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Targeted Metabolic Profiling of Preterm Neonates with Intraventricular Hemorrhage

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ABSTRACT

Background: Intraventricular hemorrhage (IVH) is a major cause of morbidity and mortality in preterm neonates. It is essential to obtain new biomarkers for its early diagnosis. This can mainly occur by understanding its pathophysiology and mechanism of occurrence.

Objective: To investigate the metabolic changes (blood amino acids and carnitine concentrations) associated with IVH in preterm neonates, and the ability to discover new biomarkers for early diagnosis of such disease that enable early intervention.

Patients and Methods: This study was conducted on twenty preterm infants with IVH in the period from July 2019 to July 2020. Preterm infants were evaluated for IVH according to Velisavljev-Fillpovie with head US scans performed within 24 h after birth, on the 3rd day of life and thereafter weekly till discharge in survivors **(Cases).** Twenty preterm infants were chosen as a control group matching with the case group in gestational age, gender, and birth weight**.**

Results: There was significant rise of alanine, aspartate, glutamate, and ornithine amino acids in IVH group than control in day 1 (P value < 0.05) in addition to glycine, valine, and proline in day 3. Alanine amino acid > 112.5 µmol/L showed the highest accuracy 80%, sensitivity 70% and specificity 90% for diagnosis of IVH in premature neonates among all tested amino acids in day 1with significant rise in C3:C2 at cut off point 0.095 (p= 0.001) and C6 cut off point 0.08 (P=0.001) and acylcarnitine cut off point >0.045 µmol/L (P=0.002) for diagnosis of IVH.

Conclusion: Preterm neonates with IVH showed significant changes in amino acids and carnitines that might guide us to new biomarkers for early diagnosis and intervention.

Keywords: IVH, Amino acids, Carnitine, Acylcarnitine, UPLC-MS/MS.

INTRODUCTION

IVH is major cause of morbidity and mortality in preterm neonates. 20-25% of infants with birth weight less than 1500 gram, 45% of infants with birth weight 500-750 g have IVH. 60% in very low birth weight newborn with hypoxic ischemic encephalopathy (HIE) also develop IVH [1].

IVH usually develop spontaneously in premature infant that may be due to trauma or asphyxia. It may occur from primary hemorrhagic disorder or congenital vascular anomaly. IVH may be associated with disseminated intravascular coagulation (DIC), isoimmune thrombocytopenia, neonatal vitamin k deficiency, inborn error of metabolism and infant born to mother receiving Phenobarbital or phenytoin [2-3].

The high prevalence of IVH and its effects in preterm newborns highlight the need for more research into the underlying processes of IVH as well as the creation of biomarkers for early diagnosis in high-risk neonates [4].

For the prediction of outcomes, metabolomics can offer useful information. Metabolites provide a distinctive profile that may be used to predict newborn illnesses, assess disease progression, and determine the impact of interventions [5-7].

There isn't a lot of metabolomics-based research on newborn brain damage that focus on term neonates and excremental models of hypoxia-induced asphyxia [8].

Some amino acids may rise in urine of premature infant having IVH as: Arginine, asparagine, glutamine, lysine, valine, proline, taurine, threonine, also elevation of acylcarnitine level in urine of preterm infant having IVH [5, 7].

Muscles are where carnitine is mostly kept. Medium- and long-chain fatty acids are transported from the cytosol into the mitochondria for oxidation and energy production with the help of carnitine. Additionally, carnitine promotes the Krebs cycle and the pyruvate dehydrogenase complex, enhancing the oxidation of branched-chain amino acids in muscles [9].

The important metabolites differed between strata regardless of IVH status. The acylcarnitine C-5OH and C-14:1 in particular were crucial to the survival of newborns with IVH, suggesting they may be a good indicator of the severity of the condition and supporting additional research [10].

Several experimental and clinical studies have been demonstrated some amino acids and certain acylcarnitine in blood level abnormality in IVH conditions [1, 5, 6].

The aim of the present study was to study the predictive ability of blood amino acids and acylcarnitine levels as potential biomarkers for early detection of IVH in premature neonates.

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PATIENTS AND METHODS

Study population:

A case-control study was conducted in our center from July 2019 to July 2020 on 20 preterm neonates (≤ 35 weeks of gestation) admitted with IVH in the first day of life according to **Velisavljev-Fillpovie *et al.*** [11] with head US scans performed within 24 h after birth, on the 3rd day of life and thereafter weekly till discharge in survivors **(cases).** A (**control**) group was recruited including 20 preterm newborns without IVH matching the case group in gestational age and gender.

