Single centre, prospective observational study to evaluate association between circulating citrullinated histone H3 and sepsis induced complications in paediatric patients with clinical sepsis.

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This is to certify that this dissertation entitled "**Single centre, prospective observational study to evaluate association between circulating citrullinated histone H3 and sepsis induced complications in paediatric patients with clinical sepsis.**" is the bonafide original work of **Dr. Akshata Pandiri** under the guidance of Dr. Sukesh Chandra Nair, Professor, Department of Transfusion Medicine and Immuno-haematology, Christian Medical College, Vellore, towards partial fulfillment of university regulations for the award of M.D. Transfusion Medicine and Immuno-Haematology Degree examination of The Tamil Nadu Dr. M.G.R. Medical University, Chennai to be held in May, 2022.

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ABBREVIATIONS

SIRS	systemic inflammatory response syndrome
PaO2	Partial pressure of oxygen
FiO2	Fraction of inspired O2
PaCO2	Partial pressure of carbon-dioxide
PAMP	Pathogen associated molecular pattern
DIC	Disseminated intravascular coagulation
PAI-1	plasminogen activator inhibitor type 1
LPS	Lipopolysaccharide
FDPs	Fibrin degradation products
ISTH	International society of thrombosis and Hemostasis
DNA	Deoxyribonucleic acid
HMGB1	High mobility group box 1
NETs	Neutrophil extracellular Traps
PT	Prothrombin time
APTT	Activated partial thromboplastin time
INR	International normalized ratio
ALT	Alanine transaminase
TLR9	Toll-like receptor 9
cfDNA	Cell free DNA
TFPI	Tissue factor pathway inhibitor
CitH3	Citrullinated histone h3

PAD2/PAD4	peptidylarginine deiminases 2 and 4
PCT	Procalcitonin
TF	Tissue factor
TM	Thrombomoduin
TGA	Thrombin Generation assay
EGF	Epidermal growth factor
MMP	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid

ABSTRACT

INTRODUCTION: It has been postulated that sepsis triggers citrullination of histone H3(CitH3) and leads to Neutrophil extracellular trap formation and release of CitH3 into circulation. This can induce Disseminated intravascular coagulation (DIC), subsequent organ failure and death. Many studies in adult population and murine studies have substantiated and proposed, CitH3 as a diagnostic and prognostic marker in sepsis.

AIMS

To evaluate whether CitH3 can be used as a prognostic indicator in paediatric patients with clinical sepsis

OBJECTIVES

1)To compare CitH3 levels between sepsis patients and non-sepsis controls

2) To evaluate whether CitH3 levels in clinical sepsis have an impact on survival in children with clinical sepsis

3) To compare the CitH3 levels in patients with and without disseminated intravascular coagulation (DIC), number of organ failures.

METHODS: Paediatric patients with clinical sepsis were recruited along with pre-operative children as controls. Clinical and lab data were collected and analysed. Complications associated with sepsis if any, were identified. Using a sandwich ELISA technique, levels of CitH3 in plasma of cases and controls were measured. The relationship between CitH3 levels and mortality, DIC, organ failure was analysed.

RESULTS: CitH3 levels [Median(minimum-maximum)] were significantly higher in 90 children with clinical sepsis [1.275ng/ml (0.01-23.10)] when compared to 38 controls [0.35ng/ml (0.15-0.94); p<0.0002)]. The area under curve (95% CI) for distinguishing sepsis from non-sepsis patients using CitH3 was 0.70 (0.61-0.80). The median Cit H3 values with Scrub typhus infection (2.480ng/ml (0.53-8.62)) and Paediatric Inflammatory Multisystem Syndrome-Temporally associated with SARS-CoV-2 (1.36ng/ml (0.16-19.6)) were significantly higher than those with controls (0.35ng/ml (0.15-0.94) and other gram-negative infections (0.24ng/ml (0.14-2.50) (p 0.0042). There was no significant difference between circulating histone H3 levels in survivors and non-survivors, patients with and without DIC or number of organ failures (p>0.05). In the logistic regression analysis, fibrinogen <242 mg/dL [odds ratio (OR) 12.388; 95% confidence interval (CI) 1.335-114.980; p = 0.027] and INR >1.43 (OR 15.740; 95% CI 1.236–200.426; p= 0.034), within the first 24 h of admission were independently associated with mortality of sepsis. However, other variables including total count, lactate, APTT and DIC score were not significant predictors of sepsis mortality.

CONCLUSION:

1. Citrullinated histone H3 levels measured at admission within 24 hours were higher in children with clinical sepsis than controls.
2. Paediatric inflammatory multisystem syndrome temporally associated with severe acute respiratory syndrome coronavirus 2(SARS-CoV-2)-PIMS-TS currently known as Multisystem inflammatory syndrome of children (MIS-C) is associated with raised circulating citrullinated Histone H3 levels when compared

to controls. This suggests a role of NETosis in the pathophysiology of this novel entity.

3. Citrullinated histone H3 levels were highest in scrub typhus, followed by PIMS-TS. The levels in scrub typhus were significantly higher than controls and other gram-negative infections.
4. Circulating histone levels were not associated with coagulopathy, Multi-Organ Dysfunction and do not predict mortality therefore cannot be used as a prognostication marker in paediatric sepsis patients.
5. Baseline raised INR > 1.43 and low fibrinogen levels < 242mg/dl in paediatric clinical sepsis patients were independent predictors of mortality in a multivariate analysis.

INTRODUCTION

Despite the availability of adequate treatment, sepsis is one of the primary causes of death. A biomarker is a measurable trait that can be used to assess normal biological processes, pathological processes, and/or pharmacological reactions to a therapeutic intervention. The use of biomarkers has been implicated in the diagnosis and especially in the prognostication of sepsis as they can provide an insight of the pathophysiology of sepsis, encouraging new diagnostics, and improved therapeutics as reviewed in the surviving sepsis campaign guidelines.(1) Therefore, biomarkers may aid in the classification of individuals who may benefit from specific medicines as well as the evaluation of treatment response. Yet, current sepsis biomarkers are hampered by their non-specificity, short half-life, and insensitivity to treatment response.(2)

Neutrophil extracellular traps (NETs) are web like structures which contain histones, DNA fibres and other microbicidal proteins present in the neutrophils. The process of formation of NETs is known as NETosis. This concept which has recently emerged is considered a host defence mechanism. Microbial infection is suspected to promote citrullination of histone H3 (CitH3), which is catalysed by peptidyl-arginine deiminase (PAD) 2 and 4. This citrullination promotes nuclear de-lobulation, disassembly of nuclear envelope and chromatin de-condensation and subsequently NETs formation. Then the plasma membrane ruptures leading to traps release along with CitH3 into circulation, culminating in coagulopathy, organ failure, and death. (3) Circulating histones are thought to play a role in each stage of sepsis, according to the literature. As a result, it's plausible that histones have a lot of potential as diagnostic or prognostic biomarkers for sepsis, and also therapeutic targets.(4)

Murine studies and prior studies on adults have supported the use of CitH3 as a sepsis marker because of its early appearance. It was more specific for sepsis than the other existent biomarkers like procalcitonin, sustained presence in the circulation, and sensitive response to therapeutic intervention.(2)(4). Studies have also shown that circulating CitH3 were also able to predict clinical outcome in sepsis such as DIC, organ failure and survival and advocated its use as a prognostic indicator and suggested that it act as an independent risk factor for predicting mortality(5), (3), (2), (4), (6).

The majority of the studies and material accessible concern adult patients that have shown association of citrullinated H3 concentration with the severity of sepsis. There are currently two studies in children which have achieved divergent results, one study was conducted in paediatric meningococcal sepsis that has generated negative results as opposed to the other study involving sepsis prognosis and severity, which has recommended CitH3 as a potential predictor.

Due to promising results in studies on adults and contrasting findings in children we sought to test the hypothesis whether this biomarker could be used as a prognostic marker in children with clinical sepsis and whether it can pose as a risk factor for non-survival in paediatric patients admitted to Paediatric Intensive care unit (PICU) with sepsis. Also, there is also a scarcity of literature, particularly in the Indian context which is why we decided to perform this study.

AIMS

To evaluate whether CitH3 can be used as a prognostic indicator in paediatric patients with clinical sepsis

OBJECTIVES

1)To compare CitH3 levels between sepsis patients and non-sepsis controls

2) To evaluate whether CitH3 levels in clinical sepsis have an impact on survival in children with clinical sepsis

3) To compare the CitH3 levels in patients with and without disseminated intravascular coagulation (DIC), number of organ failures.

LITERATURE REVIEW

The systemic inflammatory response syndrome (SIRS), immunological dysregulation, microcirculatory derangements, and end-organ failure describe sepsis, a clinical state that accompanies a severe infection. When inflammation is dysregulated, as it is in sepsis, it causes a rise in pro-inflammatory and anti-inflammatory mediators, which sets off a cascade of events that results in widespread tissue harm. (7)

DEFINITIONS

Infection- Suspected infection caused by any pathogen that has been confirmed by a positive culture, tissue stain, or polymerase chain reaction test, as well as clinical syndromes associated with a high probability of infection, such as petechiae and purpura in a child with hemodynamic instability, or fever, cough, and hypoxemia in a patient with leukocytosis and pulmonary infiltrates on chest xray.(8)

Systemic inflammatory response syndrome (SIRS): It's a widespread inflammatory response that could or could not be linked to infection. SIRS is defined by the presence of two or more of the following characteristics (at least one of which must be an abnormal temperature or a low leukocyte count) as depicted in Table 1.(8)

- >38.5°C or 36°C core temperature (measured by rectal, bladder, mouth, or central probe)
- For children younger than one year of age, tachycardia is defined as a mean heart rate that is more than two standard deviations above normal for their age, while bradycardia is defined as a mean heart rate that is less than the tenth percentile for their age.

- Mechanical ventilation for an acute pulmonary process or a mean respiratory rate more than two standard deviations above normal for age.
- >10% immature neutrophils or a leukocyte count that is high or low for age

Table 1: Age based vital signs and laboratory values

Pediatric systemic inflammatory response syndrome vital signs and laboratory values by age

Age group	Heart rate (beats/minute)		Respiratory rate (breaths/minute)	Leukocyte count (leukocytes x $10^3/mm^3$)	Systolic blood pressure (mmHg)
	Tachycardia	Bradycardia			
Newborn (0 days to 1 week)	>180	<100	>50	>34	<59
Neonate (1 week to 1 month)	>180	<100	>40	>19.5 or <5	<79
Infant (1 month to 1 year)	>180	<90	>34	>17.5 or <5	<75
Toddler and preschool (>1 to 5 years)	>140	NA	>22	>15.5 or <6	<74
School age (>5 to 12 years)	>130	NA	>18	>13.5 or <4.5	<83
Adolescent (>12 to <18 years)	>110	NA	>14	>11 or <4.5	<90

This table provides the vital sign and laboratory value modifications for the pediatric definition of the systemic inflammatory response syndrome. For the full definition, refer to UpToDate topics on the systemic inflammatory response syndrome (SIRS) and sepsis in children.

NA: not applicable.

