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ROLE OF SERUM CYSTATIN-C AS A BIOMARKER FOR PREDICTING THE NEUROLOGICAL OUTCOME AND SEQUELAE IN NEONATES ≥ 36 WEEKS WITH BIRTH ASPHYXIA

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ABSTRACT Perinatal Asphyxia stands as an important reason causing systemic and Neurological sequelae leading to Hypoxic-ischemic encephalopathy (HIE) with high morbidity and mortality in infants. This study confers that the Biomarkers such as Serum Cystatin-C in relation with renal testes like Serum Creatinine, Serum Urea may have a significant prognostic ability for predicting the neurological outcome and sequelae in HIE infants. A total of 84 patients have been enrolled into the study and were categorized in to different grades of HIE based on NICHD assessment. Baseline measurements of Serum Creatinine, Serum Urea ,Serum Cystatin -C were taken in all the subjects belonging to all the three grades of HIE with Echo findings and Hammersmith neurological examination. The serum concentrations of all the three parameters were observed at 14 days and at 3 months and by ROC curve analysis. All the observations with values suggested Serum Cystatin-C can be potentially used a prognostic biomarker in predicting the neurological sequelae in neonates ≥36 weeks with birth asphyxia.

KEYWORDS : Hypoxic-Ischemic Encephalopathy, Serum protein S-100B, Cystatin-C

Introduction

Hypoxic -ischemia encephalopathy (HIE) resulting from Perinatal asphyxia along with multi organ dysfunction like Acute kidney injury , cardiovascular events remains a major cause for neonatal mortality and morbidity. Biomarkers like Cystatin-C may play a vital role in prediction of extent of neurological sequelae , renal damage in asphyxiated neonates which helps to identify the higher risk and take appropriate clinical measures to limit the damage.

Clinical assessment of Hypoxic-ischaemic encephalopathy (HIE) with a scoring system is currently the simplest cot-side method for predicting neurological outcomes in asphyxiated infants.4 Babies with mild HIE usually have a good prognosis, however, those with severe HIE usually show poor outcomes.5 The outcome of babies with moderate HIE varies depending on the duration and persistence of the signs of encephalopathy. In some cases, the signs of encephalopathy may persist longer. Babies with signs lasting more than seven days are at a greater risk of neurological problems.4,5

Cystatin C is a cationic cysteine protease inhibitor which has low molecular weight produced by all nucleated cells in the body that is freely filtered by glomerulus and completelyreabsorbed at proximal tubules. Many studies have suggested Cystatin C can be potential indicator for AKI (Acute Kidney injury) than Sr.Creatinine and Creatine clearance due its rate of production which not affected by muscle mass, race , sex and diet unlike other indicators. Studies have shown decrease in GFR is associated with increase in Serum Cystatin-C thus signifying it as a better indicator for renal function and impairments.

In this observational study we studied the correlation between Cystatin C and its association with hypoxic-ischaemic encephalopathy (HIE) predicting the neurological outcomes (survival with sequelae) at 14 days or later after birth asphyxia and at three months of age. We have also compared serum biomarkers against the National Institute of Child Health and Human Development (NICHD) score in predicting neurological outcomes.

MATERIALS AND METHODS

Study design and setting

This was an observational correlational clinical study carried on from September 2016 to march 2017 at Department of neonatology, Madras Medical College (MMC), Chennai. Also involved are out-born unit of Institute of Child health and hospital for children (part of MMC) and inborn unit of Institute of Obstetrics and Gynaecology, Chennai (part of MMC). A written consent of willingness for enrolment of the infants in the study was obtained from the parents / care-givers of the infants selected. All 84 infants selected belonged to any of the three stages of Hypoxemic ischemic encephalopathy (HIE).

