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Hypothermic treatment alters the metabolic profile of newborns, mimicking or masking an inherited metabolic disorder: findings from a monocentric study

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Abstract

Therapeutic hypothermia, initiated within the first six hours of life, is currently the main treatment capable of reducing brain damage in newborns with asphyxia and moderate or severe hypoxic-ischaemic encephalopathy (HIE). However, it is not yet clear how the treatment alters the newborn's metabolic profile, nor whether such changes might mask or mimic a metabolic disorder, leading to false positives or false negatives in screening for inherited metabolic disorders.

The present study therefore assessed changes in the metabolites routinely analysed in the Italian neonatal screening programme. A comparative analysis was conducted between full-term newborns with asphyxia/HIE who underwent therapeutic hypothermia (TH) and a control group, through tandem mass spectrometry analysis. Treatment resulted in a number of changes being observed, including a decrease in glutamate, an increase in glycine, a reduction in certain short- and long-chain carnitines, and an increase in phosphatidylcholines.

These findings suggest that therapeutic hypothermia may significantly alter the metabolic profile of the newborns. Consequently, the findings of our research have indicated that it is recommended to take dried blood spot samples at the conclusion of therapeutic hypothermia, with the aim of reducing the risk that the treatment may affect the interpretation of screening for inherited metabolic disorders. However, further studies are required to ascertain the usefulness and necessity of incorporating this second test into newborn screening protocols.

INTRODUCTION

Newborn screening for metabolic diseases enables the diagnosis of various metabolic disorders, and the last decade has seen the launch of several screening programmes for this purpose. Many screening panels focus on metabolic diseases caused by alterations in amino acid pathways or mitochondrial β-oxidation, resulting in abnormalities in the acylcarnitine profile. It is important to note that variations in blood acylcarnitine concentrations can also be caused by other pathologies. These include sepsis, malnutrition, diabetes, obesity, intrauterine growth restriction and metabolic syndrome. [1–6]. Studies have shown that acylcarnitine levels can be altered in neonatal hypoxic-ischaemic conditions, and some authors have investigated how these levels change with hypothermic treatment. [7–9].

Therapeutic hypothermia has been demonstrated to be the only treatment proven to reduce brain injury in infants with hypoxic-ischemic encephalopathy (HIE), which remains the leading cause of infant mortality and acute neurological damage [10]. It has been recommended that, where feasible, treatment for moderate and severe HIE should be initiated within six hours of the onset of hypoxia-ischemia, as this may represent the most opportune timeframe for preventing secondary programmed cell death [11–12]. In clinical practice, there are well-established and widely recognised criteria for the diagnosis of asphyxia, as well as others that determine the eligibility of patients with moderate to severe HIE for therapeutic hypothermia treatment. [13–14]. However, it should be noted that these criteria do not apply to mild HIE cases that may or may not result in long-term trivial neuropsychological sequelae. The

combination of clinical assessment with amplitude-integrated electroencephalography (aEEG) is a well-known tool for identifying asphyxiated newborns with and without mild HIE [15]. However, there is still a need for robust diagnostic and prognostic biomarkers to fully confirm diagnoses and predict outcomes. Despite their organ-specific nature, current biomarkers for HIE are not yet fully validated and cannot provide a real-time assessment [16]. Metabolomics, on the other hand, detects the deranged metabolome that, being dynamic, changes immediately in response to disease or environmental stimuli. Over the last two decades, it has made huge advances in the consistent diagnosis of different diseases based on metabolites [17].

However, there is a paucity of studies in the literature describing how the metabolic profile varies in terms of all those metabolites routinely used in neonatal screening panels for the early detection of metabolic disorders. As a result, there is a paucity of knowledge regarding the management of the situation in the initial hours of life if a newborn is affected by a metabolic disorder and has been treated with hypothermia.

Determining whether hypothermic treatment exerts an effect on the circulating concentration of certain metabolites may facilitate the establishment of whether a potential metabolic defect is masked or enhanced by the hypothermic treatment itself, leading to false negatives and false positives respectively.

It is hypothesised that alterations in the concentrations of metabolites, which have been identified as markers for potential metabolic disorders, resulting from hypothermic treatment, could provide a rationale for the implementation of a standard protocol for the neonatal metabolic screening programmes. This protocol would involve the collection of a second blood sample from all newborns who have undergone the treatment once it has been completed. Therefore, the aim of this study is to assess possible variations in metabolite concentrations in newborns undergoing hypothermic treatment. This has been achieved by analysing their dried blood spot (DBS) samples taken during hypothermic treatment, in order to determine whether certain key metabolites, which are prognostic markers for inherited metabolic disorders, are overexpressed or underexpressed following treatment. Any change in concentration could, in fact, lead to a reassessment of each newborn’s metabolic profile some time after the end of treatment, in order to avoid possible false negatives or false positives for inherited metabolic disorders.

