, malate, propanoic acid, and succinic acid) correlated with early and/or long-term neurodevelopmental outcomes ● Citric acid, fumaric acid, lactate and propanoic acid correlated with combined outcomes of death or cerebral palsy ● Circulating metabolome has the potential to predict neurodevelopmental outcome
Solberg et al/2016[33]	Newborn piglets	Identify early brain hypoxia biomarkers	Plasma	LC-TOFMS	● Increased plasma metabolites at the end of hypoxia, reflecting a metabolic adaptation to prolonged anaerobiosis ● Metabolite levels returned to base line after resuscitation
Sachse et al/2016[34]	Newborn pigs	Identify biomarkers for subject characterization, intervention effects and possibly Prognosis	Plasma/Urine	NMR	● Plasma and urine metabolites showed severe alterations consistent with hypoxia and acidosis 2 and 4 hours after return of spontaneous circulation ● Baseline plasma hypoxanthine and lipoprotein concentrations were inversely correlated to the duration of hypoxia sustained before asystole occurred ● No evidence for a differential metabolic response to the different resuscitation protocols or in terms of survival
Blaise et al / 2017[47]	Newborn mice	Investigate the effects of excitotoxicity in metabolome	● Brain tissue ● Plasma	MS	● No difference in plasma metabolic profile ● The amino acids glutamine, proline, serine, threonine, tryptophan, valine, and the sphingolipid SM C26:1 were increased in the brain. Glycerophospholipids were decreased ● Metabolomics could identify excitotoxic effects
Brown et al /2017[35]	Newborn mice	Investigate if intrauterine inflammation alters the metabolome of the amniotic fluid, fetal and neonatal brain, and if sex makes difference	● Amniotic fluid ● Brain	LC-MS	● Intrauterine inflammation enhances amino acids and purine metabolites ● Hypoxanthine pathway metabolites were increased in amniotic fluid. They can be potential biomarkers. ● Fatty acids pattern differed in neonatal brain in a sex-specific manner

NMR: nuclear magnetic resonance (spectroscopy), MS: mass spectrometry, LC-MS: Liquid Chromatography - Mass Spectrometry, GC×GC-TOFMS: 2-dimensional gas chromatography-time-of-flight-mass spectrometry, LC-TOFMS: Liquid chromatography-time of flight mass spectrometry.

Table 2: Summary of metabolomic studies in human neonates with perinatal asphyxia-hypoxic-ischemic encephalopathy (HIE).

Author/year	Experimental model	Aim	Biological fluid	Analytical platform	Key findings
Chu et al/ 2006[56]	Asphyxiated neonates	Study the metabolomic profile in urines of neonates with severe asphyxia and subsequent neurodevelopmental handicap	Urine	High throughput MS	● Increased ethylmalonate, 3-hydroxy-3-methylglutarate, 2-hydroxy-glutarate and 2-oxo-glutarate were associated with good neonatal outcome ● Increased glutarate, methylmalonate, 3-hydroxy-butyrate and orotate were associated with poor outcome
Walsh et al/2012[42]	Newborns with HIE	Investigate the metabolomic profile	Umbilical cord blood	LC-MS/MS	● 29 metabolites showed alterations from 3 distinct classes (amino acids, acylcarnitines and glycerophospholipids) ● 9 metabolites were significantly altered in HIE ● A model of 5 metabolites clearly delineated severity of asphyxia and classified HIE infants ● Disruption to energy, nitrogen and lipid metabolism was evident in both asphyxia and HIE
Reinke et al/2013[24]	Asphyxiated neonates	Invastigate pathophysiology of HIE	Umbilical cord blood	^{1}H NMR	● 37 metabolites were significantly altered between the study groups ● Acetone, 3-hydroxybutyrate, succinate, and glycerol were significantly differentially altered in severe HIE ● A model using 3-hydroxybutyrate, glycerol, O-phosphocholine and succinate predicted HIE severity
Longini et al/2015 [21]	Asphyxiated neonates	Evaluate the effects of asphyxia on newborn metabolites	Urine	^{1}H NMR	● Lactate, glucose, trimethylamine N-oxide, threonine and 3-hydroxyisovalerate were the metabolites more characterizing the asphyxiated neonates • After 24-48 hours from resuscitation, asphyxiated neonates showed a recovery pattern but still could be differentiated from controls