Eligibility

All new borns admitted in the intramural and extramural, with perinatal asphyxia were eligible for this study

Inclusion criteria

Neonates born at ≥36 weeks of gestation and of >1800 g birth-weight, and with perinatal asphyxia were eligible. Perinatal asphyxia in patients born at the study hospital was defined as the need for resuscitation at birth, along with the presence of one or more of the following:

1. Apgar score of <6 – at 5 min after birth,
2. Continued need for resuscitation, for > 5 mins
3. umbilical cord pH or any arterial pH of <7.00 within 60 mins of birth, and base deficit of
>16 mmol/L within 60 mins of birth

Written consent was obtained from the parents/caregivers of the infants selected for willingness for enrolment in the study (Annexure

I).Exclusion criteria

1. Neonates with major congenital anomalies
2. Neonates presenting after 24 hours of birth
3. Neonates enrolled in the therapeutic Cooling Study

Primary outcome measure is to study the correlation between Serum Cystatin-C significance in predicting the neurological outcomes (survival with sequelae) at 14 days or later after birth asphyxia and at three months of age. Secondary outcome desired is the correlation of combination of Serum protein Cystatin-C, with serum protein S-100 B,Lactate dehydrogenase and Cardiac troponin I in the predictability of neurological outcomes.

Non-parametric setting for Qualitative data analysis

Pearson correlation between study variables is performed to find the degree of relationship, Pearson correlation co-efficient ranging between -1 (weak correlation) to 1 (strong correlation) between two biomarkers. The stronger the correlation, stronger is the ability to influence the other biomarker with which there is a correlation.

Classification of Correlation Co-efficient ®

Up to 0.1	Trivial Correlation
0.1-0.3	Small Correlation
0.3-0.5	Moderate Correlation
0.5-0.7	Large Correlation
0.7-0.9	V.Large Correlation
0.9- 1.0	Nearly Perfect correlation
1	Perfect correlation

ROC curve analysis is performed to find the predictability of study variables (biomarkers) in prognosis of advanced HIE stage. The biomarkers with higher sensitivity and specificity, PPV (Positive Predictive Value) and NPV (Negative Predictive Value) suggest their correlation beyond their individual cut-off values to be prognostic markers for the neurological sequelae. Diagnostic markers based on Area under curve AUROC signifies the following result –

0.9 - 1.0	Excellent test
0.8 - 0.9	Good test
0.7 - 0.8	Fair test
0.6 - 0.7	Poor test
0.5 - 0.6	Fair

Statistical Methods: Descriptive and inferential statistical analysis has been carried out in the present study. Results on continuous measurements are presented on Mean+ SD (Min-Max) and results on categorical measurements are presented in Number (%). Significance is assessed at 5 % level of significance. The following assumptions on data are made

1. Dependent variables should be normally distributed,
2. Samples drawn from the population should be random,
3. Cases of the samples should be independent

Analysis of variance (ANOVA) has been used to find the significance of study parameters between three or more groups of patients, Student t test (two tailed, independent) has been used to find the significance of study parameters on continuous scale between two groups (Inter group analysis) on metric parameters. Chi-square/ Fisher Exact test has been used to find the significance of study parameters on categorical scale between two or more groups.

Statistical software: The Statistical software namely SAS 9.2, SPSS 15.0, Stata 10.1, MedCalc 9.0.1 ,Systat 12.0 and R environment ver.2.11.1 were used for the analysis of the data and Microsoft word and Excel have been used to generate graphs, tables etc.

Results and discussion

84 infants with a gestation age of > 36 weeks of gestation were included in the study. 19% (16/84) of infants were delivered through LSCS mode of delivery while the remaining infants delivered through normal route. 90% (76/84) of infants are of birthweight 2.5kg-3.5kg and 79 infants were AGA. Respiratory support was given 79 infants.

Demographic data of the patients

Patients enrolled are of both genders – 35 male and 49 female. 39/84 patients were of gestational age less than 38 weeks. Except 8 neonates who are below 2500g, remaining 76 neonates enrolled have a birth weight between >2500g and 3500g. Only five were small for gestational age. 79 neonates required respiratory support on admission (CPAP or mechanical ventilation or O2). Oxygen saturation was still less than 90 in case of 42 neonates upon admission and in 76 neonates mean arterial pressure upon admission is <30 mm to 40 mm of Hg. Upon admission, patients were categorized based on the severity of encephalopathy – 34 patients were assessed mild (HIE1), 37 were assessed moderate (HIE2) and 13 were assessed severe (HIE3). All three grades of encephalopathy are identified among neonates upon admission – 34 neonates with mild encephalopathy (HIE1), 37 neonates with moderate encephalopathy (HIE2), 13 neonates with severe encephalopathy (HIE3). All patients were assessed with APGAR score at 1 minute, as <7 and at 5 minutes the score is <7 in 28 patients. Around 54% of patients in both HIE2 and HIE3 groups still have APGAR score <7 at 5 min. The demographic data is summarized in Table 1.