METHODS

Recruitment

The metabolic profiles of a group of 66 full-term newborns who underwent hypothermic treatment (HT) within six hours of birth [18–19] were compared with those of a control group of 69 full-term newborns (HC), for whom no treatment other than antibiotics was reported by the maternity units.

In order to ensure that the two study groups were as homogeneous as possible, only full-term newborns with a birth weight of 2,500 g or more were included in the screening programme over the last two years.

We did not include any newborns with other known medical conditions or family history of metabolic disorders covered by the panel investigated in our laboratory as reported by the maternity units. For all newborns in both groups, a blood sample was taken within the 48–72-hour time window, with all forms of feeding discontinued at least two hours prior to sampling (Table 1).

Table 1
Anamnestic data of newborn underwent hypotermic treatment (HT) and healthy control (HC)

Newborns	Sex (%)		Age at Collection (h)	Gestation (week)	Weight (g)
HT	F	41.90	62.37 ± 8.92	39.23 ± 1.90	3127 ± 483
	M	58.10			
HC	F	40.10	57.44 ± 7.94	38.92 ± 1.27	3285 ± 398
	M	59.90			

Each blood sample was absorbed onto Whatman 903 filter paper, left to dry at room temperature away from heat sources, and then sent to our laboratory, where it was analysed within 48 hours of arrival. The study forms part of the analysis of samples covered by the mandatory Italian extended neonatal screening programme. The samples were analysed and processed in accordance with Italian Law 167/2017 and the Ministerial Decree of October 13th 2016. In compliance with these regulations, the experimental protocols were approved by our organization, the University Polyclinic of Catania, when the panel of hereditary metabolic disorders subject to screening at our screening centre was updated. Informed consent was obtained from all legal guardians of the newborns involved in the study (Resolution N. 2139 – September 10, 2024).

Sample preparation and tandem mass spectrometry measurements

Each assay was carried out using the Neobase2 ^{TM} Non-derivatized MSMS kit (Revvity), according to the manufacturer's instructions, using instrument parameters that had been optimised in our laboratory as described previously [20]. Briefly, through an automatic puncher (Revvity Panthera Puncher) filter paper disks (3.2 mm in diameter) were punched out from DBS into the wells of a microplate and 125 µl of a working solution were added into each well. Each plate was shook in the incubator/shaker (TriNest incubator) at 30°C for 30 minutes at speed of 750 rpm. After incubation, 100 µl from the contents of each well was transferred to another microplate to avoid the presence of the paper disk during the injection inside the mass spectrometer. The ESI-MS/MS analysis were performed on a 210 MD QSight™ spectrometer (Perkin Elmer) equipped with a triple quadrupole. Each measurement was performed in flow injection mode using the QSight™ HC Autosampler MD (Perkin Elmer) and QSight™ binary pump (Perkin Elmer), volume of injection 10 µl, flow rate 0.02 ml/min. The MS parameters were set up as follows: capillary voltage 5 kV, source temperature 175ºC, drying gas 105 L/h and nebulizer gas 130 L/h.

The analytical measurements were performed in the multiple reaction monitoring mode (MRM), using stable isotope internal standards for quantitative analysis.

Newborn Screening Panel

A total of 57 metabolites were analysed, which can be divided into six different groups as shown in Table 2: 36 Acylcarnitines (ACs), 4 Lysophosphatidylcholines (LPCs), 2 nucleosides (ADO and D-ADO), 1 ketone (SA), 14 Aminoacids (AAs). It is important to note that all of these metabolites are primary and/or secondary biomarkers of the inherited metabolic disorders covered by the mandatory ministerial panel for neonatal screening in Italy (SI). The screening is carried out using tandem mass spectrometry.

Table 2
Metabolites analyzed in the group of newborns who underwent hypothermic treatment and in the group of healthy controls, divided by type of statistical analysis applied