Inclusion criteria: All preterm newborns (≤ 35 weeks of gestation) admitted at the first day of life with IVH diagnosed by cranial ultrasound after obtaining the approval of their caregivers.

Exclusion criteria: Preterm newborns with IVH beyond their first day of life were excluded, as well as newborns with major congenital anomalies and known metabolic disorders.

Study grouping: the included newborns were classified to:

- **Group 1:** included preterm neonates (≤ 35 weeks of gestation) diagnosed as having any degree of IVH in the first day of life diagnosed by cranial ultrasound (n=20).
- **Group 2:** included 20 preterm admitted to the unit without IVH in their first day and followed up for development of IVH till discharge. This group was chosen to be matching the case group in gestational age and gender.

Sample collection and preparation

Seven milliliters of venous blood were withdrawn from all newborns at day 1 and day 3. It was divided into three aliquots; 1st was collected in a plain vacutainer tube, used for random blood sugar measurement, liver and kidney function tests, CRP and electrolytes (measured by the Beckman Coulter, Synchron CX9 Clinical Autoanalyser, Beckman Instruments, Fullerton, California, USA), 2 ml were collected into tube containing ethylene diamine tetra acetic acid (EDTA) for CBC (Sysmex KX 21 Automated-Hematology Analyzer), then the tube was centrifuged as soon as possible and separated then the separated plasma were used immediately for measurement of ammonia and lactate. The remaining 2 mls were collected into sodium citrate tube and used in estimation of prothrombin time & partial thromboplastin time by coagulation semi-automated analyzer (PT device) (Tridema diamond, Italy). Another one ml arterial specimen for arterial blood gas (ABG) was withdrawn and analysed by easystat autoanalyser (Medica, England)**.** Blood culture was done using Egyptian Diagnostic Media when indicated.

Sample for the specific metabolites

Samples for amino acids, carnitine and acylcarnitine analyses: Blood was drawn via a clean heel puncture, spotted on filter paper (Guthrie card, GE Healthcare, NJ, USA), allowed to dry, and then stored at −80 °C till analysis.

Targeted amino acids and acylcarnitines were measured by Ultra performance Liquid Chromatography Tandem Mass Spectrometry (UPLC- MS/MS):

Chemicals and reagents:

Amino acids and acylcarnitines standards MassChrom® from (Chromsystems Instruments & Chemicals GmbH, München, Germany). The methanol was acquired from Fisher's Scientific, and the solvent was HPLC grade (Loughborough, U.K.). The remainder of the chemicals and benchmarks were bought from Sigma-Aldrich (Fluka, St. Louis, Mo, USA).

AcylCNs and amino acids assay by MS/MS: A v-bottomed plate well with a 3 mm dried blood spot disc punched into it with 100 µl of lyophilized internal standard that has been reconstituted with 25 ml of extraction buffer. Ten µl of the elute was injected into the MS/MS system (Acquity UPLC H-Class, Waters Corporation, MA, USA) every two minutes in a stream of 80% acetonitrile running at a flow rate of 200 microliters per minute that was lowered to 20 microliters per minute in 0.25 minutes. In 1.25 minutes, the flow rate rose to 600 µl/min before falling once more to 200 µl/min. Using the Neolynx program, quantitative analysis was done to get concentrations (Neolynx Inc., Glendale, CA, USA).

Imaging

Serial trans-cranial ultrasound of all newborns for detection of IVH at day1, day 2 and day 3.

Ethical consent: Menoufia University's National Liver Institute's Ethics Committee gave its approval for this study (IRB 00382/2022). The research adheres to the guidelines set out in the Declaration of Helsinki (''Recommendations for physicians in biomedical research involving human beings), along with written or verbal consents provided by the participants' parents.

Statistical analysis:

Statistical Package for the Social Sciences (SPSS) version 23 (SPSS Inc. Released 2015) was used on an IBM compatible personal computer to gather data, tabulate it, and do statistical analysis. Armnok, NY: IBM Corp., IBM SPSS statistics for Windows, version 23.0. There were two kinds of statistical analysis: For instance, descriptive statistics were expressed using the following formats: number (No), percentage (%), mean (x), and standard deviation (SD). When comparing quantitative variables between two groups of regularly distributed data, the Student's t-test is a test of significance; when comparing quantitative variables between two groups of not normally distributed data, the Mann-Whitney's test was utilized. In order to

compare several readings of data that was not normally distributed within the same group, Wilcoxon test was performed. To investigate the relationship between qualitative variables, the Chi-square test (X^2) was applied. Fischer's exact test was applied if any of the predicted cells fell below the threshold of five. To assess the clinical utility of each amino acid and acylcarnitine in discriminating IVH, the receiver operating characteristic (ROC) curve was utilized. The validity of a test is represented by the area under the curve, with 1.00 being the greatest value and 0 being the lowest. Different cut-off levels for amino acids and acylcarnitine were illustrated using ROC curves and the area under the curve. Values for the area under the curve are presented along with their 95% confidence level (CI). P value: A statistically significant P value ≤ 0.05.