Table 1 Demographic data

	Final Diagnosis			P value
	HIE 1 (n=34)	HIE 2 (n=37)	HIE 3 (n=13)	
Gender				
• Female	24(70.6%)	19(51.4%)	6(46.2%)	0.162
• Male	10(29.4%)	18(48.6%)	7(53.8%)	
Gestation age (weeks)				
• 36-37	19(55.9%)	12(32.4%)	8(61.5%)	0.070+
• 38-40	15(44.1%)	25(67.6%)	5(38.5%)	
Birth weight (g.)				
• <2500	7(20.6%)	1(2.7%)	0(0%)	<0.001**
• 2500-3000	21(61.8%)	17(45.9%)	9(69.2%)	
• 3000-3500	6(17.6%)	19(51.4%)	4(30.8%)	
Mode of delivery				
• LSCS	0(0%)	11(29.7%)	5(38.5%)	<0.001**
• Vaginal	34(100%)	26(70.3%)	8(61.5%)	
APGAR score 1 min.				
• <7	34(100%)	37(100%)	13(100%)	1.000
• >7	0(0%)	0(0%)	0(0%)	
APGAR score 5 min.				
• <7	1(2.9%)	20(54.1%)	7(53.8%)	<0.001**
• >7	33(97.1%)	17(45.9%)	6(46.2%)	
Cord ABG (pH, Base deficit)				
• <6.8	0(0%)	0(0%)	0(0%)	<0.001**
• 6.8-7.2	20(58.8%)	26(70.3%)	13(100%)	
• >7.2	14(41.2%)	11(29.7%)	0(0%)	
Severity of encephalopathy @ admission (NICHD assessment)				
Mild	34(100%)	0(0%)	0(0%)	<0.001**
Moderate	0(0%)	37(100%)	0(0%)	
Severe	0(0%)	0(0%)	13(100%)	

** Chi-Square test/Fisher Exact testAt base lines, blood samples were taken upon admission and after 24 hrs and 72 hrs to analyse for various prospective prognostic biomarkers such as SGOT, SGPT, Blood urea (mg/ dL), Serum creatinine (mg/dL), APTT, Blood sugar, Serum protein S-100B, Cardiac troponin I, Cystatin-C, Lactate dehydrogenase, PT. ECHO for myocardial dysfunction and Hammersmith neurological examination have been done. Baseline investigations of biomarkers are presented in table [2].

Table 2: Baseline investigations of Serum biomarkers

Marker	Upon admission**			24 hrs after admission**			72 hrs after admission**		
	HIE 1	HIE 2	HIE 3	HIE 1	HIE 2	HIE 3	HIE 1	HIE 2	HIE 3
Blood urea (mg/dL)	22.91 +2.45	46.54 +21.63	44.92 +3.45	27.53 +3.15	56.05 +21.74	53.62 +3.50	21.35 +3.72	57.46 +21.79	76.38 +2.53
Serum Creatinine (mg/dL)	0.49 ±0.16	1.04 ±0.20	1.56 ±0.31	0.75 ±0.11	1.32 ±0.14	1.80 ±0.50	0.84 ±0.09	1.54 ±0.35	2.29 ±0.41
Cystatin-C	1.26 ±0.04	1.86 ±0.38	1.98 ±0.41	1.30 ±0.10	1.72 ±0.26	2.37 ±0.51	1.23 ±0.04	1.74 ±0.28	5.70 ±8.12
SGOT	107.32 +2.66	145.97 +47.21	215.38 +34.75	90.97 +8.86	186.97 +67.42	269.38 +28.50	74.38 +5.41	179.27 +76.29	300.62 +45.47
SGPT	39.00 +1.87	87.03 +30.24	86.00 +5.00	50.65 +18.16	88.27 +24.63	112.31 +13.38	47.47 +22.82	76.70 +17.83	152.23 +20.48
APTT	27.06 ±1.43	26.11 ±1.51	37.69 ±3.47	26.35 ±1.54	30.05 ±2.69	41.62 ±1.80	30.71 ±1.06	32.76 ±1.79	38.38 ±6.81
Blood sugar	81.35 ±6.49*	73.97 ±18.32*	69.69 ±19.52*	90.91 ±2.22	75.68 ±23.60	64.15 ±14.5	81.62 ±6.36	73.27 ±19.55	55.54 ±17.27
Serum protein S-100B	11.50 ±1.50	19.57 ±5.24	23.69 ±1.03	10.09 ±1.22	17.07 ±2.57	22.62 ±2.33	9.35 ±1.74	11.57 ±2.10	16.77 ±2.17
Cardiac	0.04	0.05	0.07	0.04	0.06	0.08	0.04	0.04	0.07