Originally published in: Goldstein B, Giroir B, Randolph A, et al. International pediatric sepsis consensus conference: Definitions for sepsis and organ dysfunction in pediatrics. Pediatr Crit Care Med 2005; 6:2. Correction published in: Gebara BM. Values for systolic blood pressure. Pediatr Crit Care Med 2005; 6:500. Copyright © 2005 Lippincott Williams & Wilkins.

Sepsis — Sepsis is defined as a systemic inflammatory response syndrome that occurs in the presence of a suspected or confirmed infection.

Severe sepsis – When sepsis is linked to cardiovascular disease, acute respiratory distress syndrome (ARDS), or malfunction in two or more additional organ systems, as indicated in the section on multiple organ failure below, it is called severe.

Septic shock – Septic shock is defined as sepsis with persistent circulatory failure despite receiving 40 mL/kg of isotonic fluid in one hour.

Refractory septic shock – Fluid-refractory septic shock occurs when circulatory dysfunction persists despite fluid resuscitation of at least 60 mL/kg; catecholamine-resistant septic shock occurs when shock persists despite dopamine therapy of at least 10 mcg/kg per minute and/or direct-acting catecholamines (epinephrine, norepinephrine)

Multiple organ failure The International Consensus on Paediatric Sepsis developed criteria for organ dysfunction based upon several scoring systems taking into account a balance of specificity, sensitivity, and widespread availability of laboratory tests.

•**Cardiovascular** – Hypotension, or reliance on a vasoactive drug to maintain blood pressure, or two of the following: metabolic acidosis, elevated arterial lactate, oliguria, or prolonged capillary refill.

•**Respiratory** – Arterial oxygen tension/fraction of inspired oxygen (PaO_2/FiO_2) <300, arterial carbon dioxide tension ($PaCO_2$) >65 torr or 20 mmHg over baseline $PaCO_2$, need for >50 percent FiO_2 to maintain oxygen saturation ≥92 percent, or need for nonelective mechanical ventilation.

•**Neurologic** – Glasgow coma score ≤11, or acute change in mental status.

•**Hematologic** – Platelet count <80,000/microlitre or a decline of 50 percent from highest value recorded over the past three days or disseminated intravascular coagulation (DIC), a consumptive coagulopathy diagnosed by clinical findings of haemorrhage and microthrombi and laboratory abnormalities including thrombocytopenia, prolongation of clotting times (PT and aPTT), and evidence of

fibrinolysis (low fibrinogen with elevated fibrin degradation products), which is a common hematologic manifestation in sepsis.

•**Renal** – Serum creatinine ≥2 times upper limit of normal for age or twofold increase in baseline creatinine.

•**Hepatic** – Total bilirubin ≥4 mg/dL (not applicable to newborn) or alanine aminotransferase (ALT) >2 times upper limit of normal for age.

Pneumonia, bloodstream, skin, or urinary tract infections, and, less commonly, meningitis comprise the most common infections in children with sepsis.

The following Fig 1. Depicts the pathophysiology of sepsis

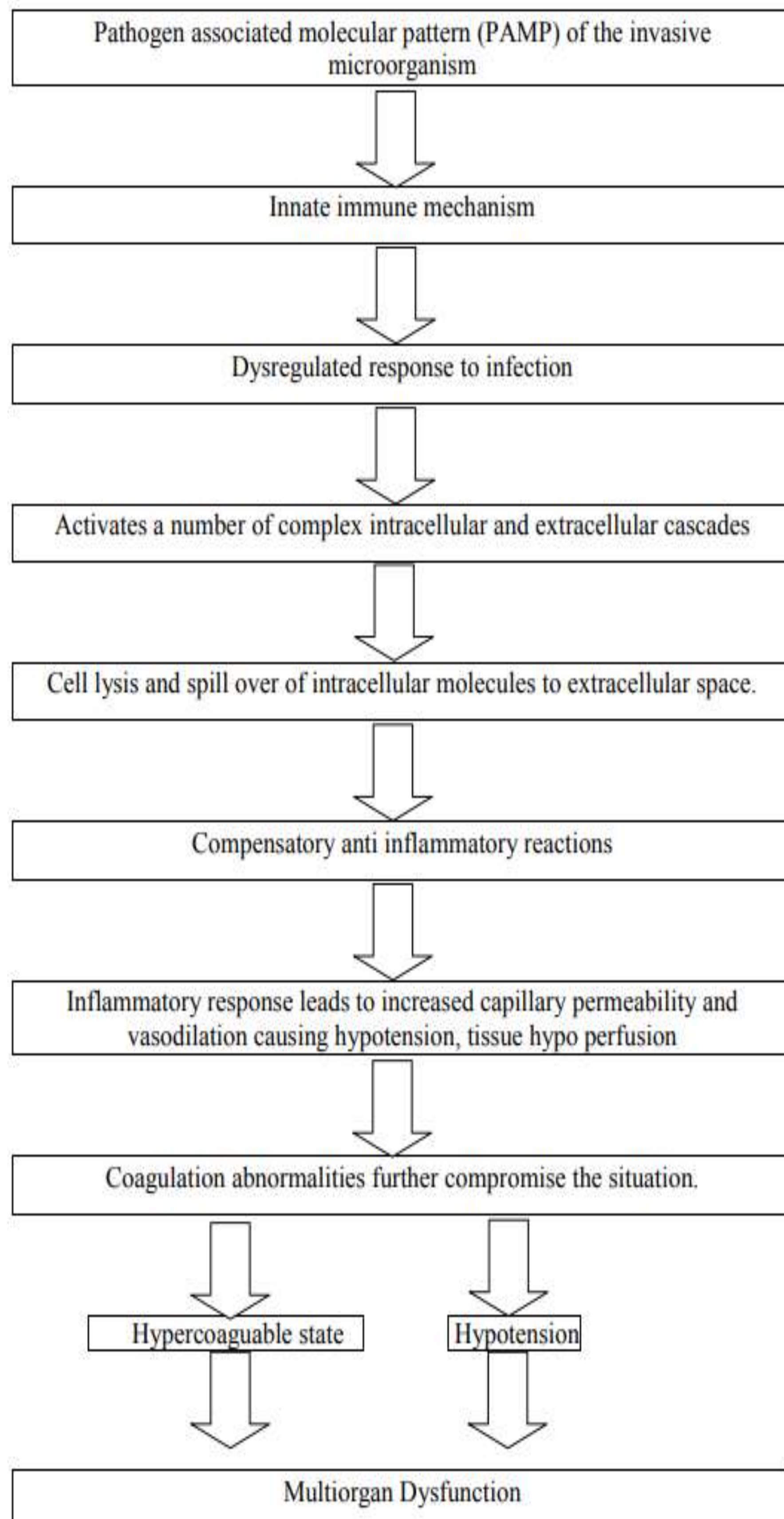

Figure 1: Pathophysiology of sepsis

DISSEMINATED INTRAVASCULAR COAGULATION (DIC)

Haemorrhage and microvascular thrombosis are two symptoms of disseminated intravascular coagulation (DIC), an acquired condition. Endothelial tissue damage caused by a number of underlying illnesses (e.g., sepsis, trauma, and cancer) initiates the coagulation cascade, which enhances fibrin synthesis and deposition as well as clotting factor consumption. The consumption of coagulation factors and platelets, suppression of natural anticoagulants and fibrinolysis, and fibrin deposition result in the clinical picture of DIC. The pathophysiology of DIC is detailed in Fig. 2.(9)

The activation of the intravascular coagulation system initiates the following processes:

- Blood exposure to procoagulants - Procoagulants are released into the bloodstream as a result of tissue damage caused by the beginning primary illness.
- Fibrin formation in the circulation - Tissue procoagulants increase fibrin formation and deposition in the microcirculation by interacting with tissue factor and Factor VII.
- Fibrinolysis - The development of fibrin initiates the fibrinolysis pathway, which results in the production of plasmin, which cleaves fibrinogen and fibrin to yield fibrin breakdown products (FDPs). FDPs prevent platelet aggregation by interfering with fibrin polymerization.
- Clotting factors and platelets are depleted due to ongoing coagulation system activation and fibrin deposition.

- End-organ injury occurs when fibrin is deposited in the microcirculation of the organs, causing tissue ischemia and damage.
- Haemolysis - Microangiopathic haemolytic anaemia is caused by intravascular fibrin strands mechanically shearing red blood cells.

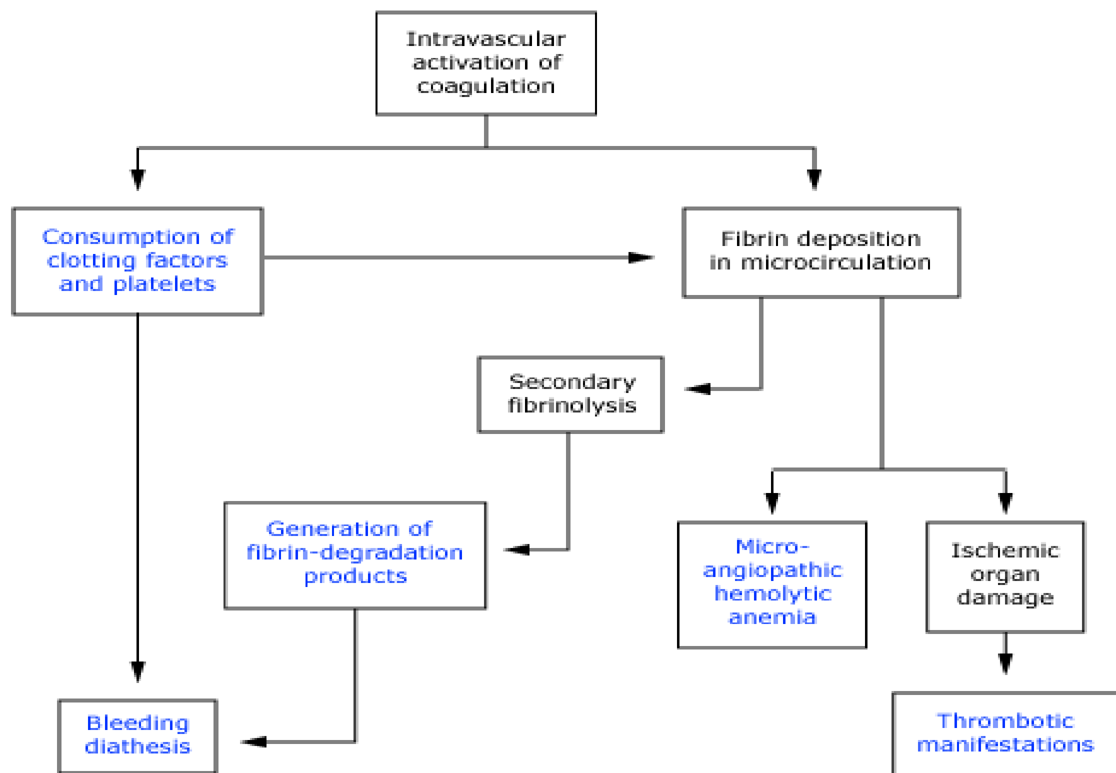

Figure 1: PATHOPHYSIOLOGY Of DIC

Pathophysiology of the clinical manifestations of disseminated intravascular coagulation- Adapted from uptodate

DIAGNOSIS OF DIC

The presence of unexplained, broad oozing or bleeding, as well as unexplained, aberrant haemostasis test results, is usually enough to raise suspicion of underlying DIC.