Metabolite	M		DS		Non Parametric ANOVA – Kruskal-Wallis Post Hoc		
	HT (N = 66)	HC (N = 69)	HT (N = 66)	HC (N = 69)	χ^2	p	ε^2
ADO	0.591	0.520	0.490	0.166	1.31	0.252	0.0100
ALA	292	293	130	53.8	3.71	0.0541	0.0277
ARG	13.2	7.18	13.7	3.67	4.82	0.0281	0.0360
ASA	0.304	0.283	0.129	0.0691	0.0259	0.8722	1.93e-4
C0	19.6	18.4	7.23	6.08	0.667	0.4142	0.005
C2	14.9	19.3	6.42	6.13	16.0	6.01e-5	0.119
C3	1.70	2.39	0.753	0.864	24.5	< 1.00e-5	0.183
C3DC\C4OH	0.0962	0.165	0.0462	0.0633	39.8	< 1.00e-5	0.297
C4	0.210	0.239	0.101	0.0882	6.27	0.0123	0.0468
C4DC\C5OH	0.224	0.203	0.0708	0.0503	4.66	0.0309	0.0348
C5	0.146	0.0848	0.0879	0.0241	33.0	< 1.00e-5	0.246
C5:1	0.0111	0.0104	0.00611	0.00205	0.359	0.549	0.00268
C5DC\C6OH	0.0802	0.0768	0.0586	0.0160	2.60	0.107	0.0194
C6	0.0474	0.0438	0.0268	0.0125	1.65	0.199	0.0123
C6DC	0.0432	0.0749	0.0221	0.0217	60.8	< 1.00e-5	0.454
C8	0.0702	0.0545	0.0357	0.0205	7.09	0.00776	0.0529
C8:1	0.0467	0.0257	0.0280	0.0147	27.9	< 1.00e-5	0.209
C10	0.0844	0.0813	0.0569	0.0309	1.56	0.211	0.0117
C10:1	0.0480	0.0406	0.0257	0.0138	1.88	0.170	0.0140
C10:2	0.0818	0.0826	0.00493	0.00382	0.0481	0.826	3.59e-4
C12	0.0788	0.0807	0.0592	0.0340	2.47	0.116	0.0184
C12:1	0.0609	0.0609	0.0506	0.0287	1.74	0.187	0.0130

In bold the metabolites with $p < 0.05$, in Italic bold the metabolites with $p < 0.05$ and $\eta^2 > 0.2$; HT: Hypotermic Treatment; HC: Healthy Controls

Metabolite	M		DS		Non Parametric ANOVA – Kruskal-Wallis Post Hoc		
	HT (N = 66)	HC (N = 69)	HT (N = 66)	HC (N = 69)	χ^2	p	ε^2
C14	0.172	0.182	0.0596	0.0484	2.18	0.140	0.0162
C14:1	0.0918	0.103	0.0573	0.0361	8.70	0.00318	0.0649
C14:2	0.0203	0.0238	0.0119	0.00597	12.7	3.60e-4	0.0950
C14OH	0.0159	0.0149	0.00976	0.00699	0.319	0.572	0.00238
C16OH	0.0247	0.0300	0.0111	0.00857	9.38	0.00219	0.0700
C16:1OH	0.0362	0.00478	0.0124	0.0116	29.8	< 1.00e-5	0.223
C18	0.725	0.894	0.246	0.195	23.7	< 1.00e-5	0.177
C18:1OH	0.0298	0.0261	0.0150	0.00732	0.304	0.581	0.00227
C18:2	0.126	0.179	0.0689	0.0838	17.1	3.99e-5	0.127
C18:2OH	0.0108	0.0133	0.00319	0.00475	12.3	4.51e-4	0.0920
C18OH	0.0133	0.0168	0.00616	0.00556	11.5	6.80e-4	0.0861
C20	0.0320	0.0281	0.0164	0.0102	0.942	0.332	0.00703
C20-LPC	0.400	0.214	0.293	0.0780	25.7	< 1.00e-5	0.192
C22	0.00985	0.0103	0.00411	0.00169	0.661	0.416	0.00493
C22-LPC	0.326	0.179	0.169	0.0542	37.0	< 1.00e-5	0.276
C24	0.0179	0.0130	0.00832	0.00464	13.4	2.49e-4	0.100
C24-LPC	0.579	0.309	0.200	0.0768	77.6	< 1.00e-5	0.579
C26	0.0118	0.0125	0.00461	0.00553	0.263	0.608	0.00196
C26-LPC	0.380	0.226	0.169	0.0524	37.7	< 1.00e-5	0.281
CIT	11.5	12.0	5.02	2.96	2.33	0.127	0.0174
D-ADO	0.0155	0.0235	0.00587	0.00590	43.7	< 1.00e-5	0.326
GLY	471	303	170	64.9	50.3	< 1.00e-5	0.375
LEU\ILEU\PROOH	109	103	54.2	18.1	0.912	0.339	0.00681
MET	20.4	13.5	16.7	2.54	13.3	2.70e-4	0.0990

In bold the metabolites with $p < 0.05$, in Italic bold the metabolites with $p < 0.05$ and $\eta^2 > 0.2$; HT: Hypotermic Treatment; HC: Healthy Controls