Noto et al/2016[57]	Asphyxiated neonates	Identify the metabolome in perinatal asphyxia and to follow changes over time	Urine	GC-MS	• The metabolomic profile of neonates who died after day 7 of life was significantly different from that of survivors
Ahearne et al/2016[58]	Infants with perinatal asphyxia and HIE	Investigate if alterations of succinate, glycerol, 3-hydroxybutyrate and O-phosphocholine can predict 3-year neurodevelopmental outcome	Umbilical cord blood	^{1}H NMR	• The metabolite index significantly correlated with outcome, predicting severe outcome and intact survival • There was no correlation between the index score and performance in the individual Bayley-III subscales (cognitive, language, motor) • The metabolite index was not superior to EEG or the Sarnat score
Deniham et al/2017[43]	Asphyxiated neonates (recovering and developing HIE)	Examine early metabolic alterations in infants recovering perinatal asphyxia vs. those who developed HIE	Umbilical cord blood	FT-ICR mass spectrometry	• Perturbed metabolic pathways and potential biomarkers specific to perinatal asphyxia and HIE were identified, which if measured at birth, may help direct treatment
Sanchez-Illana et al / 2017[50]	Newborns with HIE	Determination of lipid peroxidation biomarkers in newborn plasma samples	Plasma	LC-MS	• Isoprostanoids provide predictive power of oxidative stress related pathologies
Sarafidis et al/2017[26]	Asphyxiated term neonates with HIE	Identify metabolic changes in neonates with HIE	Urine	LC-MS/MS	• Asphyxiated neonates were clearly separated from controls • Discriminant metabolites involved pyruvic acid, amino acids, acylcarnitines, inositol, kynurenine, hippuric acid and vitamins

MS: mass spectrometry, LC-MS: Liquid Chromatography - Mass Spectrometry, NMR: nuclear magnetic resonance (spectroscopy), HIE: hypoxic-ischemic encephalopathy, GC-MS: gas chromatography mass spectrometry, FT-ICR: Fourier-transform ion cyclotron resonance.

Metabolic derangements and biological interpretation

Recent metabolomic studies in animal models and neonates have confirmed the involvement of known pathways and revealed the contribution of other heretofore unknown pathways in the development of perinatal asphyxia/HIE.

As expected, alternations in energy metabolites that indicate a shift towards anaerobic metabolism (increased lactate) and disturbance of the Kreb's cycle were the most prominent findings of the relevant metabolomic studies. Due to energy depletion (ATP)[18] and dysfunction of the respiratory chain, lactate is formed and accumulated[19-21], as are the Kreb's cycle intermediates (citrate, alpha ketoglutarate, succinate, and fumarate)[19,22-26]. As suggested by data from infants with severe HIE, increased succinate levels may play a particular role in the development of encephalopathy after asphyxia, possibly through the hypoxia-inducible factor-1α[24]. Previous animal studies have already documented increased lactate levels immediately after oxygen deprivation[27]. Moreover, lactate measured within one hour after birth has been used as an early predictor of HIE development after intrapartum asphyxia[28]. It is noteworthy that even 24-48 hours after resuscitation, neonates suffering asphyxia seem to have their own metabolic fingerprint compared to controls, suggesting ongoing metabolic alternations[21], seemingly due to delayed recovery of the Kreb's cycle[19].

Urine NMR analysis in term asphyxiated newborns also showed reduced acetate[21], a precursor of acetyl-coenzyme A, which has a central role in the metabolism of carbohydrates and fats by entering the Kreb's cycle. Interestingly, results from animal studies imply that hypothermia achieves its neuroprotective effects, through the coordinated suppression of acetyl-CoA content, which in turn down-regulates the production of acetylcholine in specific regions of the brain[29].