DIC cannot be diagnosed or ruled out by a single laboratory test.

A diagnosis of DIC should be determined based on clinical suspicion and laboratory investigations.

The laboratory tests represent a snapshot of this dynamic state; the underlying clinical condition can have an impact on the laboratory tests. DIC is a highly dynamic situation, and the laboratory tests are a snapshot of that dynamic state.

The following are the most important laboratory parameters for DIC diagnosis:; (10)

- Platelet count: <80-100,000 or a decrease of >50% from baseline
- Fibrinogen: <100mg/dl or a decrease of >50% from baseline
- Prothrombin time: >3sec prolongation more than upper limit of normal
- Fibrin degradation products: >80mg/dl
- D-dimer: moderate increase

The reference values for coagulation tests in paediatric population are depicted in Table 2 and Table 3.

Table 2: Reference values for coagulation tests in healthy premature infants (30 to 36 weeks gestation) during first 6 months of life

Reference values for coagulation tests in healthy premature infants (30 to 36 weeks gestation) during first 6 months of life

Tests	Day 1		Day 5		Day 30		Day 90		Day 180		Adult	
	M	B	M	B	M	B	M	B	M	B	M	B
PT (s)	13	(10.6-16.2)*	12.5	(10-15.3)*¶	11.8	(10-13.6)*	12.3	(10-14.6)*	12.5	(10-15)*	12.4	(10.8-13.9)
aPTT (s)	53.6	(27.5-79.4)Δ	50.5	(26.9-74.1)Δ	44.7	(26.9-62.5)	39.5	(28.3-50.7)	37.5	(21.7-53.3)*	33.5	(26.6-40.3)
TCT (s)	24.8	(19.2-30.4)*	24.1	(18.8-29.4)*	24.4	(18.8-29.9)*	25.1	(19.4-30.8)*	25.2	(18.9-31.5)*	25	(19.7-30.3)
Fibrinogen (g/L)	2.43	(1.5-3.73)*¶Δ	2.80	(1.6-4.18)*¶Δ	2.54	(1.5-4.14)*¶	2.46	(1.5-3.52)*¶	2.28	(1.5-3.6)	2.78	(1.56-4)
II (units/mL)	0.45	(0.2-0.77)¶	0.57	(0.29-0.85)Δ	0.57	(0.36-0.95)¶Δ	0.68	(0.3-1.06)	0.87	(0.51-1.23)	1.08	(0.7-1.46)
V (units/mL)	0.88	(0.41-1.44)*¶Δ	1	(0.46-1.54)	1.02	(0.48-1.56)	0.99	(0.59-1.39)	1.02	(0.58-1.46)	1.06	(0.62-1.5)
VII (units/mL)	0.67	(0.21-1.13)	0.84	(0.3-1.38)	0.83	(0.21-1.45)	0.87	(0.31-1.43)	0.99	(0.47-1.51)*	1.05	(0.67-1.43)
VIII (units/mL)	1.11	(0.5-2.13)*¶	1.15	(0.53-2.05)*¶Δ	1.11	(0.50-1.99)*¶Δ	1.06	(0.58-1.88)*¶Δ	0.99	(0.5-1.87)*¶Δ	0.99	(0.5-1.49)
VWF (units/mL)	1.36	(0.78-2.1)¶	1.33	(0.72-2.19)¶	1.36	(0.66-2.16)¶	1.12	(0.75-1.84)*	0.98	(0.54-1.58)*¶	0.92	(0.5-1.58)
IX (units/mL)	0.35	(0.19-0.65)¶Δ	0.42	(0.14-0.74)¶Δ	0.44	(0.13-0.80)¶	0.59	(0.25-0.93)	0.81	(0.5-1.2)	1.09	(0.55-1.63)
X (units/mL)	0.41	(0.11-0.71)	0.51	(0.19-0.83)	0.56	(0.2-0.92)	0.67	(0.35-0.99)	0.77	(0.35-1.19)	1.06	(0.7-1.52)
XI (units/mL)	0.3	(0.08-0.52)¶Δ	0.41	(0.13-0.69)Δ	0.43	(0.15-0.71)Δ	0.59	(0.25-0.93)Δ	0.78	(0.46-1.1)	0.97	(0.67-1.27)
XII (units/mL)	0.38	(0.1-0.66)Δ	0.39	(0.09-0.69)Δ	0.43	(0.11-0.75)	0.61	(0.15-1.07)	0.82	(0.22-1.42)	1.08	(0.52-1.64)
PK (units/mL)	0.33	(0.09-0.57)	0.45	(0.26-0.75)¶	0.59	(0.31-0.87)	0.79	(0.37-1.21)	0.78	(0.4-1.16)	1.12	(0.62-1.62)
HMWK (units/mL)	0.49	(0.09-0.89)	0.62	(0.24-1)Δ	0.64	(0.16-1.12)Δ	0.78	(0.32-1.24)	0.83	(0.41-1.25)*	0.92	(0.5-1.36)
XIIIa (units/mL)	0.7	(0.32-1.08)	1.01	(0.57-1.45)*	0.99	(0.51-1.47)*	1.13	(0.71-1.55)*	1.13	(0.65-1.61)*	1.05	(0.55-1.55)
XIIIb (units/mL)	0.81	(0.35-1.27)	1.1	(0.68-1.58)*	1.07	(0.57-1.57)*	1.21	(0.75-1.67)	1.15	(0.67-1.63)	0.97	(0.57-1.37)
Plasminogen (CTA, units/mL)	1.7	(1.12-2.48)¶Δ	1.91	(1.21-2.61)Δ	1.81	(1.09-2.53)	2.38	(1.58-3.18)	2.75	(1.91-3.59)Δ	3.36	(2.48-4.24)

All factors except fibrinogen and plasminogen are expressed as units/mL, where pooled plasma contains 1.0 units/mL. Plasminogen units are those recommended by the CTA. All values are given as a mean (**M**), followed by lower and upper boundary encompassing 95% of the population (**B**). Between 40 and 96 samples were assayed for each value for newborns.

PT: prothrombin time; aPTT: activated partial thromboplastin time; TCT: thrombin clotting time; VWF: von Willebrand factor; PK: prekallikrein; HMWK: high molecular weight kininogen; CTA: Committee on Thrombolytic Agents.
* Values indistinguishable from those of adults.
¶ Measurements are skewed, owing to a disproportionate number of high values. Lower limit that excludes the lower 2.5% of the population is given (**B**).
Δ Values different from those of full-term infants.

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Table 3: Reference values for coagulation tests in healthy full-term infants during first 6 months of life

Reference values for coagulation tests in the healthy full-term infant during the first 6 months of life

Tests	Day 1 (n)	Day 5 (n)	Day 30 (n)	Day 90 (n)	Day 180 (n)	Adult (n)
PT (s)	13.0±1.43 (61)*	12.4±1.46 (77)*¶	11.8±1.25 (67)*¶	11.9±1.15 (62)*	12.3±0.79 (47)*	12.4±0.78 (29)
aPTT (s)	42.9±5.80 (61)	42.6±8.62 (76)	40.4±7.42 (67)	37.1±6.52 (62)*	35.5±3.71 (47)*	33.5±3.44 (29)
TCT (s)	23.5±2.38 (58)*	23.1±3.07 (64)¶	24.3±2.44 (53)*	25.1±2.32 (52)*	25.5±2.86 (41)*	25.0±2.66 (19)
Fibrinogen (g/L)	2.83±0.58 (61)*	3.12±0.75 (77)*	2.70±0.54 (67)*	2.43±0.68 (60)*¶	2.51±0.68 (47)*¶	2.78±0.61 (29)
II (units/mL)	0.48±0.11 (61)	0.63±0.15 (76)	0.68±0.17 (67)	0.75±0.15 (62)	0.88±0.14 (47)	1.08±0.19 (29)
V (units/mL)	0.72±0.18 (61)	0.95±0.25 (76)	0.98±0.18 (67)	0.90±0.21 (62)	0.91±0.18 (47)	1.06±0.22 (29)
VII (units/mL)	0.66±0.19 (60)	0.89±0.27 (75)	0.90±0.24 (67)	0.91±0.26 (62)	0.87±0.20 (47)	1.05±0.19 (29)
VIII (units/mL)	1.00±0.39 (60)*¶	0.88±0.33 (75)*¶	0.91±0.33 (67)*¶	0.79±0.23 (62)*¶	0.73±0.18 (47)¶	0.99±0.25 (29)
VWF (units/mL)	1.53±0.67 (40)¶	1.40±0.57 (3)	1.28±0.59 (40)¶	1.18±0.44 (40)¶	1.07±0.45 (46)¶	0.92±0.33 (29)¶
IX (units/mL)	0.53±0.19 (59)	0.53±0.19 (75)	0.51±0.15 (67)	0.67±0.23 (62)	0.86±0.25 (47)	1.09±0.27 (29)
X (units/mL)	0.40±0.14 (60)	0.49±0.15 (76)	0.59±0.14 (67)	0.71±0.18 (62)	0.78±0.20 (47)	1.06±0.23 (29)
XI (units/mL)	0.38±0.14 (60)	0.55±0.16 (74)	0.53±0.13 (67)	0.69±0.14 (62)	0.86±0.24 (47)	0.97±0.15 (29)
XII (units/mL)	0.53±0.20 (60)	0.47±0.18 (75)	0.49±0.16 (67)	0.67±0.21 (62)	0.77±0.19 (47)	1.08±0.28 (29)
PK (units/mL)	0.37±0.16 (45)¶	0.48±0.14 (51)	0.57±0.17 (48)	0.73±0.16 (46)	0.86±0.15 (43)	1.12±0.25 (29)
HMWK (units/mL)	0.54±0.24 (47)	0.74±0.28 (63)	0.77±0.22 (50)*	0.82±0.32 (46)*	0.82±0.23 (48)*	0.92±0.22 (29)
XIIIa (units/mL)	0.79±0.26 (44)	0.94±0.25 (49)*	0.93±0.27 (44)*	1.04±0.34 (44)*	1.04±0.29 (41)*	1.05±0.25 (29)
XIIIb (units/mL)	0.76±0.23 (44)	1.06±0.37 (47)*	1.11±0.36 (45)*	1.16±0.34 (44)*	1.10±0.30 (41)*	0.97±0.20 (29)
Plasminogen (CTA, units/mL)	1.95±0.35 (44)	2.17±0.38 (60)	1.98±0.36 (52)	2.48±0.37 (44)	3.01±0.40 (47)	3.36±0.44 (29)

All factors except fibrinogen and plasminogen are expressed as units per milliliter, where pooled plasma contains 1.0 units/mL. Plasminogen units are those recommended by the CTA. AU values are expressed as mean±1 SD.

PT: prothrombin time; aPPT: activated partial thromboplastin time; TCT: thrombin clotting time; VWF: von Willebrand factor; PK: prekallikrein; HMWK: high molecular weight kininogen; CTA: Committee on Thrombolytic Agents.

* Values that do not differ statistically from the adult values.

¶ These measurements are skewed because of a disproportionate number of high values. The lower limit that excludes the lower 2.5th percentile of the population has been given in the respective figure. The lower limit for factor VIII was 0.50 units/mL at all time points for the infant.

