Neurosteroid pathway derangement in asphyctic infants treated with hypothermia: an untargeted metabolomic approach

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Summary

Background The pathobiological mechanisms associated with perinatal asphyxia and hypoxic-ischemic encephalopathy are complex and poorly understood. The metabolic effects of therapeutic hypothermia have been partially explored.

Methods We conducted a single-center longitudinal study to investigate the metabolic effects of perinatal asphyxia and hypoxic-ischemic encephalopathy on the urinary metabolome of a group of 12 asphyctic infants over time compared to 22 matched healthy newborns, using untargeted metabolomics based on mass spectrometry.

Findings Over-representation pathway analysis identified the steroidogenesis pathway as being significantly disrupted, with reduced steroid levels in the first three days of life despite treatment with hypothermia. Comparison with matched healthy newborns showed that the urinary steroid content was lower in asphyctic infants before hypothermia. The lysine degradation and carnitine synthesis pathways were also significantly affected.

Interpretation Steroidogenesis is significantly disrupted in asphyctic infants compared to healthy newborns. Given how neurosteroids are involved in neuromodulation and neuroprotection, translational research is warranted on the potential role of neurosteroid-based intervention in asphyctic infants.

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Keywords: Perinatal asphyxia; Hypoxic-ischemic encephalopathy; Metabolomics; Neurosteroids; Translational research

Introduction

Despite advances in obstetric care and in prenatal and intrapartum monitoring, perinatal asphyxia (PA) and hypoxic-ischemic encephalopathy (HIE) are still a major concern for obstetricians and neonatologists. In addition to the short-term adverse outcomes associated with these entities (death occurs in 15–20% of severely asphyxiated infants),[1] the long-term prognosis for asphyctic infants may also be burdened with severe issues (cerebral palsy, behavioural disorders, epilepsy, cognitive impairment, and neurodevelopmental disability),[2,3] particularly in those suffering severe HIE. This last clinical entity is the most detrimental consequence in survivors of severe PA,[4–6] and occurs in one to five in every 1000 cases of live births in the developed world[7,8] and even more in developing countries.[9,10] Many randomized clinical trials[11–15] have demonstrated the effectiveness of therapeutic hypothermia (TH) initiated within six hours after birth in preventing or containing HIE in newborns with PA. To date, TH represents the only approved treatment for such infants.[16] The pathobiological mechanisms associated with HIE are complex and poorly understood, and the metabolic effects of TH have only been partially explored in humans. Metabolomics is a multiparametric method

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Research in context

Evidence before this study

To assess the literature background to put our research in context, we searched the major medical databases (PubMed, EMBASE, PubMed Central) considering all relevant translational and human research exploring perinatal asphyxia using metabolomics. Considered article types were Original Researches, Reviews, and Meta-analyses; other types of publication were excluded. The time interval for literature research spanned from January 1, 1995, to December 31, 2022. Numerous metabolites (amino acids, compounds related to energy production and oxidative stress, intermediates of the tricarboxylic acid cycle, phospholipids, and many others) were found potentially related to neurological damage in asphyctic patients. The role of neurosteroids in reducing brain damage after asphyxia has been evaluated in animal models, and a disruption of steroid biosynthesis has been recently described also in human asphyctic newborns, albeit no clear trend towards rise or decrease of neurosteroid levels over time has been defined in previous works. Overall, the available data come from studies that are very heterogeneous in terms of experimental design, patient numerosity, and the analytical techniques employed; consequently, it is still impossible to generate either useful biomarkers to allow an early prediction of the entity of neurological damage in asphyctic patients, or to identify a specific metabolic set-up suitable for targeted interventions.

Added value of this study

Our exploratory study on the dynamic changes in the urinary metabolome of asphyctic newborns undergoing therapeutic hypothermia in their first three days of life showed that steroidogenesis was the most deranged pathway; the novelty of our work is that we showed that patients' urinary steroid levels were lower than in healthy matched newborns, and showed a clear trend towards steady decrease over time.