RESULTS

The characteristics of the included population:

A total of 40 preterm neonates were included in the study and were divided into 2 groups: **Group 1** included 20 (16 males and 4 females) preterm newborns ≤ 35 weeks in gestation admitted to the unit at first day of life and suffered from IVH diagnosed by cranial ultrasound scan. The mean gestational age of group 1 (IVH group) was 31.8 ± 2.2 weeks.

While **Group 2** included 20 chosen preterm neonates (16 males and 4 females) admitted to the unit in their first day of life matching the IVH group in gestational age and gender and followed up for development of IVH using the cranial ultrasound scan. The mean gestational age of this group was 33.1 ± 1.99 weeks. All the basic characteristics in the study showed no significance except for the weight of the newborn that was significantly higher in control group 1578.7 ± 295.4 gm in group 1 VS 2008.4 ± 474.5 gm in control group (P value 0.001).

The clinical characteristics of the studied groups showed significant lower temperature, systolic and diastolic blood pressure in IVH group (P value<0.05). The IVH group also had a significantly lower Apgar score at 1 min (p value < 0.001) **(table 1)**.

Table (1): The basic characteristics of the included population

	IVH group n= 20	Control group n= 20	Test of significance	P value
Gender:				
Female	4 (20%)	4 (20%)	X^2=0.0	P=1.00
Male	16 (80%)	16 (80%)		
GA (weeks):				
Mean ± SD	31.8±2.2	33.1±1.99	t= 1.879	0.068
Range	28-35	30-36		
Weight (g):				
Mean ± SD	1578.7±295.4	2008.4±474.5	t= 3.438	0.001**
Range	1009-2200	1300-3200		
Temperature ˚c				
Mean ± SD	36.8±0.3	37.03±0.2	2.541	0.016*
Range	36.5-37.7	36.5-37.6		
HR Beat/min				
Mean ± SD	139.3±11.7	145.3±8.5	1.85	0.072
Range	119-160	130-160		
Systolic BP/mmgh				
Mean ± SD	63.95±14.3	74.5±15.8	2.014	0.049*
Range	28-92	40-103		
Diastolic BP/mmgh				
Mean ± SD	40.35±8.1	53±10.5	4.25	<0.001**
Range	21-57	35-70		
RR Cycle/min				
Mean ± SD	46±9.1	45.3±6.9	0.274	0.786
Range	33-70	30-55		
APGAR score				
Mean ± SD	3.45±0.85	7.55±0.83	t=14.616	<0.001*
Range	2.0-5.0	6.0-9.0		

Results of metabolic markers:

Regarding amino acid levels in day 1:

The tested amino acids were higher in IVH group. However, the statistical significance was noticed only with the rise of alanine, aspartate, glutamate, and ornithine (P value=0.001, 0.014, 0.042, 0.037 respectively).

The mean alanine levels were 152.6 ± 36.31 µmol /L in IVH group vs 92.7 ± 20.03 µmol/L, the aspartate levels were 126.6 ± 30.65 µmol /L vs 87.4 ± 21.5 µmol /L, the glutamate levels were 243.2 ±57.61 vs 161.75 ± 39.3 µmol /L, while the mean ornithine levels were 165.49 ± 30.48 vs 114.8 ± 26.32 µmol /L **(table 2)**.

Regarding amino acid levels in day 3:

The tested amino acids showed a significant rise in serum alanine, aspartate, glutamate, glycine, valine, proline, and ornithine (P value= 0.035, 0.027, 0.027, 0.035, 0.01, 0.041, 0.042 respectively).

However, we noticed lower serum arginine levels in IVH group (23.59 ± 5.75 µmol /L) than in control group (24.8 ± 6.2 µmol /L), but this difference was not significant.