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Thrombocytopenia is sufficient to suspect DIC in the proper clinical situation. However, diseases with comparable laboratory results should be ruled out, such as severe hepatic insufficiency (with splenomegaly and splenic sequestration of platelets), heavy transfusion, primary fibrinolysis, TTP/HUS, heparin treatment, and dysfibrinogenemia. In most circumstances, a combination of tests can be utilised to identify DIC with fair certainty in a patient with a clinical condition known to be linked with it. To diagnose DIC, scoring systems have been devised that take this concept into account as detailed in Fig 3. (11)

	ISTH criteria	JMHW criteria	JAAM criteria
Underlying clinical condition predisposing to DIC	Essential	1 point	Essential
Clinical symptoms	Not used	Bleeding = 1 point; organ failure = 1 point	SIRS score ≥ 3 = 1 point
Platelet count ($\times 10^9$/L)	50-100 = 1 point < 50 = 2 points	80-120 = 1 point 50-80 = 2 points < 50 = 3 points	80-120 or > 30% reduction = 1 point < 80 or > 50% reduction = 2 points
Fibrin-related marker	Moderate increase = 2 points Marked increase = 3 points	FDP 10-20 μg/mL = 1 point FDP 20-40 μg/mL = 2 points FDP > 40 μg/mL = 3 points	FDP 10-25 μg/mL = 1 point FDP > 25 μg/mL = 3 points
Fibrinogen	< 1 = 1 point	1-1.5 = 1 point < 1 = 2 points	Not used
PT	Prolongation 3-6 sec = 1 point Prolongation > 6 sec = 2 points	PT ratio 1.25-1.67 = 1 point PT ratio > 1.67 = 2 points	PT ratio ≥ 1.2 = 1 point
DIC diagnosis	≥ 5 points	≥ 7 points	≥ 4 points

Figure 2: Diagnostic Criteria for DIC

ISTH- International Society on Thrombosis and Haemostasis JMHW- Japanese Ministry of Health and Welfare JAAM- Japanese Association for Acute Medicine

Treatment

Consumptive coagulopathy produces seeping at vascular access points and wounds in fulminant DIC, but it can also induce profuse haemorrhage in rare cases. The cornerstone of managing this illness is to take care of the underlying problem (e.g., sepsis). When there is no indication of bleeding and only minor coagulation abnormalities, additional care may not be necessary. In patients who are bleeding or are at high risk of bleeding (e.g., postoperative patients or patients undergoing an invasive operation), care guidelines recommend replacing coagulation proteins and platelets.); (12)

- Platelets: Transfusion is required to maintain a platelet count of more than 50,000/cumm.

- Source of fibrinogen (Cryoprecipitate): to maintain a fibrinogen level of >150mg/dl
- Packed red cells: to target optimum haemoglobin level
- Fresh-frozen plasma: to maintain a PT and aPTT of less than 1.5 times the standard control time

TRANSFUSION SUPPORT

The goal of blood component therapy is to achieve clinical stability rather than normal values. Different guideline in transfusion are detailed in Table 4. The use of antifibrinolytic agents in the treatment of DIC is contraindicated because the fibrinolytic system is essential to ensure the dissolution of the widespread fibrin and the recovery of DIC. Although some guidelines support therapeutic doses of

unfractionated heparin in individuals with a thrombotic phenotype (such as gangrene), clinical evidence is insufficient to make a firm recommendation. Clinical trials involving the use of natural thrombosis and fibrinolysis modulators have not consistently showed a benefit in patients with sepsis, with the exception of meningococcal purpura fulminans. The major goals of pharmacologic therapy for DIC are to stop ongoing coagulation so that coagulation factor replacement can commence, and to prevent damage. Recombinant activated factor VII (rFVIIa), a recombinant haemostatic factor, has been used to treat severe, life-threatening bleeding in DIC patients who have failed to respond to conventional treatments, as well as trauma and other medical and surgical causes of severe, life-threatening bleeding. There is no evidence that rFVIIa infusions improve outcomes as compared to usual treatment with FFP and/or plasma concentrates. (13)

Table 4: Transfusion support -Summary of Various guidelines

	BCSH	SISET	JSTH	ISTH/SSC
Transfusion of platelet concentrate	Should be considered if patient is bleeding or has a perceived high risk of bleeding and a platelet count < 50× 10^9/L, (Grade C, Level IV)prophylactic platelet transfusions not given innon-bleeding patients unless there is a high risk of bleeding (for example, platelets counts < 10-20 × 10^9/L)	Suggested for patients with active bleeding (Grade D) may be considered if patient is bleeding or has a perceived high risk of bleeding plus platelet count < 50× 10^9/L	Recommended for patients with severe bleeding or undergoing operation especially in those with platelet count < 50 × 10^9/L (JSTH Consensus)	Recommended in patients with active bleeding plus platelet count < 50× 10^9/L, or high risk of bleeding plus platelets < 20× 10^9/L(Low quality)

	BCSH	SISET	JSTH	ISTH/SSC
Fibrinogen, cryoprecipitate	May be considered for patients with severe hypofibrinogenemia (< 1 g/L) that persists despite FFP replacement (BCSH Grade C, Level IV)	Suggested for patients with active bleeding (SISET Grade D); may be considered for patients with severe hypofibrinogenemia (< 1 g/L) that persists despite FFP replacement,	NM	Recommended for patients with active bleeding plus severe hypofibrinogenemia (< 1.5 g/L) that persists despite FFP replacement (ISTH/SSC Low quality)

BCSH- British Committee for the Standards in Haematology

SISET-Guidelines of the Italian Society for Haemostasis and Thrombosis

JSTH-Japanese Society on Thrombosis & Haemostasis

ISTH- International Society on Thrombosis and Haemostasis

Predicted Death rate calculation using Paediatric index of mortality (PIM) 2 score

The PIM2 scoring system uses the logistic regression equation to calculate the anticipated death rate using standard methods as shown below:

PIM2 = [0.01395*(PaO2-120)] + [3.0791*Pupil sign] + [0.2888*(FiO2 *100/PaO2)] + [0.104* base] + [1.3352* mechanical ventilation] – [0.9282* elective admission] – [1.0244* recovery] + [0.7507*cardiac bypass] – [1.6829* high risk diagnosis] – [1.5770* low risk diagnosis] – [4.8841].

Predicted death rate = exponential (PIM2)/1 + exponential (PIM2).

It indicates the severity of the child's illness at the time intensive care was initiated.

The PIM 2 score is made up of ten variables and these variables are mentioned Annexure 8. For yes or no responses, these factors were given a score of 1 or 0. For the anticipated mortality rate to be determined, these should be entered into the system (www.sfar.org/scores2/ pim22.html).

Coding rules. These rules must be followed carefully for PIM2 to perform reliably:

1. Record SBP as 0 if the patient is in cardiac arrest, record 30 if the patient is shocked and the blood pressure is so low that it cannot be measured.

2. Pupillary reactions to bright light are used as an index of brain function. Do not record an abnormal finding if this is due to drugs, toxins or local eye injury.

3. Mechanical ventilation includes mask or nasal CPAP or BiPAP or negative pressure ventilation.

4. Elective admission.Include admission after elective surgery or admission for an elective procedure (e.g. insertion of a central line), or elective monitoring, or review of home ventilation. An ICU admission or an operation is considered elective if it could be post poned for more than 6 h without adverse effect.

5. Recovery from surgery or procedure includes a radiology procedure or cardiac catheter. Do not include patients admitted from the operating theatre where recovery from surgery is not the main reason for ICU admission (e.g.a patient with a head injury

who is admitted from theatre after insertion of an ICP monitor; in this patient the main reason for ICU admission is the head injury).

6. Cardiac bypass. These patients must also be coded as recovery from surgery.

7. Cardiac arrest preceding ICU admission includes both in-hospital and out-of-hospital arrests. Requires either documented absent pulse or the requirement for external cardiac compression. Do not include past history of cardiac arrest.

8. Cerebral haemorrhage must be spontaneous (e.g. from aneurysm or AV malformation). Do not include traumatic cerebral haemorrhage or intracranial haemorrhage that is not intracerebral (e.g.subdural haemorrhage).

9. Hypoplastic left heart syndrome. Any age, but include only cases where a Norwood procedure or equivalent is or was required in the neonatal period to sustain life.

10. Liver failure acute or chronic must be the main reason for ICU admission. Include patients admitted for recovery following liver transplantation for acute or chronic liver failure.

11. Neuro-degenerative disorder. Requires a history of progressive loss of milestones or a diagnosis where this will inevitably occur.

12. Bronchiolitis. Include children who present either with respiratory distress or central apnoea where the clinical diagnosis is bronchiolitis.

13. Obstructive sleep apnoea. Include patients admitted following adenoidectomy and/or tonsillectomy in whom obstructive sleep apnoea is the main reason for ICU admission (and code as recovery from surgery). (9)

BIOMARKERS IN SEPSIS

Standard blood culture techniques take time, with results usually taking at least 24–48 hours to arrive, emphasising the importance of quick diagnosis.

In addition, the vital sign abnormalities seen in SIRS are not exclusive to infection and might be confused with noninfectious aetiologies that cause systemic inflammation. A biomarker is a measurable characteristic that can be used to evaluate normal biological processes, pathological processes, or pharmacological responses to a therapy intervention.

Sepsis biomarkers can help in quick diagnosis, risk stratification, and prognostication. (1) Nearly 180 biomarkers have been investigated, according to a recent analysis.(ref)

Potential uses of sepsis biomarkers

- Rule out sepsis
- Provide early intervention
- Guide antimicrobial therapy
- Assess response to therapy
- Distinguish between viral and bacterial infections
- Differentiate sepsis from other non-infectious causes of systemic inflammatory response syndrome

- Predict multiorgan failure
- Predict mortality/survival

Qualities of an ideal biomarker

- Fast and specific increase in sepsis.
- Rapid decrease after effective therapy.
- Short half life.
- Easy and wide availability and quick turnaround time.
- Reliable method of determination.
- Cost effectiveness.
- Reproducibility of result.

Types of biomarkers

Acute Phase Reactants

- Amyloid
- C-reactive protein (CRP)
- Erythrocyte sedimentation rate (ESR)
- ferritin
- procalcitonin
- ceruloplasmin
- albumin
- hepcidin

Cell Markers

- CD types: CD10, CD11b, CD11c, CD18, CD40, CD 64, CD80, CD165
- mHL-DR

Receptors

- Toll-like receptors
- TNF receptors
- IL-2 receptors
- TREM-1
- CC chemokine receptor (CCR)2
- CCR 3
- C5L2
- CRTh2
- GP130

Cytokines

- interleukins (e.g. IL-27, IL1β, IL2, IL4, IL6, IL8, IL1 0, IL12, IL13, IL18
- macrophage inflammatory proteins
- monocyte protein
- TNF
- Osteoponitin
- RANTES
- MCP1 and 2

Coagulation factors

- APTT
- protein C and S
- fibrin
- antithrombin
- d-dimer
- thrombomodulin
- plasminogen activator inhibitor 1

Miscellaneous

- Vascular endothelial damage markers (ADAMTS-13, Endocan, ICAM etc)
- Vasodilation markers (Adrenomedullin, ACE activity, copeptin, nitric
- oxide, VIP organ dysfunction markers (ANP, BNP, Troponin)

CRP

It is a commonly utilised acute inflammatory marker as well as one of the most researched sepsis markers. Its sensitivity and specificity for diagnosing serious bacterial illness in non-hospitalized children were shown to be 77 percent and 79 percent, respectively, in a systematic review. CRP measurements taken in sequence have a

higher predictive value.A positive CRP increases the likelihood of infection by 11%, while a negative CRP reduces the likelihood of infection by 33%.