Implications of all the available evidence

Neurosteroids are involved in neuromodulation and neuroprotection in animal models; our study shows that steroidogenesis is significantly disrupted in human newborns suffering from perinatal asphyxia and hypoxic-ischemic encephalopathy. Translational research is warranted to assess the potential role of neurosteroid-based interventions in such infants.

for identifying low-molecular-weight metabolites (less than 1500 Da in mass) in biological systems. It has recently been applied in precision neonatal medicine, showing substantial promise to improve our understanding of the metabolic alterations associated with HIE.[17–19] Given its potential for holistic investigation of the metabolic status of an organism at a given time, metabolomics has been used in recent years in asphyctic patients, with two main goals: to elucidate and characterise the metabolic modifications that occur in PA and HIE, and to reveal metabolic markers (single metabolites or groups of metabolites) capable of predicting patients' TH needs and outcomes.[7] Most human studies used urine as the biological fluid for metabolomic analysis because samples can be collected longitudinally and noninvasively.[17,19–21] Numerous metabolites (amino acids, compounds related to energy production and oxidative stress, intermediates in the tricarboxylic acid cycle, phospholipids, and many others) were found potentially related to neurological damage and HIE.[7,18,22–25] Unfortunately, it is still impossible to generate a reliable metabolic fingerprint that meets clinical needs because the available data come from studies that are very heterogeneous in terms of experimental design, patient numerosity, and analytical techniques employed. The purpose of this exploratory longitudinal study was to investigate the metabolic effects of HIE, and the changes induced by TH on the urinary metabolome of asphyctic infants in the first days of life, to shed more light on the pathobiological mechanisms associated with PA and HIE.

Methods

Study design

We conducted a longitudinal single-center study, enrolling newborns who presented with PA and HIE at birth and received TH.

Patients

The study involved infants born at >35 weeks of gestation with HIE secondary to PA and managed with TH according to international guidelines.[26] Patients were admitted to the NICU in the Department of Woman's and Child's Health at Padova University Hospital (Italy) from May 2016 to April 2019. Asphyctic neonates were classified by gestational age, birth weight, sex, Apgar score at 1, 5, and 10 min, delivery mode, Sarnat score at 60 min of life, pH and base excess (BE) derived from cord blood. A group of 22 healthy newborns matched for gestational age, birth weight, sex and delivery mode was also enrolled (Table 1).

According to international guidelines[26] during TH asphyctic infants received 10% intravenous glucose solution, a low dose of fentanyl (0.5–1 mcg/kg/h) and initial antibiotic treatment promptly stopped whenever possible. They did not receive enteral nutrition. 10% intravenous glucose and fentanyl were both commenced along with TH and stopped simultaneously to the start of rewarming of the patients. All neonates receiving TH underwent post-rewarming brain magnetic resonance imaging (MRI).

Characteristic	Cases N = 12	Matched healthy newborns N = 22
Sex, male (female)	8 (4)	11 (11)
Gestational age, median [range] days	277 [243; 292]	273 [259; 286]
Birth weight, median [range] g	3240 [2500; 4134]	3340 [2220; 4150]
Delivery mode, vaginal (caesarean section)	7 (5)	8 (14)
SARNAT 60 min, median [range]	2 [1; 3]	0 [0]
Apgar 1 min, median [range]	3 [1; 9]	8 [7; 9]
Apgar 5 min, median [range]	5 [2; 8]	9 [8; 10]
Apgar 10 min, median [range]	7 [4; 9]	10 [9; 10]
Cord pH, median [range]	6.9 [6.8; 7.1]	7.25 [7.20; 7.44]
Cord BE, median [range] mmol/L	−15.6 [−21.5; −7.5]	−4.7 [−13.7; −2.0]

SARNAT, Sarnat score for neonatal encephalopathy; BE, base excess.

Table 1: **Characteristics of the investigated groups of subjects.**

Ethics

The study was approved by the hospital ethics committee (Azienda Ospedale-Università di Padova, Ref. 4332/AO/17), and written informed consent was obtained from the parents.

Sampling

Urine samples were collected at the following times: during the first six hours of life, before starting TH (in the 'T0' phase), during 72 h of TH (in the 'TH' phase; samples were obtained after 6 and within 24 h of life), and after rewarming (the post-treatment phase, or 'PT' phase; samples were collected within 24 h of rewarming), using the methodology previously described.[21] The same methodology was also used to collect urine samples from healthy newborns in the nursery; a single sample was collected as soon as possible, within 24 h of life.

Metabolomic analysis

Untargeted metabolomic analysis based on mass spectrometry was performed in the Mass Spectrometry and Metabolomics Laboratory of the Institute of Pediatric Research, at the Woman's and Child's Health Department at Padova University. Urine samples were analysed as previously described,[27] using an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters MS Technologies, Ltd., Manchester, UK) coupled with a Quadrupole Time-of-Flight Synapt G2 HDMS mass spectrometer (Waters MS Technologies, Ltd., Manchester, UK) operating in positive and negative electrospray ionisation mode. More details of the sample preparation, metabolomic analysis, data preprocessing, and variable annotation, are available in the Supplementary Information.

