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DNA Methylation Pattern Of Cpg Site In The Promoter Region Of CALCA-Alpha Gene As A Putative Epigenetic Biomarker In Neonatal Sepsis- A Prospective Observational Study In South India

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Introduction:

Sepsis, which is a deteriorating clinical outcome of a war between pathogens and host immune response represents one of the major public health issues in neonates. (1,2) World Health Organization (W.H.O) data from 2019 reports 6700 neonatal deaths per day and 75% of deaths occur within the 1st week of life. (3) There are 2 clinical forms of neonatal sepsis which include early-onset sepsis (EOS) (day 1 to day 7) and late-onset sepsis (LOS) (day 8 to day 28), they vary in their clinical, microbiological features, and management plans. Although blood culture is a well-established gold standard diagnostic method being implemented in the present clinical setting, it takes 48 to 72 hrs to identify the microbial pathogens and is less sensitive in terms of diagnosing true positive cases. Due to the rapid deterioration of true sepsis cases, instant broad-spectrum antibiotic therapy is the only way to combat and control the infection in suspected sepsis (4), but this empirical use has further challenged neonatologists in prescribing antibiotics due to the emergence of antibiotic resistance among prevalent bacteria. Serum markers and hematological indices are not efficient enough for the diagnosis of sepsis since these parameters also rely on physiological changes due to several other factors. (5) Recently several studies have been reported on epigenetic modifications of DNA as a predisposing factor for several diseases among neonates and adults. Identification of specific differentially methylated genes has been shown to serve as a promising marker for the diagnosis and predicting the prognosis of sepsis in neonates. (6) Out of several putative genes, the gene encoding sepsis protein markers in serum, *CALCA* gene (calcitonin-related polypeptide alpha) revealed altered DNA methylation pattern in sepsis babies when compared to healthy controls. CpG (Cytosine phosphodiester bond Guanine) sites in the promoter region of the *CALCA* gene are associated with DNA methylation (7) which has been postulated to contain microbial infection-specific response elements that are involved in transcriptional regulation of procalcitonin (PCT) during bacterial sepsis. PCT is an acute-phase protein whose concentration increases in response to

bacterial infection and a change in the methylation pattern of the *CALCA* gene could provide a hallmark in finding appropriate epigenetic biomarkers in neonatal sepsis.

It is suggested from the above description that the promoter region of this gene with its methylation pattern has great importance in sepsis. *CALCA* is a gene that codes for two alternatively spliced products, calcitonin, and calcitonin gene-related peptide (α-CGRP), and these are cleaved from larger pro-peptides PCT and proCGRP, respectively. This gene is situated on chromosome 11 mainly consisting of six exons. Exon 1 is non-coding and exon 2 codes for the signal sequences. (8) PCT is released as an acute-phase protein from leukocytes in response to the various cytokines or toxins of bacteria in the serum during infection. (8-11) PCT levels are found to be raised to the maximum in cases of bacteremia and a moderate extent in fungemia. (12) The level of the same fall as the infection is controlled suggesting a correlation with the *CALCA* gene downregulation. (13) PCT kinetics are not significantly affected by hemofiltration so can be used in practice to diagnose sepsis in those patients too. (14,15) Its levels show a higher diagnostic significance than the other established markers for sepsis (16,17), and unlike C-reactive protein (CRP) that is lowered on the administration of glucocorticoids, PCT levels remain the same as long as the infection persists. (18) The characteristic feature of differentiating systemic inflammation from infection improves the overall significance of sepsis diagnosis. (19,20)

Objective

We aimed to study the DNA methylation status in the promoter region of the *CALCA* gene in neonatal sepsis and compare it with normal healthy controls from a tertiary care centre in South India.

Materials and Methods:

A prospective observational study was carried out with 115 neonates including clinically suspected sepsis and normal healthy controls. The study was carried out in the Department of

Biochemistry in collaboration with the Department of Pediatrics. Institutional ethical committee clearance [letter no. JSSMC/IEC/3107/03 STS/2018-19] was obtained before the commencement of the study and informed consent from the parents was taken before the collection of blood samples.