Increased glucose was one of the significant changes observed in the urine metabolic fingerprint of asphyxiated neonates[20,21]. Nevertheless, circulating (and probably

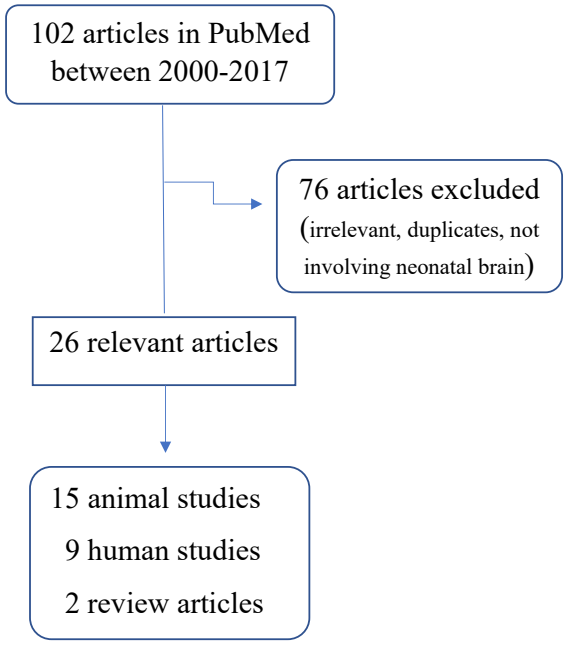

Figure 1: Flow chart of the studies retrieved from Medline (PubMed) that were finally analyzed.

urine) glucose levels may not reflect those in the brain. As a matter of fact, rats exposed to hypoxia were found to have elevated plasma glucose in spite of a profound decrease in brain glucose. Increased brain demands for glucose exceeding the supply from the periphery was speculated to explain this paradox[27]. Previous experimental data had shown that glucose supplementation reduces perinatal hypoxic-ischemic brain injury[30]. Results of a retrospective study in term infants with HIE, none of which received TH, indicated that early hypoglycemia occurring during the first six hours of life was associated with an adverse neurodevelopmental outcome at 24 months of age, irrespective of the grade of HIE. Nevertheless, this unfavorable outcome was not observed in hypoglycemia occurring after the sixth hour of life as well as in hyperglycemia[31]. Currently, although it is generally considered that normoglycemia should be maintained in asphyxiated neonates, no specific glucose targets are recommended for the post-resuscitation care of the neonates[17].

Hypoxanthine is a purine derivative and for many years has been considered as a hallmark indicator of asphyxia[32]. When metabolized by xanthine oxidase, it generates oxygen radicals that are highly destructive to the tissues, including the brain. In recent metabolomic studies involving animal models of severe neonatal asphyxia or hypoxia, plasma hypoxanthine concentrations were significantly increased[33-35]. In the latter investigations, levels of other products of purines' catabolism (e.g., inosine, uric acid, etc.) and pyrimidine were also found to be increased[33]. Allopurinol, a xanthine-oxidase inhibitor, could potentially reduce the formation of these superoxides and, thus, brain damage in HIE. However, the available data are not sufficient to determine whether allopurinol has clinically important benefits for newborn infants with HIE[36]. A European Union-funded project (the ALBINO trial) will further evaluate the efficacy and safety of allopurinol administered immediately after birth to near-term and term infants with HIE in addition to TH[37].

Metabolomic studies suggest that lipids play a crucial role in perinatal asphyxia-HIE. According to experimental and clinical studies, choline is one of the most significantly increased analytes during postnatal hypoxia/aspyxia[24,33-34]. Choline and its metabolites are very important for the structural integrity and signaling of cell membranes (phospholipids), neurotransmission (acetylcholine synthesis), lipid transport (lipoproteins), and methyl-group metabolism (homocysteine reduction)[38]. On the contrary, as shown in animal studies, arachidonic acid is characteristically elevated in asphyxia-HIE, possibly reflecting cell disruption[22,39]. This fatty acid is a component of the cell membrane found abundantly in the brain and is a precursor of biologically important metabolites such as prostacyclins, thromboxanes, and leukotrienes. Moreover, activation of cytosolic phospholipases following hypoxia-ischemia increases eicosanoid release and brings about inflammation, which represents a major mechanism of brain injury in HIE[7]. Carnitine, the transporter of the fatty acids across the inner mitochondrial membrane for the β-oxidation, is also altered in asphyxia. Plasma metabolomic analysis in studies involving asphyxia-resuscitation or hypoxia in newborn pigs revealed lower free carnitine and higher long-chain acylcarnitines after hypoxia (owing to the hypoxia-induced incomplete β-oxidation)[19,33]. In a recent study, we were also able to confirm elevated urine acylcarnitines levels in the HIE compared to controls[26]. Other derangements in metabolites include ketone bodies[24] and amino acids[19,23], both of which may serve as alternative energy sources. Moreover, ketogenesis was proposed as playing an important role in preventing neurological injury during perinatal asphyxia as acetone and 3-hydroxybutyrate were found increased two-fold in asphyxiated infants and decreased by two-fold in severe HIE[24].