Statistical data analysis

Characteristics of the recruited subjects were investigated using the Mann–Whitney test and Pearson's Chi-square test in the case of continuous and categorical attributes, respectively. Exploratory data analysis and outlier detection were performed by Principal Component Analysis (PCA).[28] Modifications in the urinary metabolome over time were investigated by univariate and multivariate data analysis. Specifically, Linear Mixed Effects (LME) modelling controlling the false discovery rate by the Benjamini–Hochberg procedure was applied to each single measured metabolite, whereas multivariate data analysis was based on Partial Least Squares (PLS) modelling following a recently introduced approach.[29] The set of relevant annotated metabolites was submitted to over-representation pathway analysis considering the 99 pathways of the Small Molecule Pathway Database (SMPDB). Furthermore, the urinary metabolome of asphyctic newborns and that of matched healthy newborns before onset of TH were compared using PLS for classification[30] with stability selection and Mann–Whitney test controlling the false discovery rate by the Benjamini–Hochberg procedure. A significance level $\alpha = 0.05$ and a level $\delta = 0.05$ were assumed in the PLS modelling and the false discovery rate, respectively. Data analysis was performed using in-house R-functions implemented by R 4.0.4 platform (R Foundation for Statistical Computing) and over-representation pathway analysis using Metaboanalyst 5.0 (www.metaboanalyst.ca). More details about statistical data analysis can be found in the Supplementary Information.

Role of the funders

There is no relevant funding source for this study.

Results

Patients' characteristics

Twelve patients had urine collected before, during and after TH for a total of 36 samples. Twenty-two healthy newborns matched for gestational age, birth weight, sex and delivery mode were enrolled. Table 1 shows the characteristics of the two groups of recruited subjects. Assuming a significance level of $\alpha = 0.05$, differences emerged in SARNAT score, Apgar score, pH and BE, as

expected. 4 out of 12 newborns undergoing TH developed neurological damage at post-rewarming brain MRI (consistent with severe HIE), while 8 did not. All but one of the patients survived to discharge. No adverse outcome has been registered for matched healthy newborns.

Untargeted metabolomic analysis

After preprocessing, a dataset of 1791 variables and 36 observations was obtained by merging the data recorded in positive and negative ionisation mode. No outliers were detected based on the T2 and Q-tests, assuming a significance level of $\alpha = 0.05$. The variable annotation led to 96 urinary metabolites being annotated at level 1. Considering the annotated variables, the score scatter plot of the PCA model ($R^2 = 0.542$, $Q^2 = 0.451$) is shown in Fig. 1. It is worth noting that asphyctic patients and matched healthy newborns occupy two different regions of the plot, which shows that their urinary metabolomes differed, regardless of the sample collection phase. Investigating the differences between cases and controls at T0, 71 of the identified metabolites showed a significant difference in the median, after controlling for the false discovery rate using the Benjamini–Hochberg procedure at the level of 0.05. Among them, 23 metabolites (Supplementary Table S1 in the Supplementary Information) proved relevant in revealing changes in the urinary metabolome during and after TH in asphyctic patients. Focusing on the cases and considering the annotated variables, LME-based univariate data analysis identified 23 metabolites that modified their level in the three phases. Multivariate data analysis based on PLS generated a model that explained 50% of the total variance associated with the phase ($p = 0.022$). The biplot of the model is shown in Fig. 2. The data representation provided by the two latent variables F1 and F2 shows a cluster structure, where samples from the same phase lie in the same region of the plot, indicating that the phase influenced the urinary metabolome. Merging the results of univariate and multivariate data analyses, 31 metabolites were found to be relevant to reveal changes in the urinary metabolome of the asphyctic infants in the three phases investigated. Table 2 shows the relevant metabolites with their fold changes. The most represented classes of compounds were amino acids (9), steroids (8), carnitine derivatives (3), and intermediates of tryptophan metabolism (2). The boxplots in Supplementary Fig. S1 show the trends of the relevant metabolites over time. Different trends emerged in the levels of urinary amino acids: the increase in L-histidine, L-cysteine, N-acetyl-L-aspartic acid, N-acetyl-L-tyrosine, N-acetyl glutamic acid, and N-acetyl valine levels persisted over time; L-lysine levels decreased steadily over the three phases; and pyroglutamic acid levels fell, then rose, while homocitrulline exhibited an opposite trend. Taking the first urine samples (obtained at T0) as a