The mean levels of the significant amino acids in IVH group vs the control group were alanine =117.2 ± 28.05 vs 85.7 ± 20.2 µmol /L, aspartate=115.9 ± 44.5 vs 86.4 ± 42.2 µmol /L, glutamate =209.7 ± 93.1 vs 152.2 ± 50.04 µmol /L, glycine = 253.05 ± 61.42 vs 162.5 ± 39.2 µmol /L, valine= 92.9 ± 23.8 vs 62.1 ± 14.3 µmol /L), proline =235.35 ± 56.4 vs 126.9 ± 24.4 µmol /L) and (ornithine =155.2±37.3 vs 117.6±27.3 µmol /L) **(table2)**.

Table (2): Comparison between the 2 groups regarding amino acids levels

Amino acids; Day 1	**IVH group n= 20 Mean ± SD**	**Control group n= 20 Mean ± SD**	**Test of significance (U)**	**P value**	**Amino acids; Day 3**	**IVH group n= 20 Mean ± SD**	**Control group n= 20 Mean ± SD**	**Test of significance (U)**	**P value**
Alanine (µmol /L)	152.6± 36.31	92.7±20.03	3.301	0.001 **	Alanine	117.2± 8.05	85.7± 20.25	2.11	0.035 *
Arginine (µmol /L)	37.06±8.39	27.46±5.85	1.664	0.096	Arginine	23.59± 5.75	24.8±6.2	t= 0.604	0.549
Aspartate (µmol /L)	126.67± 30.65	87.14± 19.54	2.462	0.014 *	Aspartate	115.9± 24.5	86.4±19.3	2.205	0.027 *
Glutamate (µmol /L)	243.2± 57.61	161.75± 39.3	2.029	0.042 *	Glutamate	209.7± 44.4	152.2± 34.04	2.217	0.027 *
Glycine (µmol /L)	270.99±6.37	172.35± 6.2	1.583	0.113	GLycine	253.05± 6.42	162.5±9.2	2.11	0.035 *
Valine (µmol /L)	84.16± 19.66	62.44± 14.32	0.5	0.617	Valine	92.9±3.8	62.1±4.3	2.57	0.01*
Proline (µmol /L)	247.55± 55.09	127.56± 26.78	1.812	0.07	Proline	235.35± 56.4	126.9± 24.4	2.043	0.041 *
Ornithine (µmol/L)	165.49± 30.48	114.8± 26.32	2.083	0.037 *	Ornithine	155.2±37. 3	117.6± 27.3	2.029	0.042 *
Leu:Ala	0.8±0.2	0.59±0.13	1.151	0.25	Leu:Ala	0.77±0.18	0.57± 0.13	1.57	0.116
Leu:Phe	2.83±0.61	2.09±0.46	1.934	0.053	Leu:Phe	2.65±0.44	2.06± 0.29	1.502	0.133

* p = 0 < 0.05

Regarding carnitine and acylcarnitine profile levels at day 1:

However, we noticed rise of the blood levels of certain metabolites and lower levels of some others in the IVH group, but only the C3:C2 ratio and C6 carnitine showed significant rise in IVH group in comparison with the control (0.13 ± 0.03 vs 0.08 ± 0.01, P value=0.001 and 0.13 ± 0.02 vs 0.06 ± 0.01, P= 0.001 respectively)**.** Also, significant higher acylcarnitine levels were also found in cases with IVH in comparison to the controls (0.13 ± 0.03 vs 0.06 ± 0.01, P value= 0.002) **(table 3).**

Regarding carnitine and acylcarnitine profile levels at day 3:

We reported significant rise of C3:C2 and C6 carnitine levels and decrease in C8:1 in IVH group in comparison with control (P value<0.05).

Other metabolites showed lower levels with IVH without significance except C16, C4-OH and C10. Also, significant higher acyl carnitine levels were also found in cases with IVH in comparison with the acyl carnitine in day3 in control (0.15 ± 0.03 vs 0.06 ± 0.01, P= 0.016) **(table 3).**

Table (3): Comparison between studied groups regarding carnitine and acylcarnitine levels