Metabolite	M		DS		Non Parametric ANOVA – Kruskal-Wallis Post Hoc		
	HT (N = 66)	HC (N = 69)	HT (N = 66)	HC (N = 69)	χ^2	p	ε^2
ORN	82.5	65.5	46.1	22.2	4.89	0.0270	0.0365
PHE	46.9	42.6	13.0	7.24	2.19	0.139	0.0164
PRO	144	107	95.3	21.4	4.57	0.0326	0.0341
SA	0.237	0.247	0.0668	0.0305	3.21	0.0734	0.0239
TYR	65.6	70.4	43.7	22.7	8.02	0.00462	0.0599
VAL	108	102	49.9	25.0	0.558	0.455	0.00402
Metabolite	**M**		**DS**		**One-Way ANOVA – Welch – Games-Howell Post Hoc**		
	HT (N = 66)	**HC (N = 69)**	**HT (N = 66)**	**HC (N = 69)**	**F**	**p**	
GLN	554	574	181	86.2	0.685	0.410	
Metabolite	**M**		**DS**		**One-Way ANOVA – Tukey Post Hoc**		
	HT (N = 66)	**HC (N = 69)**	**HT (N = 66)**	**HC (N = 69)**	**F**	**p**	**η2**
C16	2.89	3.97	1.08	0.881	36.4	< 1.00e-5	0.209
C16:1	0.173	0.268	0.0603	0.0702	70.8	< 1.00e-5	0.347
C18:1	1.09	1.46	0.316	0.345	41.9	< 1.00e-5	0.240
GLU	150	260	52.4	56.0	139	< 1.00e-5	0.510

In bold the metabolites with $p < 0.05$, in Italic bold the metabolites with $p < 0.05$ and $\eta^2 > 0.2$; HT: Hypotermic Treatment; HC: Healthy Controls

Statistical Analysis

Statistical analysis was performed using Jamovi open statistical software (2.3.28.0). The measurement data were expressed as the mean ± SD (standard deviation). A Shapiro–Wilk test and Levene test were used to determine, respectively, the suitability of the data for normal distribution and homogeneity of variance across each two independent groups. Based on the results of the Shapiro–Wilk and Levene tests, we apply the correct ANOVA tests. One-Way ANOVA was applied for normal distribution and respected assumption of homoscedasticity ($p > 0.05$). One-Way ANOVA with Welch post-hoc was

applied for normal distribution and not respected assumption of homoscedasticity ($p < 0.05$). Non Parametric ANOVA with Kruskal-Wallis post-hoc was used for those that did not show normal distribution and did not respected the assumption of homoscedasticity ($p < 0.05$). The levels of significance were set at $p < 0.05$ and the χ^2 test and ε^2 or η^2 were calculated. All the concentration values were expressed are µmol/L (µM). (Tables 2, 3a, 3b).

Table 3

a. Metabolites significantly increased in the group of newborns who underwent hypothermic treatment

Metabolite	**M**		**DS**		**χ^2 or F**	**p**	**ε^2 or η^2**
	HT (N = 66)	**HC (N = 69)**	**HT (N = 66)**	**HC (N = 69)**			
C24-LPC	0.579	0.309	0.200	0.0768	77.6	$< 1.00e^{-5}$	0.579
C26-LPC	0.380	0.226	0.169	0.0524	37.7	$< 1.00e^{-5}$	0.281
C22-LPC	0.326	0.179	0.169	0.0542	37.0	$< 1.00e^{-5}$	0.276
C20-LPC	0.400	0.214	0.293	0.0780	25.7	$< 1.00e^{-5}$	0.192
C24	0.0179	0.0130	0.00832	0.00464	13.4	$2.49e^{-4}$	0.100
C4DC\C5OH	0.224	0.203	0.0708	0.0503	4.66	0.0309	0.0348
C5	0.146	0.0848	0.0879	0.0241	33.0	$< 1.00e^{-5}$	0.246
C8:1	0.0467	0.0257	0.0280	0.0147	27.9	$< 1.00e^{-5}$	0.209
C8	0.0702	0.0545	0.0357	0.0205	7.09	0.00776	0.0529
GLY	471	303	170	64.9	50.3	$< 1.00e^{-5}$	0.375
MET	20.4	13.5	16.7	2.54	13.3	$2.70e^{-4}$	0.0990
ORN	82.5	65.5	46.1	22.2	4.89	0.0270	0.0365
ARG	13.2	7.18	13.7	3.67	4.82	0.0281	0.0360
PRO	144	107	95.3	21.4	4.57	0.0326	0.0341
3-OMD	0.921	0.441	0.988	0.127	14.4	$1.51e^{-4}$	0.133