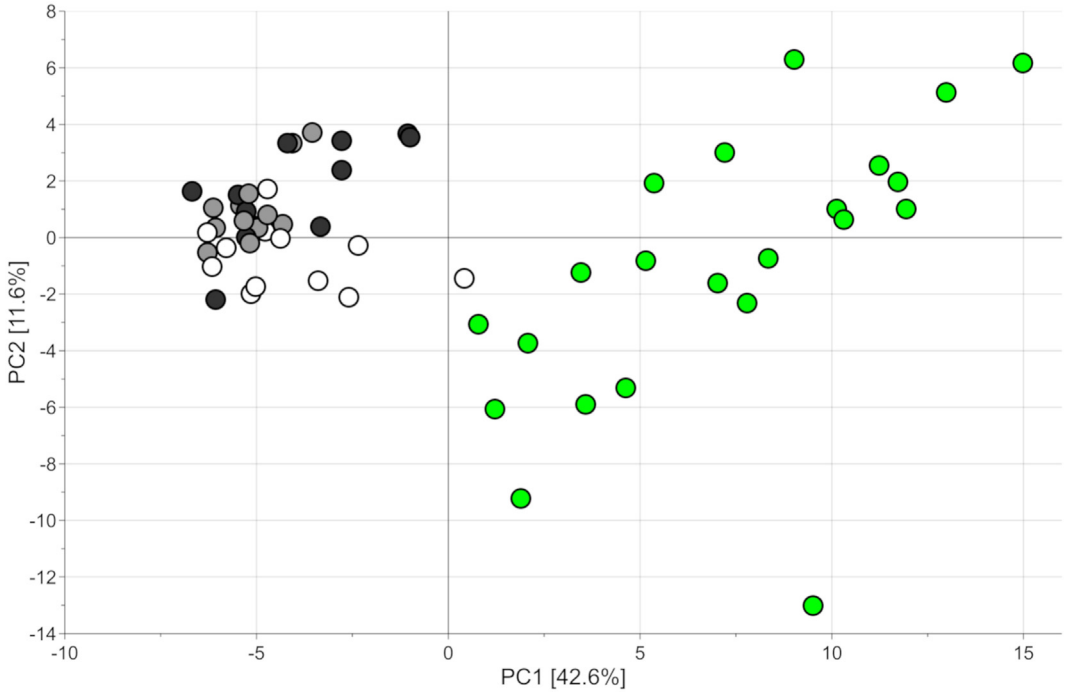

Fig. 1: Score scatter plots of the PCA models. Samples are shown as circles (green for matched healthy newborns; white at T0, light grey at TH, and dark grey at PT for asphyctic neonates).