CRP levels may be a good indicator of appropriate empirical antibiotic usage. It's a sign of antibiotic failure if CRP stays the same or rises after 48 hours of antibiotic usage.

CRP is unable to distinguish between inflammatory and infectious processes. (14)

PROCALCITONIN(PCT): (15)

The thyroid glands neuroendocrine cells ordinarily secrete PCT, which is a precursor to calcitonin hormone. It's thought that neuroendocrine cells in the lungs and intestine produce it during bacterial systemic infections. TNF- and IL-6 cytokines are responsible for its production. Interferon gamma synthesis inhibits it during viral infections. PCT levels in the blood are below 0.05ng/ml. PCT levels are detectable 3-4 hours after infection and peak 6-24 hours later. Inflammatory disorders such as systemic lupus erythematosus, gout, juvenile rheumatoid arthritis, and inflammatory bowel illness do not have elevated PCTs.However, in cases of major trauma, such as severe burns, PCT values may rise briefly. PCT serum concentrations are useful for monitoring the clinical response to sepsis therapy and for de-escalating antibiotics in the ICU.

In neonates with SIRS and suspected sepsis, studies have indicated that PCT has a reasonable diagnostic accuracy at the cutoff of 2-2.5ng/ml for diagnosis of sepsis.

Sensitivity is 0.85 (95 percent CI 0.76;0.90) and specificity is 0.54 at a cutoff of 2-2.5ng/ml (95 percent CI 0.38;0.70).

Sensitivity and specificity are 0.68 (95 percent CI 0.52;0.80) and 0.85 (95 percent CI 0.70;0.93), respectively, when using a PCT cutoff of >2.5ng/ml.

CitH3 (a NETs marker) has recently been shown in both murine and human research to be an appropriate biomarker for detecting sepsis and endotoxic shock earlier than existing clinical biomarkers like procalcitonin (PCT). Stratification of risk categories are detailed in Table 5.(16)

Table 5: Risk categories according to procalcitonin

PCT value (ng/mL)	Risk category
<0.5	Low risk of systemic bacterial or fungal infection
0.5-2.0	High risk of systemic bacterial or fungal infections
2.0-10	High risk of sepsis and progression to septic shock
>10	High risk of septic shock

NEUTROPHILS IN SEPSIS

Neutrophils are the most common leukocytes in the bloodstream, accounting for more than half of all leukocytes. They constitute the body's first line of defence against external invaders and play a role in acute and chronic inflammation. (17)

NEUTROPHIL NUCLEUS

The nucleus, which houses genetic material and serves as a biochemical factory for DNA replication and RNA synthesis, has long been regarded the cell's command centre. The nucleus, which is the largest organelle and up to 10 times stiffer than the cytoplasm, has a substantial impact on cellular biomechanics. The nuclear envelope protects and separates chromatin and nucleoplasm from the cytoplasm.(18)

NUCLEAR CHROMATIN AND HISTONES:

Chromatin is a nucleoprotein structure made up mostly of DNA. DNA is about 2 metres long and is contained in a nuclear compartment with a diameter of a few micrometers. As a result, chromatin is organised in such a way that DNA is compacted into basic units called nucleosomes. This nucleosome is made up of about 147 base pairs of DNA wrapped around a core histone octamer and 20–90 base pairs of linker DNA associated with the linker histone H1.

Histones are small basic proteins that are found in all eukaryotes.

The core histone octamer is made up of two copies of each core histone H3, H4, H2A, and H2B arranged in a (H3–H4)2 tetramer bordered by two (H2A–H2B) dimers. The H2A, H2B, H3, and H4 families have histones ranging in size from 11 to 15 kDa, whereas the H1 family's linker histones are around 21 kDa.

The nucleosome array resembles "beads on a string" with a diameter of 10 nm (nanometer).

This fibre is compressed at various levels in the cell to generate the higher-order chromatin structure. (14)

NETOSIS:

Neutrophils have been discovered to have a distinct immune response that differs from apoptosis and necrosis in the last two decades. Takei et al. were the first to notice that when dying neutrophils are triggered with phorbol myristate acetate(PMA), or Lipopolysaccharide (LPS) or Inter-leukin 8, they discharge chromatin-containing substances. The functional relevance of this unique neutrophil death pathway was successfully proven by Brinkmann et al. (2004).

Neutrophil extracellular trap formation or NETosis is the release of web-like structures by Neutrophils in response to stimulation. These structures are made up of DNA adorned with histones, granule proteins such lactoferrin, cathepsins, neutrophil elastase (NE), and myeloperoxidase (MPO), as well as cytoplasmic and cytoskeletal proteins. (19). The neutrophil nucleus' lobular form is lost first, followed by nuclear envelope disintegration. After that, the nuclear, cytoplasmic, and granular components combine, followed by a cell membrane breach and the release of intact chromatin into the extracellular environment.(20)

Mitochondrial DNA has also been discovered in NETs. NETs immobilise pathogens, inhibit their spread, and allow antimicrobial proteins to kill them. Beyond antimicrobial defence, there is mounting evidence that NETs have a role in the aetiology of a variety of disorders, owing to their excessive production and/or poor clearance, both of which are hazardous to the host.(19)

The authors were able to structurally describe the NETs as fragile and smooth fibres with the capacity to aggregate into a thick bundle of fibres with sizes up to 50 nm using scanning electron microscopy (SEM). DNA, modified histones, and cytotoxic peptides/proteins make up these web-like structures. The major component is DNA,

because treating NETs with deoxyribonuclease (DNase) is enough to disintegrate them. The presence of histone proteins on the NETs, including core histones (H2A, H2B, H3, H4), linker histone H1, and the H2A-H2B-DNA complex, has been confirmed by immunofluorescence imaging.Proteins discovered in the primary (NE, cathepsin G, MPO) and secondary/tertiary granules (lactoferrin, gelatinase) granules were also located on the NETs, suggesting that some granule proteins play a role in NETosis. NETosis is a marker for severe sepsis. The mechanism of NETs formation is depicted in Fig 4.(21)

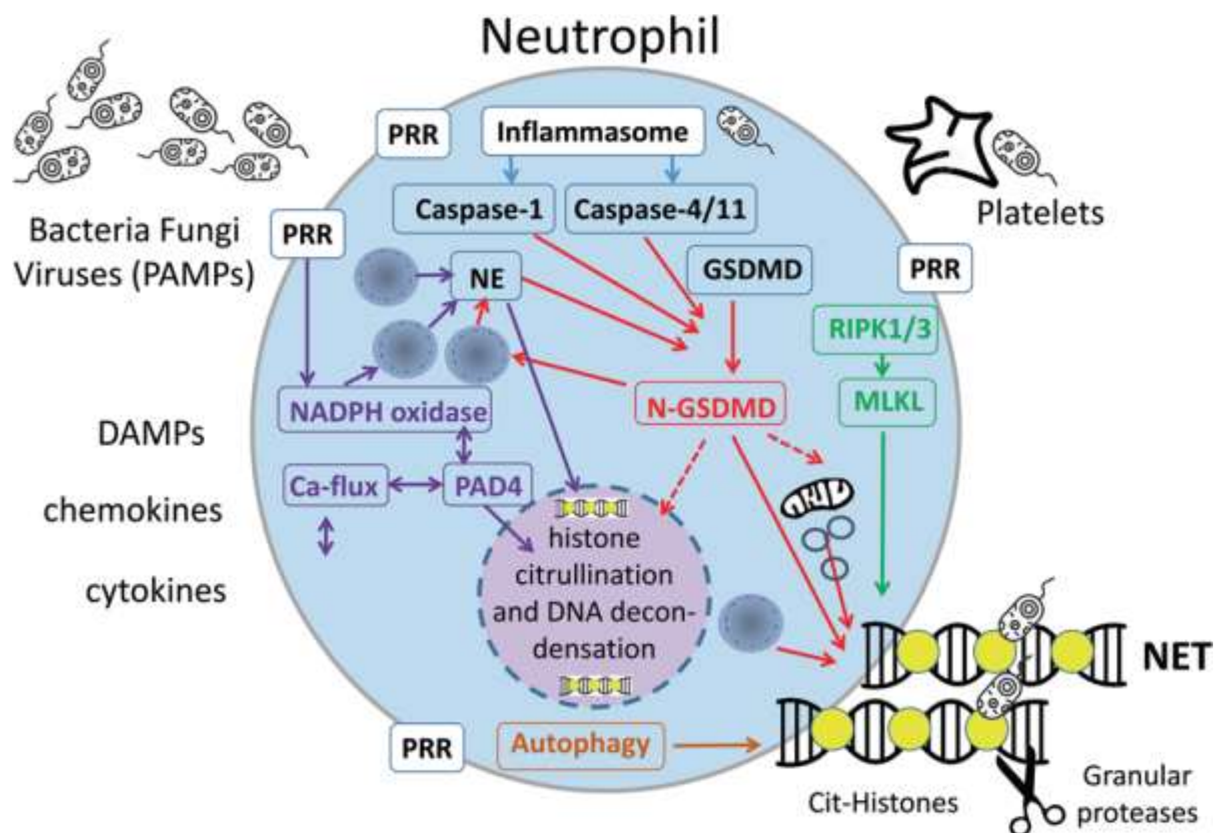


Figure 3: Mechanism of NET formation

(19)

Adapted from Ackermann M, Anders H-J, Bilyy R, Bowlin GL, Daniel C, De Lorenzo R, et al. Patients with COVID-19: in the dark-NETs of neutrophils. Cell Death Differ. 2021 Nov;28(11):3125–39.

CITRULLINATED HISTONE H3

The structure of chromatin is constantly regulated. Although the exact processes that control chromatin structure during certain nuclear activities are still being investigated, posttranslational histone modifications are recognised to play a key role. Histone acetylation, methylation, and phosphorylation control chromatin processes like transcription, as well as chromatin condensation and decondensation. (22)

HP1 is a well-known non-histone protein that affects chromatin structure by binding to modified histones, namely histone H3 Lys9 methylation.

The recruitment of HP1 to methylated histone H3 is essential for maintaining heterochromatin. HP1 is one of the most well-studied proteins linked to chromatin condensation.