Day 1 (µmol /L)	IVH group n= 20 Mean ± SD	Control group n= 20 Mean ± SD	Test of significance (U)	P value	**Day 3** (µmol /L)	IVH group n= 20 Mean ± SD	Control group n= 20 Mean ± SD	Test of significance (U)	P value
Acyl-carnitine	0.13±0.03	0.06±0.01	3.158	0.002*	Acyl-carnitine	0.15±0.03	0.06±0.01	2.411	0.016*
C0-Carnitine	15.7±3.3	13.4±2.7	191.0	0.820	C0-Carnitine	15.5±12.7	13.07±3.1	186.5	0.718
C10-Carnitine	0.109±0.02	0.12±0.03	164.5	0.341	C10-Carnitine	0.15±0.02	0.11±0.02	166.5	0.369
C16-Carnitine	0.53±0.01	0.68±0.15	198.50	0.968	C16-Carnitine	0.66±0.15	0.53±0.12	166.0	0.369
C2-Carntine	4.4±0.9	6.7±1.2	157.5	0.253	C2-Carntine	4.9±1.01	4.8±1.02	189.5	0.779
C3	0.58±0.01	0.49±0.11	164.0	0.341	C3	0.51±0.11	0.46±0.11	147.0	0.157
C3:C2	0.13±0.03	0.08±0.01	78.0*	0.001*	C3:C2	0.13±0.03	0.08±0.02	95.50*	0.004*
C4-Carnitine	0.23±0.01	0.17±0.03	137.0	0.091	C4-Carnitine	0.21±0.04	0.17±0.03	137.5	0.091
C5-Carnitine	0.30±0.06	0.21±0.04	138.5	0.096	C5-Carnitine	0.29±0.03	0.22±0.045	152.5	0.201
C4-OH (C3-DC)	0.08±0.02	0.099±0.15	177.5	0.547	C4-OH (C3-DC)	0.06±0.01	0.04±0.01	199.0	0.989
C12-Carnitine	0.04±0.01	0.05±0.11	177.0	0.547	C12-Carnitine	0.03±0.01	0.04±0.01	170.5	0.429
C14-Carnitine	0.05±0.01	0.06±0.01	199.0	0.989	C14-Carnitine	0.24±0.03	0.30±0.05	190.5	0.799
C8:1	0.108±0.16	0.107±0.02	128.5	0.052	C8:1	0.07±0.01	0.10±0.02	120.5*	0.030*
C6-Carnitine	0.13±0.02	0.06±0.013	84.0*	0.001*	C6-Carnitine	0.15±0.018	0.06±0.01	111.5*	0.015*

* $p = 0 < 0.05$

Efficacy of studied metabolites in diagnosis of IVH:

The usefulness of the studied metabolites in diagnosis of IVH was tested through receiver-operating characteristic (ROC) **(table 4** & **figures 1,2).**

At day 1, at **cut off** >112.5 alanine was able to diagnose IVH in preterm neonates with AUC=0.805, sensitivity of 70%, specificity of 90%, PPV of 87%, NPV of 75% and accuracy of 80%. While aspartate at > 84.1 points had a sensitivity of 75%, specificity of 65%, PPV of 68%, NPV of 72% and accuracy of 70%. The significant cut off point of C3:C2 ratio (P value=0.001) for diagnosis of IVH was at 0.095 with AUC=0.805, sensitivity of 80%, specificity of 70%, PPV of 70.8% and NPV of 81.2%. While C6 carnitine at a significant cut off point of 0.08 with AUC=0.790, sensitivity of 70%, specificity of 75%, PPV of 69.6% and NPV of 76.5%**.**

Serum acylcarnitine levels at a significant cutoff point ≥ 0.045 was able to predict occurrence of IVH at day 1 of life with sensitivity of 90%, specificity of 20% and AUC= 0.79 while at day 3 the significant cut off point for prediction was also ≥ 0.045 with sensitivity 85%, specificity of 20% and AUC= 0.721.