HT: Hypotermic Treatment; HC: Healthy Controls

Table 3

b. Metabolites significantly decreased in the group of newborns who underwent hypothermic treatment

Metabolite	M		DS		χ^2 or F	p	ε^2 or η^2
	HT (N = 66)	HC (N = 69)	HT (N = 66)	HC (N = 69)			
GLU	150	260	52.4	56.0	139	$< 1.00e^{-5}$	0.510
TYR	65.6	70.4	43.7	22.7	8.02	0.00462	0.0599
C2	14.9	19.3	6.42	6.13	16.0	$6.01e^{-5}$	0.119
C3	1.70	2.39	0.753	0.864	24.5	$< 1.00e^{-5}$	0.183
C3DC\C4OH	0.0962	0.165	0.0462	0.0633	39.8	$< 1.00e^{-5}$	0.297
C4	0.210	0.239	0.101	0.0882	6.27	0.0123	0.0468
C6DC	0.0432	0.0749	0.0221	0.0217	60.8	$< 1.00e^{-5}$	0.454
C14:1	0.0918	0.103	0.0573	0.0361	8.70	0.00318	0.0649
C14:2	0.0203	0.0238	0.0119	0.00597	12.7	$3.60e^{-4}$	0.0950
C16	2.89	3.97	1.08	0.881	36.4	$< 1.00e^{-5}$	0.209
C16:1	0.173	0.268	0.0603	0.0702	70.8	$< 1.00e^{-5}$	0.347
C16:1OH	0.0362	0.00478	0.0124	0.0116	29.8	< 1.00e-5	0.223
C16OH	0.0247	0.0300	0.0111	0.00857	9.38	0.00219	0.0700
C18	0.725	0.894	0.246	0.195	23.7	$< 1.00e^{-5}$	0.177
C18:1	1.09	1.46	0.316	0.345	41.9	$< 1.00e^{-5}$	0.240
C18:2	0.126	0.179	0.0689	0.0838	17.1	$3.99e^{-5}$	0.127
C18OH	0.0133	0.0168	0.00616	0.00556	11.5	$6.80e^{-4}$	0.0861
C18:2OH	0.0108	0.0133	0.00319	0.00475	12.3	$4.51e^{-4}$	0.0920

HT: Hypotermic Treatment; HC: Healthy Controls

RESULTS

Of the 57 metabolites analysed, only 33 exhibited statistically significant values ($p < 0.05$), representing just over half of the pool of analytes (57.9%).

In particular, seven amino acids, deoxyadenosine, four phosphatidylcholines and 21 acylcarnitines were found to be significant. However, when the effect size is taken into consideration in addition to the p-value, it becomes evident that the number of metabolites displaying a significant difference in concentration between the control group and the group subjected to hypothermia decreases. Specifically, only three metabolites were observed with ε^2 or $\eta^2 > 0.4$ (one amino acid, one acylcarnitine and one phosphatidylcholine) and only 11 ACs with ε^2 or $\eta^2 > 0.2$ (Table 2)

It is immediately apparent that, among the 33 statistically significant metabolites, there is one group showing higher concentration values than those of the healthy controls and another group showing lower values (Tables 3a, 3b).

It was found that the group of metabolites with higher concentrations than the controls included all the phosphatidylcholines. It is also worth noting that these show a large effect size, with the exception of C20-LPC, which nevertheless has a value close to 0.2. In the context of very long-chain acylcarnitines, statistical analysis has revealed that C24 is the only one present at concentrations that are statistically significant. In contrast, two short-chain acylcarnitines (C4DC/C5OH and C5) were found to be elevated, but only C5 has $\varepsilon^2 > 0.2$; and only two medium-chain acylcarnitines (C8, C8:1), one of which, C8:1, has $\varepsilon^2 > 0.2$.

It is worthy of note that out of the four significant amino acids, only glycine demonstrates $\varepsilon^2 > 0.2$ and $p < 1e\text{-}5$. The group of metabolites showing lower concentration values than those in the healthy control group is larger. However, it is particularly noteworthy that this group consists predominantly of acylcarnitines rather than amino acids. In fact, only glutamic acid and tyrosine have lower levels, and of these, only GLU has $p < 1e\text{-}5$ and $\varepsilon^2 > 0.4$. In addition, D-ADO demonstrates remarkably significant p and ε^2 values. The acylcarnitine component is very prominent, with four short-chain ACs, of which only one (C3DC/C4OH) has $\varepsilon^2 > 0.2$, and as many as 11 long-chain ACs, although only four have ε^2 or $\eta^2 > 0.2$. It is also important to note the high significance ($p < 1e\text{-}5$ and $\eta^2 > 0.4$) of the sole dicarboxylated carnitine, C6DC. Finally, it appears that the metabolites in the HT group are more dispersed (higher SD) than those in the HC group.