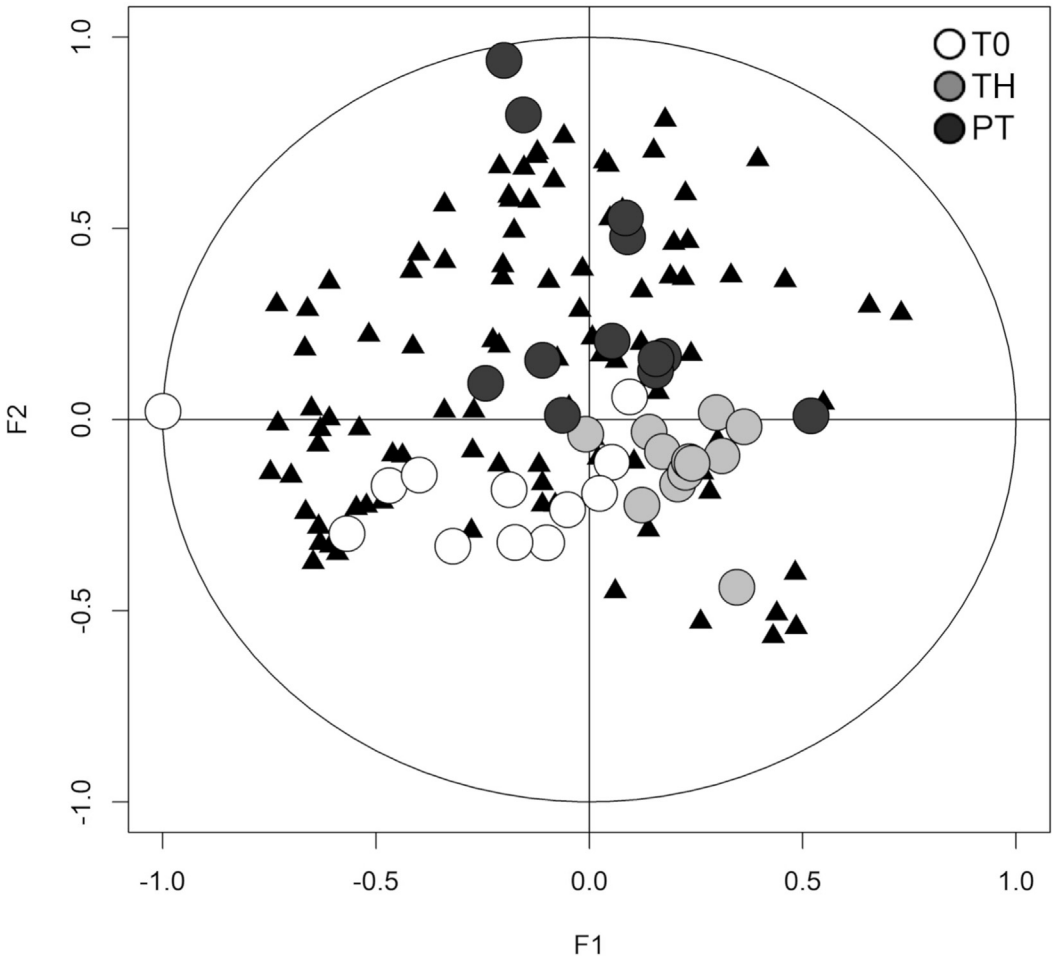

Fig. 2: Biplot of the PLS model. Asphyctic neonates' urine samples are shown as circles (white at T0, light grey at TH, and dark grey at PT), and variables as black triangles. Samples occurring close to each other have similar urinary metabolomes, while greater distances indicate greater differences between them. Variables closer to samples indicate higher levels for the samples concerned than for the others. The samples show a cluster structure by phase, with specific metabolites characterizing each phase.

reference, steroids decreased in asphyctic newborns during TH and in the PT phase (see Table 2). Regarding carnitine derivatives, L-octanoylcarnitine and isovalerylcarnitine levels gradually increased from T0 to TH, to PT. As for the intermediates of tryptophan metabolism: L-kynurenine levels steadily increased over time, while kynurenic acid levels constantly decreased. The set of relevant metabolites was investigated using over-representation pathway analysis to see which metabolic pathways were disrupted in the three phases. As presented in Fig. 3, the findings showed a $p < 0.10$ for three pathways: steroidogenesis, lysine degradation, and carnitine synthesis. The differences between cases and controls at T0 were investigated. After combining the results of the univariate analysis (which identified 71 relevant metabolites) and the multivariate analysis (the PLS model showed a Matthew correlation coefficient in cross-validation of 0.936, with $p < 0.001$, and 31 metabolites were found as relevant), 79 metabolites were found to be relevant for distinguishing between the two groups. Among them, 23 metabolites (see Supplementary Table S1 in Supplementary Information) were also relevant in revealing changes in the urinary metabolome of asphyctic patients during and after TH. The asphyctic infants had a lower urinary steroid content at T0 than the matched healthy newborns. The same was true for tryptophan metabolites and lysine metabolites (in particular, aminoadipic acid, involved in the lysine pathway degradation, was significantly lower in asphyctic than in healthy neonates with a fold change-calculated as the ratio between the median of the variable before therapeutic hypothermia and the median of the same variable in the healthy subjects-of 0.24; see also Supplementary Table S2 in the

Supplementary Information), while intact lysine proved to be not significantly different between asphyctic and healthy newborns at T0. Urinary gamma-butyrolactone levels in asphyctic infants were strikingly higher than in healthy newborns.

Discussion

This exploratory study investigated the dynamic changes in the urinary metabolome of asphyctic newborns with HIE undergoing TH. One of the main findings, and a novel element compared with previous literature, is that steroidogenesis was the most perturbed pathway, with a clearly decreasing trend in asphyctic patients over time. In addition, comparison with matched healthy newborns showed that the urinary steroid content was lower in asphyctic infants at T0. Steroids are involved in neuromodulation and neuroprotection, which is one of the major issues in the clinical management of HIE. Among the 31 metabolites identified as relevant for revealing changes over time in the urinary metabolome of asphyctic newborns, eight metabolites (aldosterone, pregnenolone [a precursor of neuroactive allopregnanolone],[31] 17a-hydroxypregnenolone, tetrahydrodeoxycorticosterone, cortolone, 21-deoxycortisol, 7a-hydroxydehydroepiandrosterone and 3a,21-dihydroxy-5b-pregnane-11,20-dione) belong to the steroid family. The derangement of the steroid pathway did not disappear during and after TH: the urinary steroid content of asphyctic newborns gradually decreased during TH and in the 'PT' period (see Table 2). The differences detected between the urine metabolomes of asphyctic and healthy newborns (see Supplementary Table S1 in the Supplementary Information) serve as a proof of concept,