troponin I	±0.00	±0.01	±0.01	±0.00	±0.02	±0.01	±0.00	±0.00	±0.02
Lactate dehydrogenase	234.79 ±8.35	290.73 ±135.99	617.23 ±133.47	318.74 ±69.56	444.32 ±80.09	762.62 ±103.02	327.32 ±51.12	594.14 ±92.56	828.00 ±60.43

P<0.001**, ANOVA test, P=0.03*

Increase in mean concentrations of Serum Creatinine levels were observed as the severity of the HIE increased.15.3% increase from admission to 24hrs and 46.7% increase after 72hrs of admission.Significant increase in mean concentrations of serum Cystatin-C is observed with 19.6% increase after 24 hrs and 187.8% increase after 72 hrs from baseline value for HIE 3 group.Similarly with blood urea the mean concentrations have increased as the severity of HIE progressed, 19.3% and 70.03% at 24 hrs and 72 hrs respectively from admission for HIE 3 group patients. Increase in mean concentrations of SGOT is seen in HIE2 and HIE3 groups after 72hrs, a similar significant increase of SGPT is witnessed in HIE 3 category patients only. Similarly, an increase in mean concentration of Lactate dehydrogenase increase in HIE3 group and an increase in each grade at 24 and 72 hours after admission. Serum protein S-100B increase was correlated to severity of HIE, as there is a gradual decrease of the same witnessed over 72 hours across three grades of HIE patients (80%-124% increase) in mean concentrations as the severity progresses and over 72 hours. While cardiac troponin levels are observed to be almost constant across the groups and over 72 hours.Upon admission, Pearson correlation was used to assess the strength of correlation between individual markers versus Cystatin-C, and at baseline, there is a strong correlation observed among the four biomarkers of interest.

Table 3: Pearson Correlation: Cystatin C with other clinical variables

Pearson Correlation	R value	P value
Cystatin –C vs Lactate dehydrogenase	0.202	0.065+
Cystatin –C vs SGOT	0.483	<0.001**
Cystatin –C vs SGPT	0.785	<0.001**
Cystatin –C vs Blood urea(mg/dl)	0.680	<0.001**
Cystatin –C vs Serum Creatinine (mg/dl)	0.713	<0.001**
Cystatin –C vs APTT	0.220	0.044*
Cystatin –C vs Blood Sugar	0.398	<0.001**
Cystatin –C vs Serum protein S-100B	0.840	<0.001**
Cystatin –C vs PT1	0.285	0.009**

From Pearson correlation coefficient, it was observed that Cystatin-C had the strongest correlation with Serum protein S-100B amongst all the markers and a weaker correlation with Lactate dehydrogenase. Results show Cystatin-C elevation has very strong correlation with SGPT, blood urea, Serum protein S-100B and serum creatinine.To observe these patients and to assess prognostic value of the four prospective biomarkers in predicting neurological sequelae, blood samples were again taken for neonates after 14 days and infants at 3 months of age, and were observed for Cystatin-C, Serum protein S-100B, Lactate dehydrogenase, Cardiac troponin, the biomarkers of interest in the current study. The mean serum concentrations (with standard deviation) of these four biomarkers were studied in normal and abnormal subjects as assessed by USG cranium findings to assess correlation between abnormal serum concentrations of the biomarkers to oedema or haemorrhage in infants, abnormal ECHO findings (possibility of myocardial dysfunction) and MRI findings were recorded during final diagnosis.