Inclusion criteria:

The presence of two or more below-mentioned symptoms was taken as a case of suspected/probable sepsis with or without accompanying bacteremia in the first 4 weeks of life. The symptoms include:

- Lethargy or loss of consciousness, poor/stopped feeding, weak/stopped crying in a previously normal baby, fever (Temp 37.5°C or more) or low body temp (< 35.5° C), abdominal distension/vomiting, fast breathing (60bpm or less), severe chest in-drawing or grunting, pus draining from umbilicus/multiple skin pustules.

Exclusion criteria:

Neonate with confirmed intrauterine viral infection (toxoplasmosis, cytomegalovirus, syphilis, and herpes), neonates with visible congenital anomalies and inborn errors of metabolism detected by new born screening were excluded from the study.

Study plan:

Neonates recruited for the study were grouped into suspected sepsis and age, location, and gestational matched healthy controls. Baseline investigations such as complete blood count (CBC), CRP, and blood culture and sensitivity were done for each neonate under the study as a regular new born sepsis screening program. Positive cases were isolated from suspected cases based on positive blood culture and sensitivity reports. DNA was extracted from EDTA whole blood that was collected as a part of baseline hematological examination.

1. Blood genomic DNA extraction: The extraction was done by the non-enzymatic salting-out

method adopted from Suguna et al. (21). About 200µl of EDTA blood was subjected for RBCs lysis using TKM1 low salt buffer for 3 times followed by the lysis of cellular pellet using high salt TKM2 buffer along with 10% sodium dodecyl sulfate (SDS). Lysis was done at 37^0C in a shaker incubator. Further, the lysate was treated with 10µl of RNase A and incubated at room temperature followed by centrifugation at 8000rpm for 5minutes. The supernatant was transferred to a new 1.5ml microcentrifuge tube and an equal volume of isopropanol was added and mixed gently by finger flicking. The sample mixture was incubated at -20^0C for 1 hour followed by centrifugation at high speed (14000 rpm for 15minutes) under cold conditions (4^0C). The supernatant was discarded and DNA precipitate at the bottom of the tube was washed 2 times with 70% ice-cold ethanol. Finally, excess alcohol was drained off and the precipitate was air-dried and then dissolved in 80µl of 1X-TE Buffer which was stored at -20^0C for further use.

2. DNA Quantification and Purity assessment: The isolated DNA was quantified using nanodrop (DeNovix). Absorbance at 260nm was recorded along with purity ratios A260/280nm and A260/230 against blank i.e. 1X-TE. Concentrations of each DNA sample (ng/µl) were also recorded.

3. DNA separation by Agarose Gel Electrophoresis: Blood genomic DNA was checked for their intactness and quality by gel electrophoresis in which 1µg of DNA was subjected for electrophoresis using 1.5% agarose gel. 1X-TAE Buffer with pH 8.2 was used to prepare agarose gel as well as for running buffer. About 4µl of Ethidium bromide (10mg/ml) was used as an intercalating dye for 100ml of melted agarose gel before casting into the gel tray with appropriate combs. Casted agarose gel was allowed to polymerize at room temperature for 30 minutes and combs were removed along with sealers and then submerged into the 1X-TAE running buffer present in the electrophoresis tank. DNA was mixed with 1X bromophenol blue loading dye or tracking dye along with 1X-TE buffer as a sample diluent. DNA-dye mixture

was loaded into the sample wells in an equal volume having equal concentrations of DNA. Electrophoresis was carried out for 1 hour at 50V using a power pack. After electrophoresis, the processed gel was exposed to UV-light using a gel documentation unit and DNA bands were observed. The quality of DNA bands was analyzed for any degradation or solvent contaminations that possess inhibiting properties in downstream applications.