Annotation	HMDB	FC [TH/T0]	FC [PT/T0]	Metabolic class
Pregnenolone	HMDB0000253	0.61	0.46	C21 steroids/progestins
17a-Hydroxypregnenolone	HMDB0000363	0.77	0.49	C21 steroids/progestins
Aldosterone	HMDB0000037	0.53	0.83	C21 steroids/mineralocorticoids
Tetrahydrodeoxycorticosterone	HMDB0000879	0.62	0.42	C21 steroids/corticoids
Cortolone	HMDB0003128	0.56	0.66	C21 steroids/corticoids
21-Deoxycortisol	HMDB0004030	0.73	0.83	C21 steroids/corticoids
3a,21-Dihydroxy-5b-pregnane-11,20-dione	HMDB0006755	0.89	0.92	C21 steroids/corticoids
7a-Hydroxydehydroepiandrosterone (7-a-OH-DHEA)	HMDB0004611	1.13	1.35	C19 steroids/androgens
Butyrylcarnitine	HMDB0002013	0.43	0.37	acylcarnitine
Isovalerylcarnitine	HMDB0000688	1.52	1.72	acylcarnitine
L-Octanoylcarnitine	HMDB0000791	1.47	1.31	acylcarnitine
Glycocholic acid	HMDB0000138	1.49	2.32	bile acids
L-Lysine	HMDB0000182	0.91	0.25	L-alpha-amino acids
L-Cystine	HMDB0000192	1.66	2.05	L-alpha-amino acids
Homocitrulline	HMDB0000679	1.05	0.81	L-alpha-amino acids
L-Histidine	HMDB0000177	2.49	5.92	L-alpha-amino acid
L-Kynurenine	HMDB0000684	1.04	2.05	Tryptophan Metabolism
Kynurenic acid	HMDB0000715	0.67	0.93	Tryptophan Metabolism
Pyroglutamic acid (5-oxoproline)	HMDB0000267	0.88	1.36	Alpha amino acids and derivatives/Glutathione Metabolism
N-Acetyl-L-aspartic acid	HMDB0000812	1.51	3.02	N-acyl-alpha amino acids
N-Acetyl-L-tyrosine	HMDB0000866	1.74	3.36	N-acyl-alpha amino acids
N-Acetylglutamic acid	HMDB0001138	1.12	1.74	N-acyl-alpha amino acids
N-Acetylvaline	HMDB0011757	1.04	1.32	N-acyl-alpha amino acids
Aminoadipic acid	HMDB0000510	1.99	2.39	Carboxylic acids and derivatives/pathway of lysine
Oxoglutaric acid	HMDB0000208	1.31	2.85	Keto acids and derivatives/TCA Cycle
1-Methyluric acid	HMDB0003099	0.75	0.33	Purines and purine derivatives
Pseudouridine	HMDB0000767	1.22	1.36	Nucleoside and nucleotide analogues
5-Hydroxymethyluracil	HMDB0000469	1.91	2.22	Pyrimidines and pyrimidine derivatives
p-Hydroxyphenylacetic acid	HMDB0000020	3.74	4.6	Phenols/Tyrosine Metabolism
Glycolic acid	HMDB0000115	3.88	3.16	Alpha hydroxy acids and derivative
Gamma-Butyrolactone	HMDB0000549	2.15	2.03	Lactones

Annotation indicates the name of the metabolite, and *HMDB* the *Human Metabolome DataBase* identifier; *FC[TH/T0]* is the fold change calculated as the ratio between the median of the variable during therapeutic hypothermia and the median of the same variable before therapeutic hypothermia; *FC[PT/T0]* is the fold change calculated as the ratio between the median of the variable after therapeutic hypothermia and the median of the same signal before therapeutic hypothermia; *metabolic class* is the chemical classification based on the HMDB. In calculating the fold change, the random effect due to individual differences was removed from the data.A fold change >1 indicates a rise in the metabolite's concentration with respect to T0; a fold change <1 indicates a drop in its concentration with respect to T0.

Table 2: **Annotated metabolites found relevant in revealing changes in the urinary metabolome across the three phases investigated (T0, TH, and PT).**