DISCUSSION

Of the two amino acids that showed a significant decrease, only glutamate exhibited a strong effect. The decrease we observed is consistent with the findings reported by Sohn et al. in 2024 [19]. In their study, the trends of 140 metabolites were investigated in normothermic and hypothermic subjects. These were expressed in terms of lo2(V/A), that is, the ratio of venous to arterial concentrations of the various metabolites. In their study, glutamate was among the few metabolites to exhibit negative values in hypothermic subjects. This finding was interpreted as glutamate uptake by the brain, resulting in a

decrease in circulating levels. Glutamate is an excitatory neurotransmitter; therefore, in conditions of hypothermia, the authors observed that the uptake of glutamic acid to the brain meant that it was taken up from the intravascular space to the extravascular space of the brain, which may increased the extracellular level of the brain. The uptake of glutamate – and consequently its reduction in the bloodstream – plays a protective role, in that excessive extracellular glutamic acid results in excitotoxicity and neuronal apoptosis. It is well established that a significant number of brain diseases are associated with neurotransmitter abnormalities, primarily elevated concentrations of glutamic acid. The substantial accumulation of glutamic acid gives rise to a continuous depolarisation of neurons due to the activation of the NMDA receptor-gated ion channels, which in turn results in a considerable increase in the intracellular calcium concentrations. The resultant positive feedback effect leads to a significant increase in the release of glutamic acid in neurons. Hypothermia has been demonstrated to reverse this trend. Wisnowski et al. also observed a decrease in glutamate levels during hypothermic treatment, a finding that is in accordance with the results of our own study. Furthermore, a decline in creatine, aspartate and GABA was observed. The authors posit that these findings provide evidence to support the hypothesis that hypothermia improves energy homeostasis by decreasing the availability of excitatory neurotransmitters and thereby reducing cellular energy demand. [21].

The findings of the present study demonstrate a contrasting trend for glycine, which exhibits the highest level of significance among elevated amino acids in hypothermic patients. It is well known that during hypoxic-ischemic events, glycine functions as an obligatory co-agonist that binds to the GluN1 subunit of NMDA receptors, working alongside excessive extracellular glutamate and membrane depolarisation, resulting in the full opening of the channel and the subsequent drive to excitotoxic calcium overload. In fact, it is well known that in addition to the site for agonist and for glutamate, the NMDA receptors also possess specific domains for glycine, Mg^{2+}, Zn^{2+} and phencyclidine-like drugs. The evidence that has been accumulated thus far indicates that there are definite relationships between glycine and NMDA-sensitive glutamate receptor recognition sites. The activation of the NMDA receptor by glutamate has been demonstrated to necessitate the presence of glycine at a strychnine-insensitive binding site. As reported in the literature, therapeutic hypothermia has been shown to significantly reduce brain glycine levels following ischemia, thereby decreasing hyperexcitability caused by glutamate. This results in a decrease in the brain and an increase in the bloodstream. In fact, TH blocks the release of excitatory neurotransmitters such as glutamate, aspartate, and dopamine. Additionally, it inhibits the action of glycine in the NMDA receptor, increases angiogenesis, and inhibits the ERK pathway by stimulating the formation of BDNF. TH also prevents the transformation and activation of astroglial cells after the process of ischaemia [22–23].

Glycine, along with GABA, is one of the major inhibitory neurotransmitters, serving as the primary inhibitory transmitter in the spinal cord and brainstem. However, the descriptions of how its inhibitory or co-agonist function prevails and by what mechanism at the various stages of hypoxia-ischaemia and during subsequent hypothermic treatment are not entirely comprehensive, particularly in terms of how an individual's response differs from that of another. For instance, in the study by Sohn et al. [19], it was

observed that, despite a significant decrease in extracellular glutamate levels during hypothermic treatment, the concentration of glycine remained virtually constant.