A cell can strictly regulate the active or inactive state of a gene via HP1 binding to specific locations on chromatin, depending on the need for specific cellular functions.(22)

Post-translational alteration of an arginine to a citrulline residue is known as citrullination or deimination. The guanidino group of arginine is hydrolyzed, resulting

in the formation of a ureido group and the loss of an ammonia molecule converting to citrulline that is mediated by the calcium dependent enzymes peptidylarginine deiminases (PAD4 and PAD2) and is implicated in the NETosis/METosis signalling cascade. Kindly refer to Fig 5.(23)

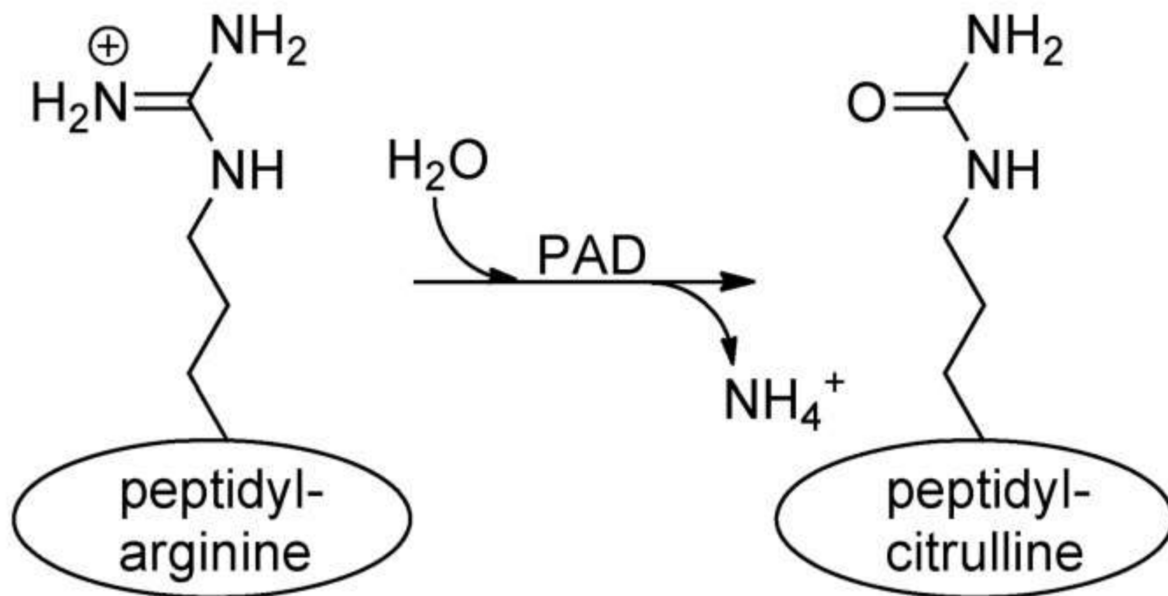


Figure 4: Citrullination of Histone protein

Adapted from Rohrbach AS, Slade DJ, Thompson PR, Mowen KA. Activation of PAD4 in NET formation. Front Immunol. 2012 Nov 29;3:360.

Peptidyl arginine deiminase enzymes, or PADs, catalyse citrullination.

The loss of positive charge and a rise in mass of about 1 Da result from this action. While this change may appear minor, the loss of positive charge and hydrogen bond acceptors can have a significant impact on cell signalling since these interactions are necessary for protein–protein, protein–DNA, and protein–RNA interactions to remain stable.

Furthermore, this PTM may disrupt intramolecular interactions, potentially causing substantial conformational changes in a protein, as well as affecting intermolecular interactions and reducing protein stability.

And as the positive charge of the histone residue decreases, the binding to the negatively charged DNA becomes weaker, resulting in chromatin de-condensation.

Humans and mice both express five PAD enzymes, with tissue localisation appearing to be the most significant distinction between them. PADs 1, 3, and 6 are found in the uterus, hair follicles, and the egg, ovary, and embryo, respectively. PAD4 has been found in granulocytes, malignant cell lines and tumours, and, most recently, mammalian oocytes and the preimplantation embryo. PAD2 is detected in the CNS, skeletal muscle, and immune system cells and has a significantly broader tissue expression profile. The only PADs expressed in the hematopoietic lineage are PADs 1, 2, and 4, which are of particular immunological significance.

Only PAD2 and PAD4 with nuclear localization can citrullinate histone H3 and cause NETosis.(23)

All of these enzymes require calcium concentrations greater than those found in homeostatic cytoplasm to catalyse, implying that calcium flux or a calcium-producing event is required to promote activity, at least in vitro. A PTM or interacting protein, on the other hand, may lower the calcium concentration necessary for activation to normal values.(23)

It is thought that gene regulation is important in NET formation, and that the histone-modifying enzyme PAD4 is one of the key mediators of this immunological process. (22)

Therefore, PAD over expression In non-granulocytic cells, in conditions like sepsis causes extensive chromatin decondensation and prevents HP1 binding to chromatin which further contributes to chromatin decondensation leading to neutrophil extracellular trap formation. The mechanism of DIC and muti-organ failure is shown Fig 6 and Fig 7. (22)

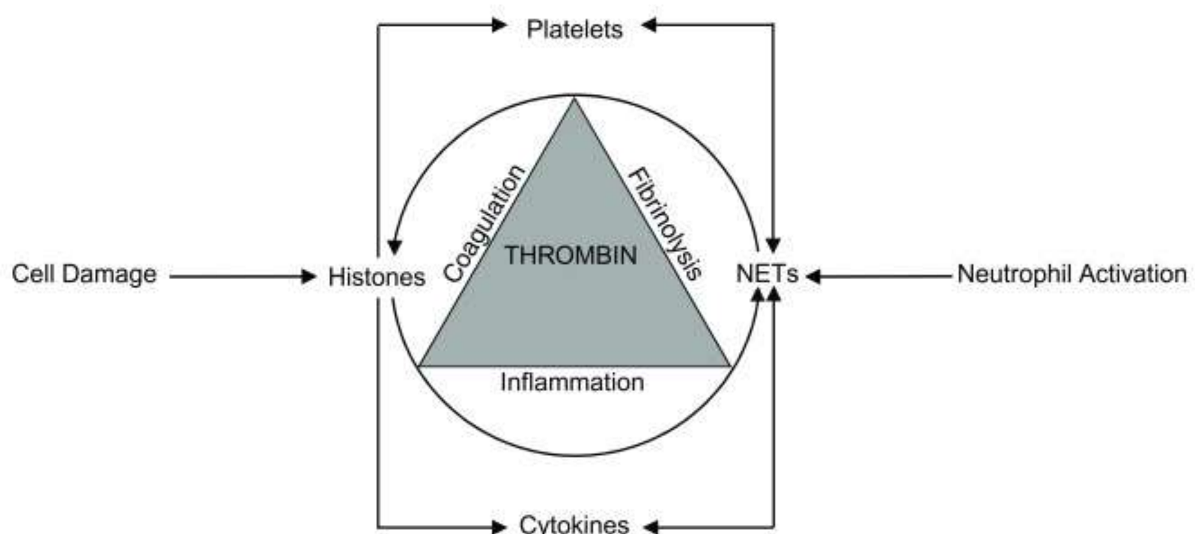


Figure 5: NETs and Histones cause DIC and Multi-organ failure

Adapted from Alhamdi Y, Toh C-H. Recent advances in pathophysiology of disseminated intravascular coagulation: the role of circulating histones and neutrophil extracellular traps. F1000Research. 2017 Dec 18;6:2143.

Between NETs and histones, there is a bi-directional interaction. For starters, NETs have exposed histones (along with a slew of other powerful enzymes like elastase) on their meshwork, facilitating local histone-mediated cytotoxicity, pro-coagulant, and pro-inflammatory actions. Histones can also be released into the circulation by NETs in order to spread the disease's negative consequences. Second, histones can directly

stimulate neutrophils to form NETs, creating a vicious cycle that is triggered by cellular injury and then propagated by this bi-directional relationship between histones and NETs, promoting further thrombin generation and contributing to DIC pathogenesis.

Proposed immunopathological roles of circulating histones in sepsis.

In sepsis, pathogen associated molecule patterns (PAMPs, such as bacteria and LPS) and damage associated molecule patterns (DAMPs, such as IL-18, TNF-, and high mobility group box 1 etc.) activate NETosis/ETosis, necroptosis, necrosis, and pyroptosis via pattern recognition receptors (toll-like receptors, C-type lectin receptors, and NOD-like receptors). Histones are then released into the extracellular area. Histones activate the Toll-like receptor (TLR) 2/4/9 or NLPR3 inflammasome pathways, resulting in the production of pro-inflammatory cytokines (IL-1, IL-1, IL-6, IL-18, TNF-, etc.) that induce neutrophils to produce extracellular traps and blood monocytes to upregulate tissue factor (TF) expression. Histones bind to endothelial cells, causing permeabilization, calcium influx, and damage to the endothelium. Endothelium injury also results in barrier dysfunction, leakage of plasma-protein-rich fluid into tissues, changes in blood flow dynamics, recruitment and activation of circulating leucocytes, and the release of excessive cytokines, as evidenced by increased TF, prostacyclin (PGI2), and superoxide expression, as well as decreased thromboxane A2 (TXA2) and nitric oxide release. Extrinsic coagulation pathway activation of prothrombin can result in disseminated intravascular coagulation when TF levels are high (DIC). Furthermore, by attaching to prothrombin segments 1 and 2 and forming a histone-prothrombin-FXa complex, histones can directly drive thrombin production. TLR2 and TLR4 pathways

are also used by Histones to cause platelet aggregation, activation, and platelet-dependent thrombin production. (4)

Following are some other postulated mechanisms by which histones contribute to DIC:

1. **Reduced endogenous anti-coagulant activity:**

Histones have been found to disrupt the Protein C(PC) pathway by downregulating Thrombomodulin (TM) or dampening TM-dependent PC activation, according to two investigations. This would limit Activated PC's capacity to proteolytically break histones, as well as its anticoagulant, anti-inflammatory, and cytoprotective effects. Anti-Thrombin and Tissue Factor Pathway Inhibitor are both degraded by NET-associated elastases. Reduced production by the liver and loss to the extravascular space as a result of increased vascular permeability are two other possible pathways for endogenous anti-coagulant loss. Histones may have a role in both of these pathways by causing liver injury and inflammation, as well as a large increase in vascular permeability through endothelial damage

2. **Intrinsic pathway:**

Histones can activate the intrinsic pathway via an FXII-dependent mechanism, and histone-DNA complexes play a significant role in elevated FXII levels in patients with overt DIC. Histone-induced platelet polyphosphate production can boost factor XI auto-activation and speed up its thrombin-mediated activation in an indirect manner.