4. Bisulfite conversion of DNA: After quantification and purity assessment of genomic DNA, the same was subjected to bisulfite conversion by a manual method in which 2µg of DNA was used from each of the DNA samples (22). In brief, 2µg of genomic DNA was mixed with 8.84 µl of bisulfite lysis buffer in the total volume of 18 µl and incubated at 37^0C in a water bath for 1 hour followed by the alkaline denaturation of DNA by adding 2 µl of 3M NaOH. The alkaline denaturation was carried out at 37^0C for 15 minutes after which DNA was incubated at 90^0C for 2 minutes using a dry block. The denaturation process was then stopped by immediately shifting it over ice where it was incubated for 5 minutes. The sample was then centrifuged and then subjected to bisulfite deamination by adding 208 µl of saturated metabisulphite along with 12 µl of 10mM quinol. The reaction mixture was mixed gently by inversion and centrifuged to make the contents settle down to the bottom of the tube which was then overlaid with 100 µl of mineral oil and incubated at 55^0C in a shaker incubator for 8 hours. The tubes were covered with aluminium foil to avoid photo-oxidation after which the DNA bisulfite sample mixtures were allowed to pass through the silica column to remove salts. The column was then washed with washing buffer and the treated DNA was eluted in 50 µl of preheated molecular biology grade water. The DNA elute was further mixed with 5.5 µl of 3M NaOH for desulfonation. The content was incubated at 37^0C for 15 minutes followed by the addition of 1 µl of glycogen (10mg/ml). About 10M ammonium acetate was added to the reaction mixture in a final concentration of 3M and incubated at -80^0C for 1 hour. The reaction tubes were then cold centrifuged at 14000 rpm for 15 minutes. The supernatant was removed, and the precipitate

was washed with 70% ethanol. Finally, it was airdried and then dissolved in 0.1 TE buffer (10mM Trsi-HCL, 0.1mM EDTA, pH 8.0) and incubated at -20^{0}C for 1 hour to completely dissolve the bisulfite-converted DNA. The bisulfite converted DNA was again quantified using nanodrop and 30 ng of it was used for methylation-specific PCR (MS-PCR).

5. Methylation-specific PCR for *CALCA* gene: MS-PCR was performed on thermal cycler gradient (Takara) using the standard protocol. MS-PCR is a sensitive technique that utilizes primers for *CALCA* gene methylation as well as unmethylation which anneals with the corresponding complementary sequences present in the bisulfite-treated DNA. MSP can distinguish methylated from unmethylated DNA after bisulfite modifications taking advantage of the changes in the sequences due to treatment with bisulfite as originally reported by Herman et al (23). The primers that amplify unmethylated (F-5'-GTTTTGGAAGTATGAGGGTGACG-3' & R-5'- TTCCCGCCGCTATAAATCG-3'- 105bp amplicon size) and methylated (F-5'-TTTTAGGTTTTGGAAGTATGAGGGTGATG-3' & R-5'-TTCCCACCACTATAAATCA-3'- 100bp amplicon size) sequences of *CALCA* promoter were synthesized by GeNoRime, based on Paixao et al (24). MS-PCR was carried out using an optimized concentration of the reagents at a final concentration of 1X-EpiMark Hot start Taq reaction buffer, 1U EpiMark Hot start Taq DNA polymerase, 0.2μM of each forward and reverse primer sets, PCR enhancer, 0.2mM dNTPs mix in the final volume of 20 μl. About 1 μl (30 ng) of bisulfite-converted DNA was included in each of the reactions. PCR reaction was carried out under the following condition that included, initial denaturation 95^{0}C for 5 minutes, 3 steps cycling for 30 cycles included denaturation 95^{0}C for 45 secs, annealing 57^{0}C for 45 secs and extension 72^{0}C for 45 secs followed by a final extension at 72^{0}C for 5 minutes with a final hold at 4^{0}C. PCR amplified products were then subjected to electrophoresis using 2.5% agarose using an isolated power supply of 50V for 90 minutes. DNA 100bp marker was also included in the wells to estimate the sizes.

6. EpiTect HRM analysis: High-resolution melting (HRM) analysis was utilized to study the melting properties of both methylated as well as unmethylated products. Methylated samples after PCR were subjected to HRM to establish the melting pattern of control. EpiTech HRM kit (Qiagen) and the above-mentioned primers were used for HRM. About 20ng of bisulfite converted DNA was taken as a template in each PCR reaction. HRM was carried out in Rotor-Gene Q-5PLEX HRM–Qiagen under the following reaction conditions, initial denaturation: 95^0C for 5 minutes, 3 steps of preamplification phase for 35 cycles included denaturation: 95^0C for 10 secs, annealing: 56^0C for 30 secs, extension: 72^0C for 15secs followed by HRM analysis in which amplicons were melted from 60^0C to 90^0C in higher resolution mode with 0.1^0C increment in temperature.