Table (4): Efficacy of studied metabolites in diagnosis of IVH

Amino acids; Day 1	Cut-off point	AUC	Sensitivity	Specificity	95% CI	P value	Amino acids; Day 3	Cut-off point	AUC	Sensitivity	Specificity	95% CI	P - value
Alanine	> 112.5	0.805	70%	90%	0.662-0.948	**0.001***	**Alanine**	> 92.2	0.695	60%	60%	0.53-0.86	**0.035***
Arginine	≥ 20.3	0.654	85%	15%	0.47-0.837	0.096	**Arginine**	≥ 26.8	0.561	75%	45%	0.377-0.745	0.508
Aspartate	> 84.1	0.727	75%	65%	0.572-0.883	**0.014***	**Aspartate**	> 85.1	0.704	70%	65%	0.539-0.869	**0.027***
Glutamate	> 167.5	0.688	60%	55%	0.521-0.854	**0.042***	**Glutamate**	> 165	0.705	60%	70%	0.543-0.867	**0.027***
GLycine	≥ 106.5	0.646	90%	25%	0.469-0.823	0.114	**GLycine**	≥ 113	0.695	95%	35%	0.529-0.861	**0.035***
Valine	≥ 33.7	0.546	75%	20%	0.358-0.734	0.617	**Valine**	> 67	0.737	60%	70%	0.582-0.893	**0.01***
Proline	≥ 87.75	0.668	75%	30%	0.49-0.845	0.07	**Proline**	> 152	0.688	65%	70%	0.515-0.863	**0.041***
Ornithine	> 128.5	0.692	60%	70%	0.528-0.857	**0.037***	**Ornithine**	> 118	0.688	65%	70%	0.52-0.855	**0.042***
Leu:Ala	≥ 0.465	0.606	70%	35%	0.421-0.791	0.25	**Leu:Ala**	≥ 0.45	0.645	75%	35%	0.46-0.83	0.117
Leu:Phe	≥ 1.775	0.679	90%	30%	0.509-0.848	0.053	**Leu:Phe**	≥ 1.99	0.639	80%	40%	0.461-0.817	0.133
Acyl carnitine	≥ 0.045	0.79	90%	20%	0.642-0.938	**0.002***	**Acyl carnitine**	≥ 0.045	0.721	85%	20%	0.556-0.887	**0.017***
C3:C2	0.095	0.80	80%	70%	0.670-0.940	**0.001***	**C3:C2**	0.095	0.76	75%	70%	0.612-0.910	**0.004***
C6 carnitine	0.08	0.79	70%	75%	0.642-0.938	**0.001***	**C6 carnitine**	0.065	0.72	70%	65%	0.556-0.887	**0.015***

* p = 0 < 0.05

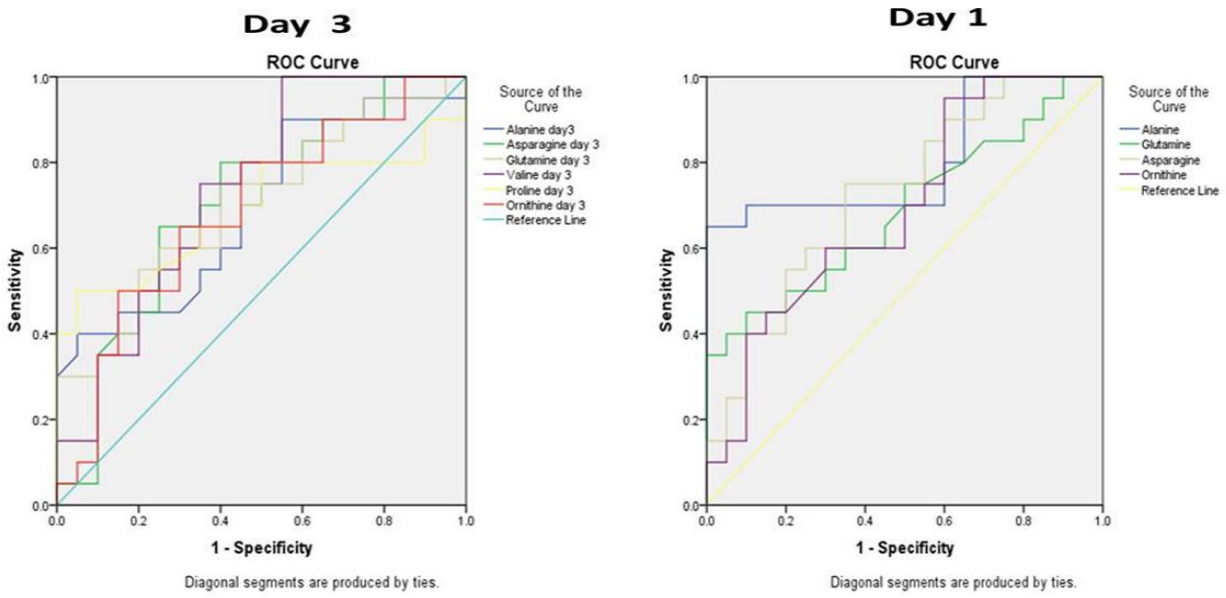

Figure (1): Roc curve analysis of amino acids in prediction of IVH.