Inositol is a precursor for phosphorylated compounds that are involved in signal transduction[40] widely distributed in human tissues and cells. Its most widely occurring stereoisomer, *myo*-inositol, has been documented to increase in the blood following perinatal asphyxia or HIE in animals[22] and humans[24,26]. 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[41].

Finally, perturbations of the amino acids seem also to be the case in hypoxia and HIE. Nevertheless, in some animal[19] and humans studies[42,43], a significant increase in the levels of several amino acids was noted, whereas others in humans showed elevated or reduced amino acids[26,44]. Among the latter metabolites, glutamine merits specific consideration, as is a crucial link between carbon metabolism of carbohydrates and proteins, and also plays an essential role in the growth of several cells. Most importantly, glutamine is related to glutamate[45], the major excitatory neurotransmitter in perinatal brain injury[46]. Previous studies documented significantly elevated glutamine levels in the plasma and brain of newborn animals following hypoxia[33,47].

B. Translation of experimental data to clinical practice

Oxygen concentration and neonatal resuscitation

A clinically important point in neonatology is how to optimize resuscitation to prevent morbidity and mortality. Cardinal clinical studies have shown that neonates resuscitated with ambient air (21 % oxygen) had a faster recovery as indicated by the significantly higher heart rate at 90 seconds after birth and Apgar score at five minutes compared to those resuscitated with 100 % oxygen[48]. Ensuing metabolomic investigations in asphyxiated piglets resuscitated with 21 % versus 100 % oxygen provided a biological explanation of the findings of the aforementioned clinical studies, documenting not only metabolic variations with different oxygen concentrations[49] but also an earlier recovery of the mitochondrial function (decline of the Krebs cycle intermediates) with 21 % oxygen[19]. As derived from another metabolomic study in animals, resuscitation with oxygen at 21 % seems to be associated

with optimal cellular function and maintenance whereas lower (18 %) and higher oxygen concentrations with carbohydrate metabolism (increased glucose and lactate) and free radical scavenging, respectively[20]. Progress in analytical techniques allowed the identification of novel lipid peroxidation biomarkers (isoprostanoids) related to hypoxia and reoxygenation in neonates with HIE using very small volumes of plasma[50] that is clinically challenging. Altogether, these data corroborate recent resuscitation guidelines in term neonates, in which initiation of resuscitation with room air is suggested[17]. Interestingly, an animal study evaluating six different neonatal cardiopulmonary resuscitation protocols with the parallel use of plasma and urine metabolomics, although confirmed the presence of severe metabolic alterations in hypoxia-induced cardiac arrest up to four hours after recovery of the circulation, yet provided no evidence for a differential metabolic response to the various resuscitation protocols or in terms of survival[34].