Statistical analysis

Epi info version 7.2 was used for all statistical analyses. The difference between mean biochemical parameters between the two groups was assessed by inferential statistical tests like unpaired t-test. Descriptive statistical measures were used to represent data as percentages or mean and standard deviation. The differences were considered statistically significant at $P< 0.05$. Tables and figures are used to present the data wherever necessary.

Results:

A total of 115 neonates were included in the study out of which 63 were normal healthy controls and 52 were diagnosed to have sepsis by the neonatologist based on the clinical presentation. Out of the 52 neonates suspected to have sepsis only 7 had positive blood culture. The mean age of the controls was 5.0±8.1 days and the mean age of the cases was 12.0±11.0 days. Both the groups had more males, 41 in controls and 40 in cases. There was no significant difference between the birth weight and gestational age of the neonates in the two groups. Among the cases, 46 neonates improved whereas 3 neonates expired and 3 more were discharged against medical advice. The clinical characteristics of the study subjects are shown in **Table 1**.

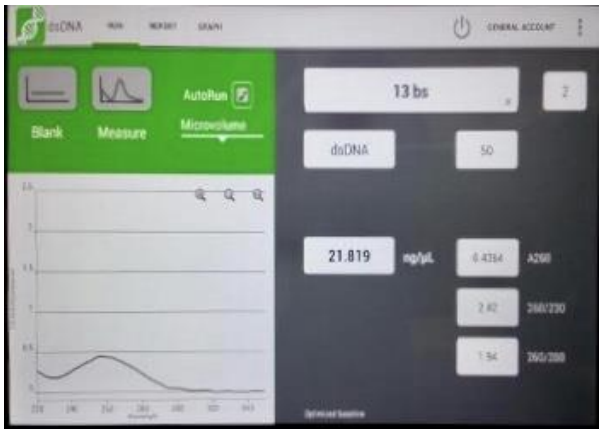
Table 1: The clinical characteristics of the study subjects

Study parameters	**Controls (n=63)**	**Cases (52)**	**P-value**
Age (days)	5.0±8.1	12.0±11.0	0.000
Sex (F/M)	22/41	12/40	
Birth weight (grams)	2332±733	2285±791	0.740
Gestation (weeks)	35.7±2.7	34.6±3.5	0.074
EOS/LOS	0	18/34	
Proven sepsis (Culture +/-)	0	7/45	
Outcome (Survived/expired)	62/1	46/3 (3 more were discharged against medical advice)	
DNA methylation *CALCA* gene (-/+)	62/1	47/5	

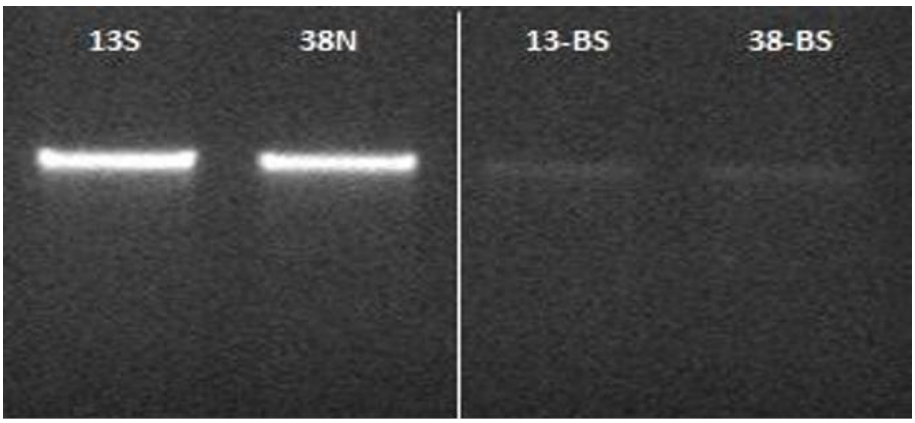
The genomic DNA extraction from neonatal whole blood adopting the non-enzymatic salting-out method revealed good purity DNA having an average purity ratio of 1.75 (A260/280 ratio) and 2.71 (A260/230) which is an ideal ratio for the bisulfite treatment (figure 1A). Bisulfite treatment although resulted in the loss of DNA the purity of DNA was not compromised and purity ratios were within the acceptable limit as shown in figure 1B. During bisulfite conversion of DNA, almost 70 to 80% of DNA was lost, apart from this no major changes were observed

in terms of purity as shown in figure 1B for a representative sample. The genomic DNA was then subjected to 1.5% agarose gel electrophoresis and the quality of DNA was found to be good with the intact band and no signs of DNA degradation were observed in the native form as shown in figure 1C.