3. Impaired fibrinolysis:

All research on the effects of histones, cfDNA, and NETs on the fibrinolytic system have consistently found that they are anti-fibrinolytic. This impact is achieved via plasmin-mediated increased clot resistance to fibrinolysis and tPA-mediated inhibition of plasminogen activation.(24)

Finally, multiple organ dysfunction (MODS) is caused by dysregulated inflammation response, impaired endothelial barrier, immunothrombosis, and DIC, which results in circulatory failure, acute respiratory distress syndrome (ARDS), renal failure, and liver failure, as well as death in the most severe form of sepsis.(25)

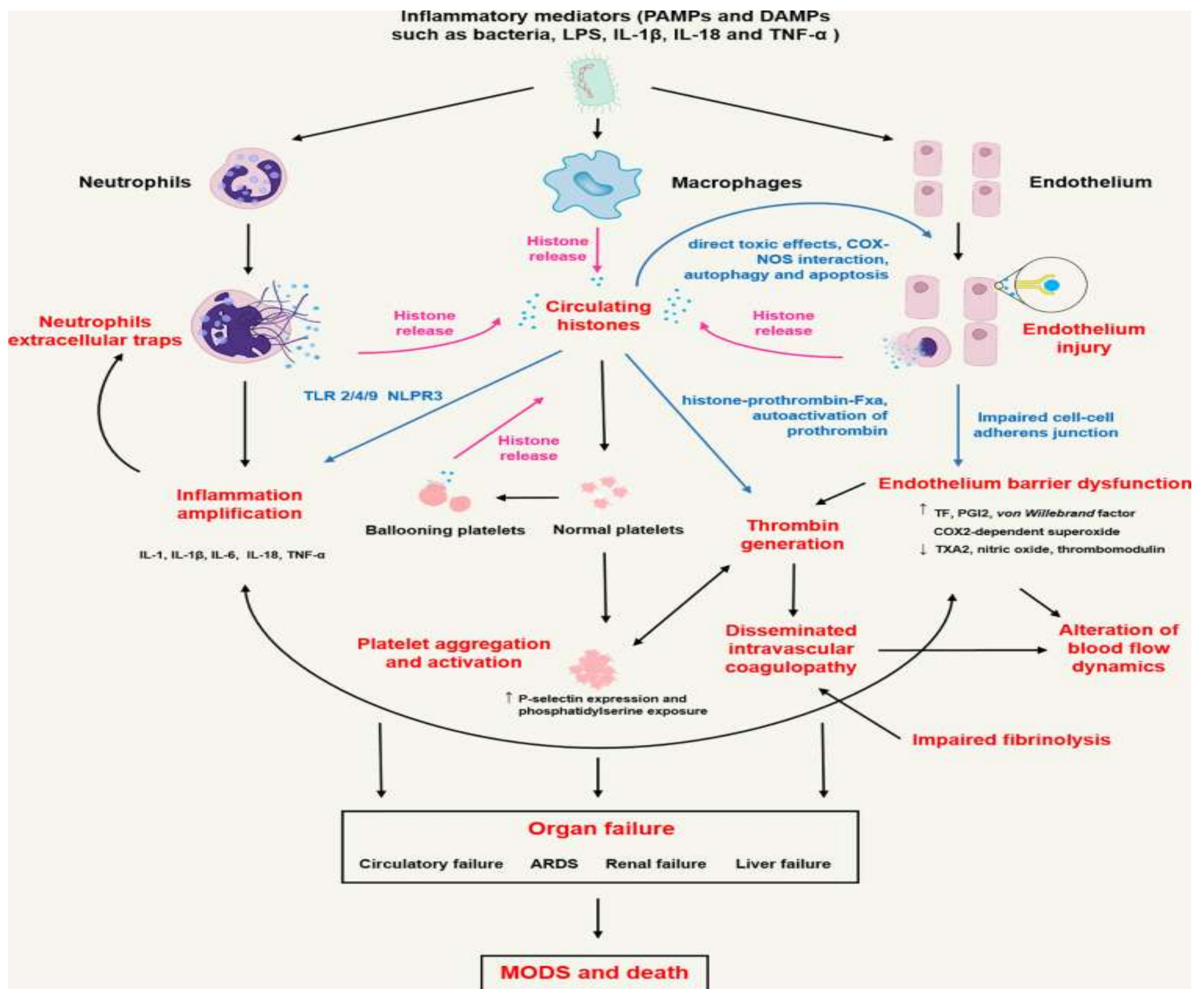


Figure 6: Role of NETS and Histones in MODS

Adapted from Li Y, Liu Z, Liu B, Zhao T, Chong W, Wang Y, et al. Citrullinated Histone H3 – A Novel Target for Treatment of Sepsis. Surgery. 2014 Aug;156(2):229–34.

Although the ELISA approach has been validated, there are no standard assays for measuring histones yet.

OTHER STUDIES

Pan et al used mice which were injected with lipopolysaccharide forming the LPS model and those in which haemorrhage was induced to compare cit h3 levels. Both lipopolysaccharide (LPS)-induced shock (LPSS) and hemorrhagic shock (HS) in mice were reported to release NET components. CitH3 specific enzyme-linked immunosorbent assay was utilised to quantify CitH3 in NETs. Only in LPSS, but not in HS, was circulating CitH3 shown to be increased. CitH3 levels in the blood were observed 30 minutes after the LPS insult and remained higher for 24 hours (period of the highest mortality). The peptidylarginine deiminase-2/4 inhibitor YW3-56 dramatically reduced CitH3 levels in the blood of endotoxic mice. Interleukin-1 did not respond to LPS immediately, and levels of interleukin-1 and interleukin-6 varied despite treatment. Only 24 hours after the LPS insult did procalcitonin begin to rise. In comparison to CitH3, these biomarkers were elevated in LPSS and HS in a non-specific manner. The inference was that, in the model of deadly lipopolysaccharide (LPS)-

induced shock, their findings showed that **CitH3 appeared earlier, was more selective,** and that the therapeutic intervention YW3-56 (PAD2/4 inhibitor) dramatically enhanced survival (LPSS). **This study suggested that cit histone h3 can be early and better biomarker of sepsis than procalcitonin**.(2)

Similar results were obtained on mice used as sepsis models by Nomura et al.(26)

A combination of in vitro and in vivo tests on mice were used by Li et al to show that blocking Cit H3 can be protective in the situation of fatal sepsis. First, while boosting histone protein acetylation, the Histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) lowered their citrullination. Second, in the Cecal ligation and puncture model, inhibiting peptidyalarginine deiminase with Cl-amidine, a pan-PAD inhibitor, decreased Cit H3 generation and improved survival (septic shock). Finally, neutralising circulating Cit H3 with an anti-Cit H3 antibody reduced the model's mortality considerably.(25)(citrullinated histone a novel target for treatment of sepsis)

In a retrospective analysis by Tian et al(27) CitH3 levels were determined in the serum of 160 critically ill patients in septic and non-septic shock, as well as healthy volunteers, using a sandwich enzyme-linked immunosorbent assay (ELISA) that recognised PAD 4 and PAD 2 citrullination of R2 + R8 + R17 + R26 on the CitH3 protein. Patients' clinical and analytical data were analysed. Septic shock patients (n = 102) had significantly higher levels of circulating CitH3 at enrolment than Non-Infectious controls (NIC) patients (n = 32, $p < 0.0001$). CitH3 had an area under the curve (95 percent confidence interval) of 0.76 (0.65–0.86) for identifying septic shock from NIC, while procalcitonin had an area under the curve (95 percent confidence interval) of 0.57 for separating septic shock from NIC (0.51-0.64). CitH3 was found to have a positive

relationship with PAD2 and PAD4 concentrations, as well as the Sequential Organ Failure Assessment Scores [total score (r = 0.36, p 0.0001)]. CitH3 serum levels were substantially greater in the septic patients who did not survive at 24 hours (p 0.01) and 48 hours (p 0.05). **Septic patients have higher levels of citrullinated histone H3 than healthy volunteers and non-infectious controls, according to this study.**

In the case of sepsis, citrullinated histone H3 outperforms procalcitonin as a potential diagnostic biomarker. In advanced sepsis, citrullinated histone H3 could be a predictive factor. The concentration of citrullinated histone H3 in the blood is linked to the SOFA score. In septic patients, there is a link between the level of citrullinated histone H3 and PAD2 and PAD4. (27)

In a study conducted by Yokoyama et al (2019), blood histone H3 levels were evaluated in 85 patients hospitalised to the critical care unit due to infectious disorders using a novel enzyme-linked immunosorbent test for histone H3 detection. The researchers then looked at the links between circulating histone H3 levels and organ failure, coagulopathy, and death**. They found that circulating histone H3 levels were considerably greater in patients with coagulopathy and were positively associated to the number of organ failures in their investigation.** The levels of histone H3 in the bloodstream were likewise linked to death. The prediction performance of circulating histone H3 levels for mortality was higher than that of traditional inflammatory markers such as white blood cell count, C-reactive protein, and cell-free DNA, according to receiver-operating characteristic studies.(28)

In a study published in August 2021 by Chandra et al in Indonesia, the median Cit-H3 level was 1,210 (800-32,160) ng/mL among clinically septic children, with an optimum

cut-off value of 1200 ng/mL (sensitivity 83.3 percent and specificity 75.7 percent) to diagnose sepsis . Survivors had lower median Cit-H3 levels at 0 hour (p=0.016). With an optimal cut-off point of 1,200 ng/mL, Cit-H3 level was able to predict mortality with 72.2 percent sensitivity and 57.1 percent specificity (AUC of 69.2%; p=0.017). Cit-H3 levels were found to be substantially linked with death (p=0.023; hazard ratio of 3.45) in a survival analysis, suggesting that Cit-H3 levels may be used to predict paediatric sepsis episodes and outcomes.(29)

COVID -19 AND NETOSIS

Even under steady-state settings, mature neutrophils transit from the bone marrow to the circulation and from the circulation to the tissues. Neutrophil trafficking and responsiveness to pathogens are regulated by the 24-hour clock. The influx of neutrophils from the circulation into tissues occurs in various organs, but especially in highly vascularized organs like the lungs and kidneys, which are the primary targets of coronavirus illness 19 (COVID-19). The most important independent risk factor for severe COVID-19 has been found as the neutrophil to lymphocyte ratio. (19)

Neutrophil Elastase also cleaves gasdermin D (GSDMD), a molecule crucial to pyroptosis and a feed-forward loop to enhance granule and plasma membrane permeabilization, during NET formation. After cytosolic LPS sensing and caspase-11-dependent activation of GSDMD, however, NET formation is enhanced. Disulfiram inhibits the SARS-COVID-19 infection cycle's papain-like proteases and has been demonstrated to alter Cys191 Cys192 in GSDMD, reducing pore and possibly NET release. (19)

The role of citrullination of histones in COVID 19 illness

As previously stated, physiological NET formation is frequently linked to PADI4 activation. In protein substrates, such as core histones, PAD4 transforms positively charged arginines to neutral citrullines. Citrullination releases the energy stored in coiled DNA, causing NETs to be ejected in a catapult-like fashion. PAD4 keeps its enzymatic activity in the extracellular environment and changes proteins such as extracellular matrix proteins and coagulation factors. COVID-19 and influenza-infected mice both have citrullinated histone accumulation. Because pan-PADI and PADI4 inhibitors like Cl-amidine, BB-C Lamidine, YW-56, or GSK484 have shown efficacy in the treatment of NET-mediated pathologies like lethal lung endotoxemia and cellular damage due to hypoxia, the administration of such inhibitors may be beneficial for the treatment of COVID-19. (19).