With regard to the ACs, only four carnitines – two medium-chain and two long-chain – have shown increased levels. However, the increase in C5OH is negligible. The rise in C5 may also be attributable to the administration of antibiotics, which, due to the presence of pivalic acid, generate a false positive in the mass spectrum due to the overlap of the 246.20/85.00 transition. Unfortunately, in some cases it is not possible to rule out the administration of antibiotics for newborns due to a lack of clear communication with the maternity units. While there is a significant elevation in octanoylcarnitine levels, the effect size is relatively modest and the only noteworthy finding is the corresponding unsaturated C8:1. Short-chain carnitines, particularly the dicarboxylic carnitines C3DC and C6DC, which have a high ε^2 value, are significantly reduced. A considerable percentage of the reduced acylcarnitines is comprised of long-chain saturated, unsaturated, hydroxylated, and non-hydroxylated ones. As is well established in the literature, levels of acylcarnitines, in particular the long-chain ones, tend to increase during neonatal hypoxic-ischaemic events. [9, 19, 24–25]. Indeed, a systemic hypoxic trauma leads to disruptions in oxidative metabolism and the switch to anaerobic metabolism. This results in metabolic acidosis and alterations in amino acid and acyl-carnitine profiles. In fact, the rate of β-oxidation is likely constrained by the modification in the NADH/NAD and $FADH_2$/FAD ratios, consequent to a diminished oxygen supply. As a result, the reduced rate of β-oxidation becomes the rate-limiting step for fatty acid metabolism. This leads to an increase in matrix long-chain acyl-CoA and their upstream precursors, the long-chain acylcarnitines. Consequently, an increase in acyl-CoA and long-chain acylcarnitine levels can cause further disruption to oxidative metabolism and can be directly toxic [25–26]. It has been observed that in six infants with severe perinatal asphyxia, there appeared to be significantly higher levels of total and long-chain acylcarnitines. These findings were also noted in newborns with mild acidemia and hypoxia. It is therefore speculated by the authors that levels of long-chain acylcarnitines could potentially serve as a useful parameter for perinatal asphyxia [27].

Strangely, contrary to what these authors report, Lopez-Suarez's study shows a decrease in long ACs during the hypoxic-ischaemic event and an increase during hypothermic treatment [7].

In our study, however, we observed a decrease in the concentration of acylcarnitines, particularly the long-chain ones, during hypothermic treatment. This finding may be consistent with the hypothesis that hypothermic treatment acts by improving the cell's energy homeostasis, thereby reversing the dysregulatory mechanism underlying hypoxic-ischaemic injury. Indeed, it is well documented that mild hypothermic treatment increases blood glucose levels, elevates gluconeogenesis, and promotes fatty acid oxidation (FAO), indicating a high metabolic state, and also enhances Peroxisome Proliferator-Activated Receptor Alpha (PPARα) expression. In addition, the PPARα promotes FAO and gluconeogenesis, thereby amplifying the protective effects of mild therapeutic hypothermia (TH) on hepatocytes. Furthermore, mild hypothermia has been shown to promote the physical interaction and binding between Forkhead box O1 (FoxO1) and PPARα transcription factors. This structural interaction enhances the transcription of two primary target downstream metabolic genes: CPT1a (Carnitine

Palmitoyltransferase 1A) provides for the enzyme CPT1, which restores mitochondrial fatty acid oxidation (FAO), and PCK1 (Phosphoenolpyruvate Carboxykinase 1), which drives gluconeogenesis to maintain necessary cellular blood glucose levels [28].

Our research also indicates a substantial increase in all the phosphatidylcholines that were analysed, with a particular emphasis on C24LPC, which also displays the highest ever recorded value for ε^2.

This significant increase in LPCs is consistent with the data from the literature, which reports an increase in LPCs on dried blood spot analysis. In fact, a study has shown that newborns undergoing therapeutic hypothermia for moderate-to-severe HIE show a distinct rise in circulating lysophosphatidylcholine and phosphatidylcholine species over the cooling period. While LPCs increase, plasma triglycerides (TGs) and sphingomyelins (SMs) concurrently decrease during successful therapeutic temperature management. The authors hypothesise that this may be indicative of a compensatory mechanism intended to mitigate the consequences of HIE. However, they do not provide additional information regarding the potential mechanisms of action or the rationale behind this hypothesis [29]. Nevertheless, lysophosphatidylcholine levels are well known to reflect adaptive changes in membrane structure and signalling. These are critical for neuronal survival and inflammation resolution [30].

In fact, plasma LPC levels are influenced by several factors, including the rates of *de novo* synthesis and hydrolytic degradation. Specifically, LPC hydrolysis in plasma is mediated by enzymes such as lysophospholipase A1, which is present in neutrophils. Clinically, poor outcomes in ischemic stroke correlate with increased plasma neutrophil levels. This leads to reduced plasma LPC due to enhanced hydrolysis. Furthermore, it is plausible that, during a brain injury, the rate of LPC uptake into the brain exceeds its rate of recirculation, thereby reducing plasma LPC availability. In addition, the breakdown of the Lands cycle in the ischemic brain, which regulates the conversion between phosphatidylcholine (PC) and LPC, could lead to increased PC-to-LPC conversion, decreased reverse conversion, and a consequent reduction in the plasma LPC concentration [31].