A.



B.



C.

Figure 1: A) Genomic DNA-Quantification and purity assessment by nanodrop (Sample: 13). B) Bisulfite treated DNA-Quantification and purity assessment by nanodrop (Sample: 13BS). C) Left side: Genomic DNA bands (13S and 38N) Right side: Bisulfite treated DNA bands (13-BS and 38-BS)

The amplification product of MS-PCR for methylated and unmethylated DNA was resolved by using 2.5% agarose gel electrophoresis. Methylated DNA was separated and resolved at 100bp whereas unmethylated DNA at 105bp is shown in **figure 2** for a representative sample of 13bs**.**

DNA methylation of the *CALCA* gene was detected in one healthy control and 5 neonates in the sepsis group.

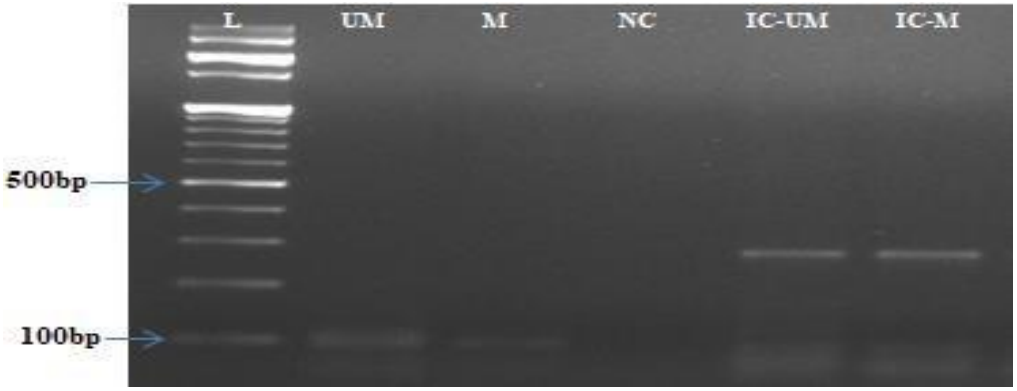


Figure 2: Methylation-specific PCR amplified bands for Unmethylated DNA (UM-105bp) and Methylated DNA (M-100bp) along with Negative Control (NC) and Internal controls (GAPDH-227bp). L – 100bp DNA marker. A representative image of samples (UM- 3BS and M-113BS)

EpiTect HRM was performed for positive samples using EpiTech HRM kit (Qiagen) which was carried out in Rotor-Gene Q-5PLEX HRM–Qiagen. Differences in the melting pattern were indicated between methylated and unmethylated DNA and are shown in figure 3.