Therapeutic hypothermia

The theoretical background of TH involves the protection of the nervous system by lowering body temperature and, thus, the metabolic rate of the brain. With a series of pioneer studies, Liu et al using the NMR analysis of rodent brain tissue documented the important role of metabolomics for the evaluation of different hypothermia strategies on the outcome[18,25,51]. In any case, though, early recognition of neonates with HIE is crucial as the efficacy of TH is time-dependent. HIE-affected, full-term neonates could be discriminated from non-affected ones on the basis of umbilical cord serum metabolome. Nevertheless, only separation of the severe HIE cases could be accurately made in the latter studies[24,42]. Clinical parameters such as the combination of umbilical arterial lactate and neonatal resuscitation level were reported to accurately identify at birth neonates that may benefit from neuroprotective therapies as TH, that is, those with moderate-severe HIE[52]. Major cooling trials[4] and subsequent guidelines[6,7,17] excluded babies with mild encephalopathy from receiving therapeutic hypothermia, as prognosis in these cases is generally considered "good". The issue, however, is precisely the neonates with mild HIE. These infants may subsequently develop moderate-severe HIE that has escaped initial diagnosis with the existing clinical tools (e.g., the Thompson Score)[53] and, in general, require a high level of expertise. Additionally, emerging evidence indicates that mild HIE may not be as safe as assumed. As it is proven in animal studies, cerebrospinal fluid levels of lactic acid, alanine, phenylalanine, lysine, tyrosine, branched chain amino acids, and hypoxanthine were increased even in cases with mild hypoxia compared to control[41]. These findings are in agreement with recent clinical data reporting a larger than expected proportion of infants with mild encephalopathy and abnormal outcomes[54]. It is reasonable, then, that the existing clinical practice not to cool neonates with mild encephalopathy is considered a matter of controversy[55]. Recent metabolomic studies indicate potential biomarkers that, if measured at birth, could distinguish neonates recovering from perinatal asphyxia from those developing HIE, helping thus to direct treatment[43].

Prognosis of outcome

In one of the earliest works in the field of metabolomics, using Gas Chromatography-MS (GC-MS), Chu et al showed that the urine metabolic profile of the asphyxiated neonates with a good neurodevelopmental outcome is different from that of the neonates who develop HIE or die. In Chu's study, eight organic acids involved in distinct biochemical pathways were significantly associated with neurodevelopmental handicap having high sensitivity and specificity[56]. Similarly, preclinical studies documented an association between specific metabolites (arachidonic acid, butanoic acid, citric acid, fumaric acid, lactate, malate, propanoic acid, and succinic acid) and early or long-term neurodevelopmental outcome[39]. Moreover, in cooled asphyxiated neonates, the urine metabolic profiles of those who died after seven days of life were closely comparable to each other and significantly different from those of survivors[57]. Overall, metabolomic studies performed so far on neonatal asphyxia/HIE, although limited in number, support the possibility of developing an HIE biomarker in the future using metabolomics.

C. Metabolomics and novel HIE biomarkers

A unique metabolomic "fingerprint" might be used in the future for the development of diagnostic and prognostic biomarkers, thus individualizing treatment and allowing more accurate prediction of important clinical outcomes. Currently, our urgent need to develop HIE biomarkers is mainly based on the following scientific gaps: a) our inability to differentiate mild HIE from perinatal depression without encephalopathy or to identify those neonates who are at risk of progressing to moderate-severe HIE; b) our inability to predict response, for instance to TH, whenever it is applied, and possibly add or apply other neuroprotective interventions; and c) the need to prognosticate short- and long-term outcomes related to the severity of the disease, coexisting perinatal conditions and management.

So far, only a few metabolites have been proved to be robust enough, diagnostically, in neonatal asphyxia/HIE. Most likely, a panel of metabolites will offer higher diagnostic accuracy than a single one[24,42]. It is to be hoped that an easy-to-apply test will be available for clinical practice soon, contributing significantly to the improvement in neonatal care. A ^{1}H-NMR-derived metabolomic index based on early umbilical cord blood alterations of succinate, glycerol, 3-hydroxybutyrate, and O-phosphocholine has shown potential for the prediction of HIE severity. Although the latter metabolite index outperformed other standard biochemical markers at birth for prediction of neurodevelopmental outcome at three years, it was not found to be superior to EEG or the Sarnat score[58].

Conclusions

Metabolomics is a valuable tool for the exploration of the multiple biochemical alternations observed in HIE. A better understanding of the disease, potentially, may allow the discovery of new neuroprotective interventions as well as of novel diagnostic-prognostic biomarkers, thus individualizing the management of the affected neonates. So far, relevant research is limited. Large-scale studies are needed to prove the utility of metabolomics in perinatal asphyxia/HIE, ultimately clarifying important clinical dilemmas and questions. Nevertheless, as highlighted in a previous review article by Deniham et al, issues related to the highly unpredictable nature of HIE, and thus patient recruitment, renders metabolomic research in neonatal HIE a difficult task[16].

Conflict of interest

Authors declare no conflict of interest.

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