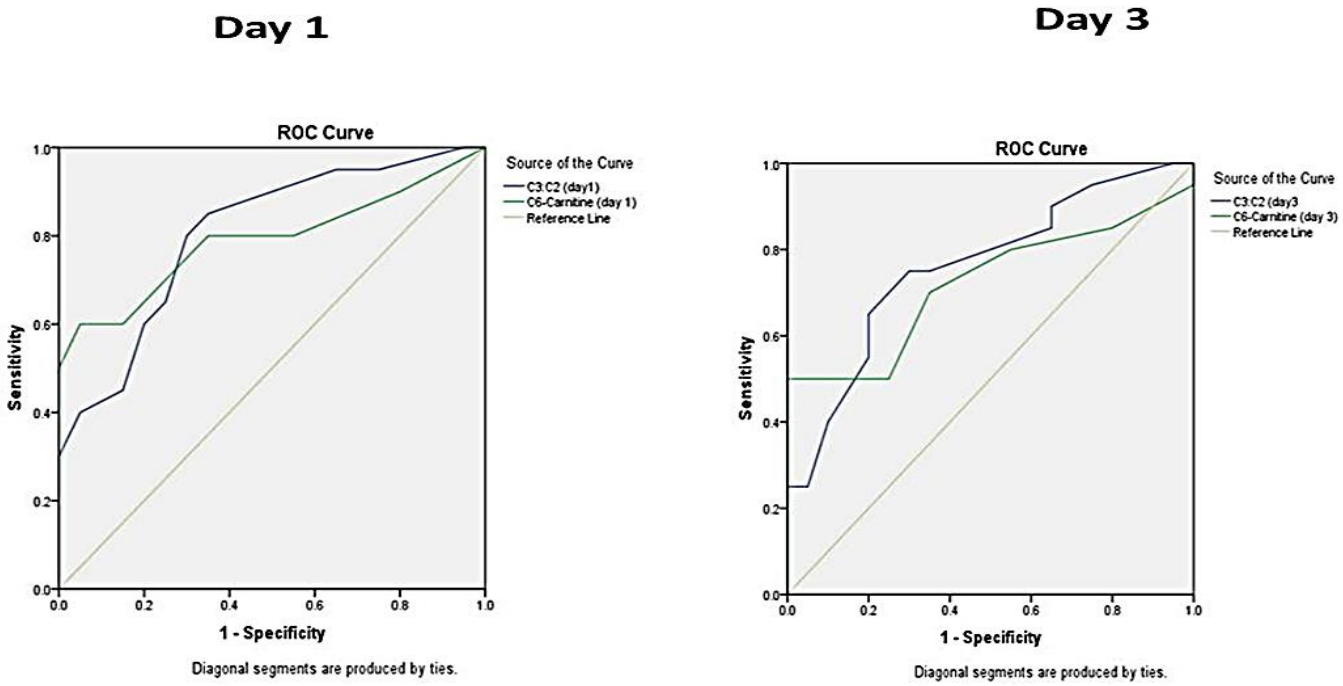

Figure 2: Roc curve analysis of acylcarnitine and carnitine in prediction of IVH.

DISCUSSION

Since we need to expand the metabolomics based investigations in preterm neonates in relation to the occurrence of (IVH), so we aimed to investigate the amino acids and acylcarnitine profile in IVH neonates to provide possible new available biomarkers for prediction of IVH.

The results in this study signify the anaerobic metabolism and the mitochondrial dysfunction as a result of hypoxic injury that was obviously a primary leading cause for development of IVH. The low gestational age of the IVH cases, the lower birth weight and the lower Apgar score in these cases were considered risk factors for IVH. The low Apgar score and lower PH in these cases signify the occurrence of intra-partum hypoxia, which implies organ hypo-perfusion. This is consistent with the body's response to hypoxia, which is the acceleration of anaerobic

glycolysis in order to give the brain the oxygen and energy it needs to survive [12].

Certain amino acids were elevated in blood of the IVH group. This might be explained by the fact that hypoxia and ischemia may trigger a disturbed metabolism of the amino acids. Alterations of the amino acid's metabolism might be attributed due to the lowered energy generation, reduced synthesis of neurotransmitters, and the probable brain damage [6, 10]. Elevated certain amino acids in relation to hypoxia and hypoxic ischemic encephalopathy (HIE) were also noticed in other studies as in **El-Farghali *et al.*** [10] study (alanine, phenyl alanine, valine, leucine were significantly higher in HIE group). A review done by **Debuf *et al.*** [13], reported that the analysis of umbilical cord blood showed significant higher levels of certain amino acids in patients suffering from asphyxia (alanine, isoleucine, leucine, methionine, phenylalanine, and valine are the most significantly observed). The higher levels of glutamate observed in this study in the 1st and the 3rd day could reflect the higher need for the precursors following hypoxia/ischemia. Another theory of the rise in serum glutamine could be the relation between the brain injury and glutamate toxicity, as glutamate and glutamine cycle provide glutamate neurotransmitter, consumption of α-ketoglutarate during the glutamate production process results in reduced oxidation of acetyl-CoA and oxaloacetate [14]. These findings were also supported by **Sarafidis *et al.*** [5] as they noticed rise of urinary glutamine in the same manner in their study.