All the four biomarkers have been measured in normal and abnormal patients as assessed by Hammersmith neurological examination to understand the possible correlation to predict neurological outcomes with such assessment of these biomarkers

Cystatin –C in relation to ECHO findings

Cystatin -C	Echo findings Absent	Echo findings Present	P value
Admission	1.53±0.41	1.84±0.40	0.001**
24 hrs	1.47±0.27	2.02±0.52	<0.001**
72 hrs	1.43±0.30	3.59±5.78	0.006**

Cystatin –C in relation to Hammersmith neurological examination @ neonates (at 14 days)

Cystatin -C	Echo findings Absent	Echo findings Present	P value
Admission	1.28±0.08	1.91±0.39	<0.001**
24 hrs	1.31±0.12	1.90±0.45	<0.001**
72 hrs	1.25±0.08	2.83±4.48	0.038*

Cystatin –C in relation to Hammersmith neurological examination @ infants (at 3 months)

Cystatin -C	Echo findings Absent	Echo findings Present	P value
Admission	1.54±0.39	1.93±0.42	<0.001**
24 hrs	1.47±0.27	2.19±0.47	<0.001**
72 hrs	1.47±0.34	4.17±6.60	<0.002**

Assessment of possible correlation Cystatin C correlation to Echo findings.

The infants with abnormal Echo findings had significant elevation of 20% in mean Serum Cystatin-C concentration than normal infants up on admission.After 72 hrs there is an 95% increase in the value from admission in mean serum Cystatin -C concentrations in abnormal patients with echo findings. Where as for normal infants the mean concentration of Serum Cyatatin-C value decreased from admission to 72 hrs after admission.

Assessment of Cystatin -C in correlation to Hammersmith neurological examination of Neonates and Infants at 3 months.

Mean serum concentrations of Cystatin-C are increased in abnormal group of patients. At 72 hours, the elevation of concentrations in abnormal group is 48% in neonates and 116% elevation in infants as compared to the respective serum concentrations upon admission.

Serum protein S-100B in relation to MRI changes

Serum protein S-100B	Normal	Abnormal	P value
Admission	13.08±4.40	21.20±4.28	<0.001**
24 hrs	12.01±3.81	18.51±3.84	<0.001**
72 hrs	0.785	<0.001**	<0.001**

Serum protein S-100B in relation to Hammersmith neurological examination @ neonates (14 days)

Serum protein S-100B	Normal	Abnormal	P value
Admission	11.57±1.49	20.98±4.69	<0.001**
24 hrs	10.26±1.41	18.73±3.38	<0.001**
72 hrs	9.31±1.70	13.10±3.04	<0.001**

Serum protein S-100B in relation to Hammersmith neurological examination @ infants (3 months)

Serum protein S-100B	Normal	Abnormal	P value
Admission	14.84±5.26	23.24±2.30	<0.001**
24 hrs	13.18±3.96	20.86±3.02	<0.001**
72 hrs	10.13±2.00	15.52±2.52	<0.001**

Assessment of possible Serum protein S-100B correlation to MRI changes

The infants who showed abnormal on MRI had significant elevation of mean serum protein concentration of 62% compared to normal infants upon admission and 54% elevation after 24 hrs and 39% elevation is still evident after 72 hrs which is almost equivalent to normal serum protein concentrations upon admission. It is observed that over 72 hours period, the serum protein concentrations decrease both in normal and abnormal MRI groups.

Assessment of Serum protein S-100B in relation to Hammersmith neurological examination of neonates (14 days old) and infants (at 3 months of age)

Upon admission it is observed that abnormal neonates show 81% higher mean serum protein concentration than normal neonates which is persistent at 24 hrs but is still 41% elevation at 72 hrs in abnormal neonates. At 3 months of age, the higher mean serum protein concentrations of abnormal infants is around 57% compared to normal upon admission. While the mean concentration of serum protein decreases in both normal and abnormal groups over a period of 72 hrs, a 51% elevation is still seen in abnormal group of patients as compared to normal.