Consequently, it is our conclusion that, during hypothermic treatment, which curbs hypoxia-ischaemia damage, all the processes listed above tend to return to normal. Therefore, rather than observing a reduction in circulating LPC, we observe an increase in their levels. Another noteworthy finding of our study is that D-ADO levels were lower than in the control group. It is widely acknowledged that during hypoxic-ischemic events (such as stroke or perinatal asphyxia), oxygen and energy deprivation triggers rapid oxidative stress, leading to early DNA damage and progressive catabolism [32–33]. It is also known that neonatal HIE leads to an inflammatory response that becomes clearly evident one week after the insult and a concomitant increase in the activity of ADA1, which is accompanied by a decrease in the levels of ADK and A1Rs [34]. Therefore, it is not straightforward to ascertain which process is favoured during the hours of brain damage progression, and how the pool of deoxynucleosides behaves during hypothermic treatment.

Given that DBS samples for extended neonatal screening programmes are collected within the 48–72-hour window – that is, whilst infants undergoing hypothermic therapy are in the midst of their treatment – the data discussed so far clearly highlight a key factor: the metabolites involved in the various biochemical pathways that become dysregulated in metabolic disorders undergo changes in concentration during the hypothermic therapy phase. This means that a lower or higher concentration of a particular metabolite or a group of metabolites could potentially result in false negatives or false positives. Incidentally, many metabolic disorders have an onset that closely resembles a hypoxic-ischaemic event [35]. Among the inborn errors of metabolism with features of hypoxic–ischemic encephalopathy, we might, for example, mention non-ketotic hyperglycaemia or fatty acid β-oxidation disorders. Therefore, if a newborn, for example, already had baseline glycine levels close to the laboratory cut-off value, and these increased during hypothermic treatment (as observed in our study), the cut-off values themselves could certainly be exceeded. This would result in the screening centre staff having to carry out a recall test, which would cause stress for the families involved.

Conversely, if a child were to have a late-onset form of β-oxidation deficiency but, during hypothermic treatment, the values fell below the laboratory's threshold levels, the staff would not issue a recall test and the child might be 'missed' by the screening programme. For this reason, we recommend introducing a second mandatory blood sample as part of the protocol for all children undergoing hypothermic treatment. This could be taken at least two days after the end of treatment to prevent such gross errors of assessment. Our study is severely limited by the small number of samples and the inability to ascertain the extent to which nutrition affects the values obtained, despite the temporary suspension of feeding. Furthermore, it was not possible to collect a second sample from all the children involved in the study at least 48 hours or more after the initial sample to assess the trends in metabolites.

CONCLUSION

Our study reports on trends in the metabolites routinely monitored as part of Italy's mandatory screening programme. This was conducted in a sample of full-term, normal-weight newborns with moderate-to-severe asphyxia or hypoxic-ischaemic encephalopathy. These newborns underwent hypothermic treatment within six hours of birth. The study also includes a control group. Our data demonstrate that, during hypothermic treatment, the levels of the two main amino acids involved in neurotransmission – namely glutamate and glycine – decrease and increase, respectively. Furthermore, we observed a significant decrease in certain short-chain carnitines and in several long-chain carnitines (both saturated and unsaturated, as well as hydroxylated). Conversely, there was a notable increase in phosphatidylcholines. Our findings appear to be consistent with the literature.

The key point we wish to emphasise is the need, within neonatal screening programmes for inherited metabolic disorders, to recommend a DBS sample be taken after the end of hypothermic treatment. Despite the limitations of the present study, it is evident that the concentrations of numerous metabolites exhibit substantial variations. In the event of an inherited metabolic disorder, the biochemical profile of a baby undergoing hypothermic therapy may change significantly, potentially

resulting in false positives or negatives. For this reason, we hope that further studies on this subject will be carried out to confirm the need for the additional follow-up test recommended in our protocol.

Declarations

COMPETING INTERESTS

None.

ETHICS APPROVAL

This study forms part of the analysis of samples covered by the mandatory Italian extended neonatal screening programme. The samples were analysed and processed in accordance with Law 167/2017 and the Ministerial Decree of 13 October 2016. All data processing was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments (Helsinki Declaration 1975, revision 2024).

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Author Contribution

Dr. M.A. Messina: Conceptualization, data interpretation and curation, statistical analysis, manuscript writing and correspondence. Dr. F. Raudino: laboratory analysis. Dr. C. Meli: manuscript formatting and final proofreading. Prof. M. Ruggieri: manuscript formatting, final proofreading and literature review. The authors read and approved the final manuscript.

Data Availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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