Regarding acylcarnitines, animal studies showed higher levels of acylcarnitines after hypoxia [15]. Similar to this, newborns with hypoxia have considerably greater amounts of various acylcarnitines in their cord blood [13]. According to metabolomics-based animal studies of hypoxia, there is less free carnitine floating about and more long chain acylcarnitines after hypoxia, which may be explained by insufficient -oxidation [15]. According to the **Oltman *et al.*** [8] investigation, some acylcarnitine was discovered to be crucial for the survival of children with IVH.

A rise of C4, C5, C6, C6-OH, C8, and C12 was noticed in HIE patients in the study of **López-Suárez *et al.*** [16], but decreased levels of C2, C4-OH, and most of long chain acylcarnitines were noticed using dried blood in 3rd day of life and hypothermia treatment was offered to most of their patients. In contrast to a study by **Walsh *et al.*** [17] who found significant increased levels of C2, C4, C4-OH, C6, C12, C14, C14:1, C14:2, C16, C16:1, C18:1, C18:2 as they investigated the cord blood drawn at birth. The differences in our results might be due to difference in timing of obtaining the blood sample. Another finding in a study by **Sarafidisa *et al.*** [5] was the increased urine acetyl-carnitine in the IVH neonates. In a research by **Wainwright *et al.*** [18], acylcarnitines were elevated in babies with both asphyxia and HIE relative to controls, whereas amino acids were mostly raised in those with HIE. They proposed that elevated acyl-CoA levels could be "the first and a key irreversible event in ischemia," occurring before the production of nitric oxide and oxygen free radicals. Additionally, they offered a possible explanation for the rise in acylcarnitine levels observed in both case groups in their study, suggesting the changes in the amino acid profile occurred later than changes in the acylcarnitine profile following an asphyxia incident [18].

In our study the significant cut off point of C3:C2 ratio (P value=0.001) for diagnosis of IVH at day 1 was at 0.095 points with AUC=0.80, sensitivity 80%, specificity 70%, PPV 70.8% and NPV 81.2%. While C6 carnitine at a significant cut off point of 0.08 points with AUC=0.790, sensitivity 70%, specificity 75%, PPV 69.6% and NPV 76.5%. Multiple logistic regression analyses of metabolites were performed in **Pietrzak *et al.*** [19] study, and the results showed that L-carnitine (20:3/22:6) was a significant predictor of ICH and may be utilized as a biomarker. L-carnitine and mitochondrial metabolism and operation are tightly connected. By bringing long-chain fatty acids into the mitochondria and eliminating extra short- and medium-chain fatty acids from the mitochondria to control the oxidation rate and vital cellular processes like apoptosis, it plays a crucial role in fatty acid oxidation and energy metabolism [19–20].

Carnitine can control the amounts of fatty acyl coenzyme A and is important for transporting long-chain acyl CoA to the mitochondrial matrix, where it will be used for oxidation. Carnitine deficiency can lead to an increase in fatty acyl coenzyme A, which can reduce mitochondrial function and increase oxidative stress and inflammation. Fatty acid oxidation may be reduced by any lack of carnitine availability or a carnitine-dependent transport pathway in the mitochondria [21].

Early AA infusion is crucial in reducing the number of hyperglycemic and hypoglycemic patients while improving glycemic variability since prolonged hyperglycemia in the first 96 hours of life is linked to serious IVH in preterm newborns [22, 23].

In this study we are providing new tools for suspicion and diagnosis of IVH using serum alanine, aspartate, C3:C2 ratio and C6 as good predictors for IVH in preterm neonates, which might open a new era for metabolomics to study new biomarkers in brain injury and IVH in preterm neonates. Significant metabolic changes are seen in preterm newborns with IVH, according to a metabolomics investigation. The utilization of a particular panel of metabolites as biomarkers for IVH occurrence and/or follow-up in high-risk preterm newborns is significant.

Limitations of this study are lack of older research in this view as most of the research was focusing on hypoxia. The need to investigate other metabolic precursors and their relations to the IVH is necessary.

Relatively small sample size and that is based on two days amino acids determination rather than consecutive samples collected through the first days of life. Targeted metabolites measurement limits the discovery of other subtle metabolic changes.

CONCLUSION

IVH in preterm neonates was associated with various metabolic changes regarding amino acids and carnitine, acylcarnitine profile. Of which serum alanine and aspartate showed the most significant predictors of IVH. Also, the acyl carnitine profile revealed the significant role of C3:C2 ratio and C6 as predictors of IVH.

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