Lactate dehydrogenase in relation to MRI findings

Lactose dehydrogenase	Normal	Abnormal	P value
Admission	249.57±57.98	394.58±210.22	<0.001**
24 hrs	346.89±89.76	548.20±173.61	<0.001**
72 hrs	380.07±115.7	678.83±133.11	<0.001**

Lactate dehydrogenase in relation to

Hammersmith neurological examination @ neonates (at 14 days)

Lactose dehydrogenase	Normal	Abnormal	P value
Admission	235.97±12.81	380.60±199.61	<0.001**
24 hrs	319.75±68.65	535.00±163.28	<0.001**
72 hrs	338.00±68.80	660.58±132.93	0.038*

Hammersmith neurological examination @ infants (at 3 months)

Lactose dehydrogenase	Normal	Abnormal	P value
Admission	269.71±104.31	465.33±227.1	<0.001**
24 hrs	377.10±97.40	639.71±186.63	<0.001**
72 hrs	440.25±143.26	768.57±91.29	0.002**

As per the serum concentrations of lactate dehydrogenase recorded in abnormal patients as per MRI findings, there is an elevation of 60-70% of the enzyme in abnormal group as compared to normal. As per Hammersmith neurological examination of neonates and infants, the elevation of serum lactate dehydrogenase is 60% (at admission) to 95% (at 72 hrs after admission) in neonates and around 74% elevation in infants as compared to normal group.

Cardiac troponin I in relation to ECHO findings

Cardiac troponin I	Echo Absent	Echo Present	P value
Admission	0.04±0.01	0.06±0.02	<0.001**
24 hrs	0.04±0.02	0.06±0.01	<0.001**
72 hrs	0.04±0.00	0.05±0.02	<0.001**

Cardiac troponin I in relation to

Hammersmith neurological examination @ neonates (at 14 days)

Cardiac troponin I	Normal	Abnormal	P value
Admission	0.04±0.00	0.05±0.02	<0.001**
24 hrs	0.04±0.00	0.06±0.02	<0.001**
72 hrs	0.04±0.00	0.05±0.02	0.002**

Cardiac troponin I in relation to

Hammersmith neurological examination @ infants (at 3 months)

Cardiac troponin I	Normal	Abnormal	P value
Admission	0.04±0.01	0.07±0.02	<0.001**
24 hrs	0.05±0.02	0.07±0.01	<0.001**
72 hrs	0.04±0.00	0.06±0.02	<0.001**

Among other three biomarkers, which are secondary outcomes, Lactate dehydrogenase (60-80% higher levels of lactate dehydrogenase in abnormal groups over a period of 72 hrs in abnormal groups assessed by MRI findings and Hammersmith neurological examination of neonates and infants) and Serum S-100 showed significant difference in mean serum concentrations between normal and abnormal groups.

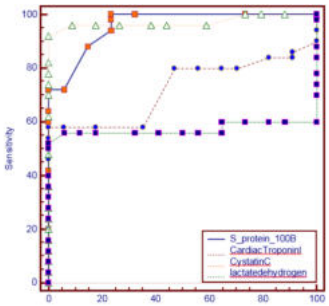
ROC curve analysis to predict advanced stage

ROC curve analysis was done to have sensitivity value, specificity value, the cut off value of each biomarker and the AUROC (Area under ROC curve) in predicting the neurological sequelae in patients with birth asphyxia. ROC curve analysis was shown in Table 6.

Table 6: ROC curve analysis

Variables	ROC results to predict Advanced Stage				Cut-off	AUROC	SE	P value
	Sensitivity	Specificity	LR+	LR-				
SGOT	86.00	100.00	0.0	0.14	>112	0.880	0.046	<0.001**
SGPT	92.00	100.00	0.0	0.080	>42	0.978	0.012	<0.001**
Blood urea(mg/dl)	86.00	100.00	0.00	0.14	>27	0.964	0.0168	<0.001**
Serum Creatinine (mg/dl)	88.00	100.00	0.00	0.12	>0.8	0.981	0.0108	<0.001**
APTT	26.00	100.00	0.0	0.74	>29	0.505	0.061	0.938
PT	100.00	35.29	1.55	0.00	>11	0.644	0.060	0.017*
Blood Sugar	34.00	100.00	0.00	0.66	≤60	0.557	0.062	0.364
Serum protein S-100B	100.00	76.47	4.25	0.00	>12	0.958	0.018	<0.001**
Cardiac troponin I	58.00	100.00	0.00	0.42	>0.042	0.722	0.056	<0.001**
Cystatin-C	92.00	100.00	0.00	0.08	>1.32	0.972	0.0188	<0.001
Lactate Dehydroge nase	52.00	100.00	0.00	0.48	>250	0.573	0.069	0.292

Amongst the four biomarkers of interest, it was observed that two biomarkers, Serum protein S-100B and Cystatin-C exhibit high specificity and sensitivity values which makes them more reliable biomarkers for predictability of neurological events. It was observed from ROC curve analysis that Serum protein S-100 B has a cut off value of >12 and that of Cystatin-C is more than 1.32. It can be inferred that these cut off values serve as prognostic biomarker concentrations that can predict the possibility of neurological sequelae.