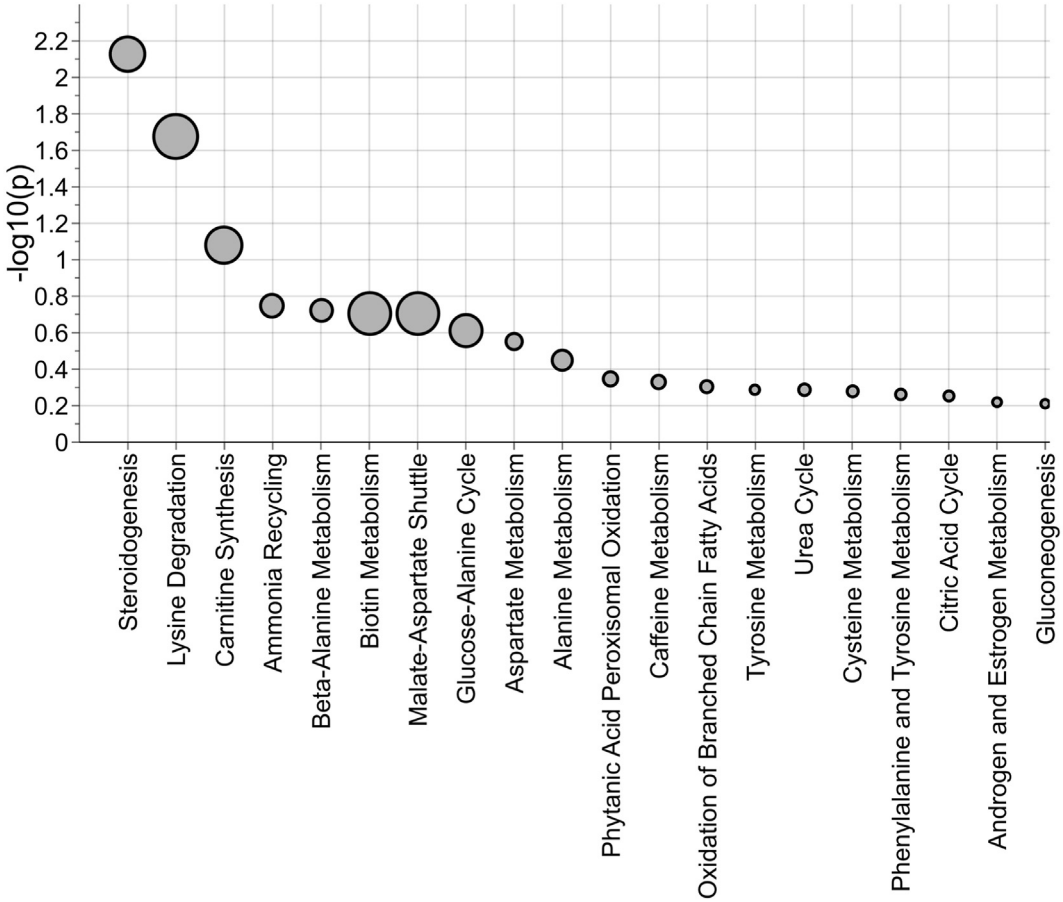

Fig. 3: Over-representation pathway analysis. Pathways are shown according to their negative logarithm of p (–log10 [p]) using a symbol size proportional to their enrichment ratio.

confirming the power of metabolomic analysis to distinguish between different classes of subjects. The gradual drop in steroid levels in the urine of asphyctic patients during the TH and PT phases appears to be consistent with a persistent derangement of normal neuroprotective mechanisms in neonates with HIE after PA.

In a similar study on asphyctic infants treated with TH, Piñeiro-Ramos and coworkers recently described a significantly altered steroid biosynthesis along with changes involving also amino acid pathways and lipid metabolism.[18] In these authors' work, a clear trend in urinary steroid levels over time was not reported. Another difference with our results is that steroid hormone biosynthesis was only significantly affected in the first 24 h of life in the cohort of Piñeiro-Ramos and coworkers, while we found that changes persisted throughout the TH period. Their study did not include healthy controls.

During the second half of human gestation, the placenta produces increasing amounts of progesterone.[32] This precursor is then converted in the foetal peripheral tissues and brain into other neuroactive steroids (NAS), creating a particular hormonal axis between the foetus and the placenta. High levels of NAS during late gestation protect the foetal brain against hypoxia/ischemia, promote neural development,[33,34] and aid in repair processes by stimulating oligodendrocyte maturation and myelination,[35,36] whereas low NAS levels coincide with increased asphyxia-induced brain injury.[37] These complex biochemical and metabolic effects in the developing human brain take place through allosteric modulation of GABA, NMDA, serotonin, and alpha-1 receptors.[38–41]

NAS have been divided into three categories: pregnane NAS, androstane NAS, and sulfated NAS.[42] Pregnane NAS, the most explored, are a class of progesterone derivatives encompassing allopregnanolone, pregnanolone, pregnenolone, and others. Androstane NAS include androstanediol and etiocholanone. Sulfated NAS consist of dehydroepiandrosterone sulphate and pregnenolone sulphate.[43,44] One factor that may contribute to the decrease of neuroprotective