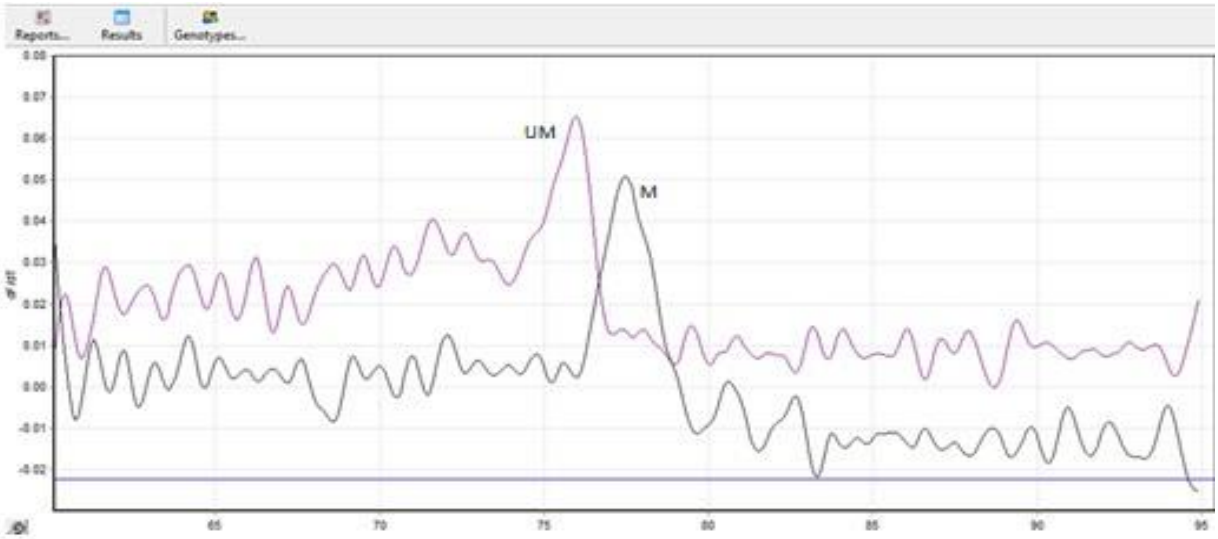


Figure 3: EpiTect HRM- Melting pattern of methylated (M) and unmethylated (UM) DNA- A representative image of samples (UM- 3BS and M-113BS).

Discussion:

DNA methylation is considered as a non-genetic change in which only the gene expression changes due to methylation without any changes in the sequence of the gene at a particular locus of the chromosome, usually CpG Island. *CALCA* promoter region is more prone for methylation in several disease conditions including neonatal sepsis but DNA methylation as a biomarker for neonatal sepsis has not been extensively studied. It is a specific feature of the *CALCA* gene and studies have shown that other genes activated during sepsis did not show any DNA methylation changes in their promoter region (26). Tendl et al. tried to evaluate the DNA methylation status of the *CALCA* gene in the preterm neonates and 4 novel changes were identified. 1. partial methylation at -769 CpG in 5 EOS, and 3 LOS cases; 2. demethylation of 8 CpGs in 1 EOS case; 3. demethylation of 7 CpGs in 2 EOS cases and; 4. -771 C:G>T:A; 5′ de novo -778 CpG mutation on both alleles in 1 EOS case. None of the controls showed any of the above-described methylation changes. In neonatal sepsis, immunosuppression is the major clinical manifestation due to incomplete maturation of immune cells especially among preterm infants which predisposes to several abnormalities that have been found extended to the level of gene expression. This study was taken up to find the occurrence of DNA methylation in the promoter region of the *CALCA* gene among neonatal sepsis in a tertiary care hospital of South India. The differences in genomic DNA methylation among cases and controls were observed and this change in genomic DNA methylation level could be due to microbial infection which is evident from the previous studies where the effect of Lipopolysaccharides (LPS) on DNA methylation has been described. (25) The pathophysiology of sepsis in an individual not only depends on the pathogen causing the infection but also on the genetic and epigenetic

background of the host. During sepsis, there is a lot of burden on the immune system which affects the functioning of the myeloid cells, and effects on epigenetic signatures in these cells are seen. This leads to the differentiation and maturation of the immune cells with key changes in their genes. The mortality in sepsis may be due to hyper-inflammation which leads to immune paralysis or immunosuppression. Several studies have shown epigenetics to be a driving force involved in short-term and long-term immunosuppression. (26) Hence, epigenetic therapy might control the immune response and produce important effects on the host immune system that may prove to be beneficial during the septic episode. Diet-induced epigenetic modifications have also been observed in sepsis cases due to poor nutritional support to the mother during her gestational period which could be a possible predisposing factor for early-onset sepsis, but further studies are needed to support nutrigenomics in correlation with DNA methylation. The study carried out on preterm infants having epigenetic changes in the *CALCA* gene promoter region has proposed the need to explore whether DNA methylation changes represent a cause or consequence of preterm birth which has been proved as one of the factors predisposing to sepsis among new-borns.

Limitation of the study is that we have not confirmed the presence or absence of the DNA methylation changes in the *CALCA* gene by sequencing. We have also not compared the DNA methylation status with the serum PCT levels or with the type of bacteria affecting the neonates.

Conclusion:

The study was carried out to identify the occurrence of methylation in the CpG dinucleotide promoter region of the *CALCA* gene using MS-PCR. The number of sepsis neonates reported with DNA methylation in the promoter region of the *CALCA* gene were more when compared to that of normal healthy controls. Hence, this may serve as a useful biomarker of sepsis in the future. Further, we would like to study the changes in the DNA methylation status at different

time points during the course of hospitalization to understand if it could prove to be useful in assessing the prognosis for better management of sepsis.

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