ROC curve analysis of four biomarkers of interest

Variables	ROC results to predict Advanced Stage				Cut off	AUROC	SE	P value
	Sensitivity	Specificity	LR+	LR-				
Cystatin–C	92.00	100.00	0.0	0.08	>1.32	0.972	0.0188	<0.001**
Serum protein S-100 B	100.00	76.47	4.25	0.00	>12.0	0.958	0.0180	<0.001**
Cardiac troponin I	58.00	100.00	0.0	0.42	>0.042	0.722	0.056	<0.001**
Lactate dehydrogenase	52.00	100.00	0.0	0.48	>250	0.573	0.069	0.292

Based on the result of AUROC analysis (AUROC value of 0.958 for Serum protein S-100B and value of 0.972 for Cystatin-C) and because of high sensitivity and specificity values, Serum protein S-100B and Cystatin-C could possibly be two reliable biomarkers beyond the cut off values of which have prognostic value in predicting neurological sequelae. The observations are highly significant for both these biomarkers. These observations are backed by strong Pearson correlation seen amongst these two biomarkers.

Apart from understanding the predictability of individual biomarkers, the combination of these biomarkers have also been studied by ROC curve analysis.

Combination of Serum protein S-100B, Cardiac troponin I, Cystatin –C and Lactate dehydrogenase to predict the HIE 2 & 3

Combination of 4 markers	HIE1 (n=34)	HIE2&3 (n=50)	Total (n=84)
Negative	26(76.4%)	0	26(30.9%)
Positive (any one positive)	8(23.5%)	50(100.0%)	58(69.1%)

Sensitivity %	100.00
Specificity%	76.47
PPV%	86.21
NPV%	100.00
Accuracy%	90.48
Significance	c2=55.375; P<0.001**

A high sensitivity and specificity values of the combination of four markers suggest to check for the abnormalities of these biomarkers together in various grades of HIE. The high PPV (Positive predictive value) and high NPV (Negative predictive value) signifies a high degree of correlation between the combination of these four biomarkers and the predictability of neurological outcome such as HIE and other disorders.Conclusion:

As observed during the study the elevated levels of Serum Cystatin-C in neonates at 14 days and in infants at 3 months, if witnessed beyond the cut-off value, could be independent prognostic marker to predict abnormal neurological outcomes in neonates / infants.Significant increase in the Serum Cyatatin-C levels after 72 hours of admission among the infants having abnormal echo findings infers its potential as prognostic biomarker for understanding different neurological sequealae among HIE infants.Altogether this study has observed clinically significant increase in Serum Cystatin-C levels among the infants categorized with severe HIE during admission, progressively after 72 hrs and on observation after 14 days and 3 months. Additionally mean concentrations of Serum Creatinine and Blood Urea made a significant importance in severely categorized Hypoxic Ischemic Encephalopathic subjects.

Serum protein S-100B and Serum Cystatin-C exhibited a strong correlation with significant and reliability both individually and together can be used in prognosis of neurological outcome and sequelae. While the highly abnormal concentrations of Lactate dehydrogenase is witnessed with an increasing severity of HIE which can't be ignored, this study could neither find a strong correlation of the enzyme with other biomarkers nor the reliability or significance as per ROC analysis. Cardiac troponin I is to be further investigated in detail for its prognostic ability as biomarker in predicting neurological sequelae. As a combination however, all these four biomarkers have stronger positive correlation amongst each other and are significant compared to other biomarkers to contribute to cumulative predictable effect on neurological outcomes such as HIE. The high PPV, NPV, sensitivity and specificity as measured by ROC analysis suggests that the combination of all four biomarkers could be useful for prognosis of neurological sequelae.

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