steroid concentrations over time in asphyctic patients is their concomitant use in the above-mentioned repair and neuromodulation processes in response to hypoxic-ischemic insult. This would also explain why healthy newborns have higher urinary concentrations of steroids at T0 than those with HIE after PA. Translational studies support investigations of the potential neuroprotective role of exogenously administered NAS in asphyctic infants. Yawno et al. found that administration of the synthetic pregnane analog alfaxalone reduced spiking EEG activity after brief *in utero* asphyxia in an animal model.[45] The same molecule was also found to play a role in diminishing post-asphyctic cell necrosis in the cerebellum and hippocampus.[37] Ganaxolone, a synthetic 3b-methyl by-product of allopregnanolone,[46] also acts as a GABA agonist by modulating the activity of GABAergic interneurons through the benzodiazepine-binding site on GABA receptors. Its neuroprotective properties have been extensively studied in rodents,[47,48] and it has also been shown to be active in adult human patients with partial-onset seizures, and in children with infantile spasms.[49] There is evidence that GABA receptors mediate general excitatory activity in human neurons in most of the gestation period, and particularly in the early ontogenetic phases of neuron migration and synapsis formation.[50] Shortly before delivery, however, oxytocin-mediated reduction of [Cl-] causes a shift in the GABA receptors of the central nervous system, making them effectors of neuromodulation rather than excitatory effects, exerting a neuroprotective action and potentially reducing the severity of anoxic episodes.[51] This 'GABA shift' also modulates ascending electrical pathways of pain signaling, increasing the newborn's pain threshold.[52] Taken together, these findings depict the term or near-term asphyctic newborn as a promising candidate for assessing the neuroprotective effects of exogenously administered NAS (particularly, synthetic analogues of the pregnane family).[53]

Other pathways found to be significantly affected were lysine degradation and carnitine synthesis. In contrast to all other amino acids investigated, we found a decline over time in intact lysine and, in parallel, a rise of lysine degradation products (ie, aminoadipic acid) in the urines of newborns with HIE undergoing TH (Supplementary Fig. S1 of Supplementary Information). The aminoadipic acid, involved in the lysine pathway degradation, showed a steady increase from T0 to TH and PT, opposite to lysine and was significantly lower in asphyctic than in healthy neonates at T0.

The above-described trend of intact lysine in asphyctic infants is consistent with the known neuroprotective and neuromodulatory role of this amino acid.[54–56] Our analysis showed a rise in acylcarnitines and all N-acyl-alpha amino acids investigated. Accumulation of acylcarnitine has been found in animal models of PA,[56,57] and in previous series of asphyctic infants,[24,54,56] corroborating the hypothesis of a mitochondrial 'stunning' in these patients. The intracellular accumulation of long-chain fatty acids due to β-oxidation being unable to run properly may further contribute to neuronal toxicity in HIE.[58]

During TH, asphyctic patients received concurrent treatments (see "Methods" section); however, the degradation metabolites of these drugs do not pertain, to our knowledge,[59,60] to the metabolites emerged in our study.

Our study has some limitations. Given the sample size, it was impossible to divide patients into those who developed damage on brain MRI and those who did not. The short sampling time frame also prevented us from investigating the long-term trend of urinary steroids in our patients. On the other hand, a strength of the study lies in its experimental design: we analysed the urinary metabolome over time and included a matched group of healthy newborns. In a near future the integration of genomic, transcriptomic, proteomic and metabolomic tools (multi-omic technology) will represent the new frontier with a view to enabling a high-throughput molecular characterisation of neonatal encephalopathy associated with PA.

Conclusions

Our exploratory study on the dynamic changes in the urinary metabolome of asphyctic newborns undergoing TH in their first three days of life showed that steroidogenesis was the most deranged pathway: patients' urinary steroid levels were lower than in healthy newborns at T0, and decreased steadily over time despite hypothermia. Because neurosteroids are involved in neuromodulation and neuroprotection, translational research is warranted to assess the potential role of neurosteroid-based interventions in asphyctic infants.

Contributors

E.V.: writing–original draft, writing–review and editing, investigation, methodology; M.S.: data curation, software, formal analysis, writing–review and editing; P.P.: investigation, methodology, validation, writing–review and editing; I.D.E.: investigation, visualisation, software, methodology, writing–review and editing; L.B.: investigation, methodology, validation, writing–review and editing; A.G.: writing–review and editing; G.G.: conceptualisation, supervision, project administration, resources, writing–review and editing; E.B.: conceptualisation, supervision, project administration, resources, writing–review and editing. E.V., M.S., P.P., G.G., and E.B. have directly accessed and verified the underlying data reported in the manuscript. All authors read and approved the final version of the manuscript.

Data sharing statement

The data sets generated during and/or analysed during the current study are available at the following URL: https://data.mendeley.com/datasets/5749x5p2nz/1.

Declaration of interests

The authors have no competing interests to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2023.104636.

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