COVID-19 and MIS-C/PIMS-TS IN CHILDREN

COVID-19 tends to be moderate in children. Children can, however, be seriously impacted in rare situations, and clinical signs may differ from those seen in adults. A severe shock-like illness in children with features of incomplete Kawasaki disease (KD) or toxic shock syndrome was reported in the United Kingdom and Italy in April of 2020, according to studies. Following that, children from all around the world have reported being affected in the same way. Multisystem inflammatory syndrome in children is the term coined to the condition (MIS-C; also referred to as paediatric multisystem inflammatory syndrome [PIMS-TS], paediatric inflammatory multisystem syndrome temporally associated with SARS-CoV-2 [PIMS-TS], paediatric hyperinflammatory

syndrome, or paediatric hyperinflammatory shock). Fever, rash, conjunctival injection, and gastrointestinal symptoms were among the clinical characteristics, which sometimes progressed to multi-organ failure and necessitated admission to the paediatric intensive care unit (PICU) (30)

Compared to adult patients, COVID-19 in children appears to be less severe. A review of 72,314 cases by the Chinese Center for Disease Control and Prevention showed that less than 1% of the cases were in children younger than 10 years of age. (30) Symptoms in children were milder than those in adults, with the commonest symptoms being cough and fever in 60-73%. (31)(32) There was a slight male preponderance (56-57%), and the median age of infection was 7-8 years. (31,32) 20% needed hospitalization, with 0.6-2% needing ICU admission. 23% had underlying conditions including chronic lung disease, cardiovascular disease and immunosuppression. Underlying conditions were present in 77% of hospitalized children compared to only 12% of those not hospitalized. (33) Laboratory abnormalities reflect those in adults with lymphopenia, thrombocytopenia and elevated ESR, CRP, procalcitonin, D-dimer and LDH although much less commonly seen compared to adults, even in hospitalized children.(33) Radiological findings, although similar to those in adults, were also seen in a good percentage of asymptomatic children. (34)The proportion of severe and critical cases was 10.6%, 7.3%, 4.2%, 4.1% and 3.0% for those aged <1, 1-5, 6-10, 11-15 and ≥16 years respectively.[7] Need for ICU admission and mechanical ventilation were also commoner in younger children. (34) Mortality rate in large series of hospitalized children have ranged from 0.6% in China to 0.69% in Europe.(35)

Starting with a single case report of typical Kawasaki disease in an infant with COVID-19 in April 2020, (36) clinicians in Italy and France noticed a 30-fold increase in Kawasaki disease compared to pre-COVID times, and reported children with COVID-19 presenting with both complete and atypical Kawasaki disease and a hyperinflammatory syndrome with and without Kawasaki disease-like manifestations.(37,38) Compared to pre-COVID Kawasaki, these children were older, had higher incidences of myocarditis and pericarditis, lymphopenia and thrombocytopenia and were more likely to need second-line treatment after a standard dose of IVIG. (37,38) Labelled Multi-System Inflammatory Syndrome in Children (MIS-C) by the WHO and in the US, and Pediatric Inflammatory Multisystem Syndrome Temporally associated with SARS-CoV-2 infection in children (PIMS-TS) in Europe and the UK, this was temporally found to occur 2 to 4 weeks after the peak of SARS-CoV-2 infection in a community, and therefore often in children negative on PCR testing but positive for SARS-CoV-2 antibodies. Clinical presentations were varied and included fever with elevated inflammatory markers, shock and Kawasaki disease. GI manifestations were much more common compared to respiratory symptoms, and lymphopenia, elevated inflammatory markers, evidence of myocardial dysfunction and elevated cardiac enzymes were also much more common (up to 100% in some series) in these children compared to those without MIS-C. (39–41) Although children with MIS-C account for only 10% of all COVID-19 cases (42) they are 5 times more likely to be admitted to critical care compared to those without, (43) with a higher mortality at 2% in several large case series (39–41)compared to 0.6% in all children with SARS-CoV-2 infection. Children with MIS-C make up approximately 50% of

admissions to critical care. Mortality in ICUs has been reported to range from zero (43,44) to 4% (45) in children with and without MIS-C.

JUSTIFICATIONS

Sepsis still remains one of the primary causes of death and this has mitigated the need for understanding the pathophysiology of sepsis at the molecular level. One of the molecular mechanisms proposed is the Neutrophil extracellular trap formation and exposure to citrullinated histones as an aetiology for sepsis related complications. Literature review has revealed that majority of studies so far, especially in the adult population indicated a positive link between sepsis illness severity and increased circulatory levels of citrullinated histone H3. There is paucity in the literature concerning children with only a few studies in the paediatric group and also in the Indian population.

Despite similar studies in the past, we wanted to see if the claims on these biomarkers were replicable and reproducible. In order to negate or affirm these intriguing findings of other research we decided to perform this study in order to generalise this principle, and pave way into further research for newer diagnostics and therapeutics with Citrullinated Histone H3 as targets for a potentially better outcome in sepsis.

METHODS

MATERIALS AND METHODS

Institutional review board and Ethics committee approval

This proposal was accepted by the Institutional review board and Ethics Committee of the institution. The proposal was presented to the committee and queries raised by the committee were clarified, and permission was obtained. Scanned copy of IRB form attached. (ANNEXURE 1)

Study Period: June 2020 to September 2021

Study design: A single centre prospective observational study

Study Setting: The study was conducted in the department of Transfusion Medicine and Immuno-haematology and Paediatric Intensive care unit (PICU), Department of Paediatrics, Christian Medical College and Hospital, Vellore, a tertiary care centre in South India

Study population: Children with clinical sepsis, between 28 days to completed 15 years of age admitted in PICU/PHDU were included in the study after excluding children less than 28 days, children with any type of trauma, malignancy, or autoimmune diseases.

Also 38 non-sepsis controls (pre-operative children, posted for minor surgeries) were recruited from paediatric surgery department to establish baseline Histone H3 levels prior to testing variations in patients.

Inclusion criteria:

All Children (>28days to completed 15 YEARS) admitted with clinical suspicion of sepsis.

Exclusion criteria:

1. Neonates (less than 28days of age at admission)
2. Children with any type of trauma, malignancy or autoimmune diseases
3. Children for whom informed consent was not obtained

Sample size: Based on the paediatric ICU admission census, 50% of sepsis patients developed Multiple organ failure (MODs) and this data was taken for the sample size calculation. With the expected proportion 0.50, 10% precision, 95% desired confidence level, the study required totally 96.

Single Proportion - Absolute Precision

Expected Proportion	0.50
Precision (%)	10

Desired confidence level (1- alpha) %	95
Required sample size	96

Formula (REF):

$$n = \frac{Z_{\alpha/2}{}^{2} * 2 * PQ}{d^2}$$

We have recruited 90 patients as cases and additionally, we have enrolled 38 non sepsis (pre-op children, posted for minor surgeries), to study the baseline value of Histone H3.

Data Sources/measurement:

The clinical and laboratory data obtained during hospitalization regarding age, gender, clinical and confirmed diagnosis, complete blood count, renal function tests, liver function tests, lactate, respiratory status, ventilation, coagulation profile and calculation sepsis screening tool, ISTH DIC criteria and transfusion support were followed up and collected from hospital records.

Definitions of variables and diagnostic criteria followed:

1. Age:

- ✓ Neonate: 0 days to 28 days
- ✓ Infant: 29 days to 1 year
- ✓ Toddler and preschool: >1 to 5 years
- ✓ School age child: >5 to 12 years
- ✓ Adolescent: >12 to <16 year

2. Infection — Infection is defined as a suspected or proven infection caused by any pathogen. It includes clinical syndromes associated with a high probability of infection, such as petechiae and purpura in a child with hemodynamic instability, or fever, cough, and hypoxemia in a patient with leucocytosis and pulmonary infiltrates on chest radiograph

3. **SIRS/ suspected sepsis**-The presence of two or more of the following criteria (one of which must be abnormal temperature or leukocyte count) defines SIRS, Refer to Table 6 for age specific cut-offs for SIRS:
    - Core temperature (measured by rectal, bladder, oral, or central probe) of >38.5°C or <36°C
    - Tachycardia, defined as a mean heart rate more than two standard deviations above normal for age, or for children younger than one year of age, bradycardia defined as a mean heart rate <10th percentile for age
    - Mean respiratory rate more than two standard deviations above normal for age or mechanical ventilation for an acute pulmonary process
    - Leukocyte count elevated or depressed for age, or >10 percent immature neutrophils

Table 6: Age specific cut-offs for Systemic inflammatory response syndrome

Age group	Heart rate (beats/minute)		Respiratory rate (breaths/minute)	Leukocyte count (leukocytes x $10^3/mm^3$)	Systolic blood pressure (mmHg)
	Tachycardia	Bradycardia			
Newborn (0 days to 1 week)	>180	<100	>50	>34	<59
Neonate (1 week to 1 month)	>180	<100	>40	>19.5 or <5	<79
Infant (1 month to 1 year)	>180	<90	>34	>17.5 or <5	<75
Toddler and preschool (>1 to 5 years)	>140	NA	>22	>15.5 or <6	<74
School age (>5 to 12 years)	>130	NA	>18	>13.5 or <4.5	<83
Adolescent (>12 to <18 years)	>110	NA	>14	>11 or <4.5	<90

4. **Confirmed Sepsis:** The systemic inflammatory response syndrome in the presence of suspected or proven infection constitutes sepsis.
5. **Probable sepsis:** Suspected sepsis along with elevated inflammatory markers such as CRP or procalcitonin.
6. **Septic shock:** The clinical diagnosis of septic shock is made in children who,

1) have a suspected infection manifested by hypothermia or hyperthermia and
2) have clinical signs of inadequate tissue perfusion
    a. decreased or altered mental status
    b. prolonged capillary refill greater than 2 seconds
    c. diminished pulses
    d. mottled cool extremities or flash capillary refill
    e. bounding peripheral pulses and wide pulse pressure
    f. decreased urine output less than 1 mL/kg/hr
    g. may or may not be hypotension

Septic screening tool: Refer to Annexure 5A

8. **MULTIORGAN DYSFUNCTION:** MODS is defined as the simultaneous occurrence of at least two organ dysfunctions based on 2002 international paediatric sepsis consensus conference. The details are given in Annexure 7
9. **ISTH DIC CRITERIA:** A score of more than 5 indicates overt DIC and the scoring should be repeated daily whereas a score of less than 5 indicates a non-overt DIC or a low grade DIC and the scoring can be repeated after 2 days
   The variables to calculate the DIC score are given in Annexure 6.

10. Transfusion support: ISTH/SSC recommendations

- ✓ Platelet rich concentrate: 50ml RDP/10 kgs Recommended in patients with active bleeding plus platelet count $< 50 \times 10^9$/L, or high risk of bleeding plus platelets $< 20 \times 10^9$/L
- ✓ FFP: 15ml/kg-Recommended in patients with active bleeding or requiring an invasive procedure and either prolonged PT/aPTT ($> 1.5 \times$ normal)
- ✓ Cryoprecipitate: 1 unit /10kgs decreased fibrinogen (< 1.5 g/L).

11. PDR score-Paediatric index of Mortality (PIM 2) scoring system

One of the severity grading systems used to predict the outcome of patients admitted to Paediatric intensive care units (PICUs) is the paediatric index of mortality (PIM) 2 score. Shann developed it in 1997 (modified in 2003) to predict the outcomes of children admitted to PICUs. PIM2 is derived from data acquired when a kid is hospitalised to the intensive care unit (ICU).

It specifies the severity of the child's illness at the time when intensive care was started. Ten variables make up the PIM 2 score. These variables were given a score of 1 or 0 for yes or no responses. These should be entered into the system (www.sfar.org/scores2/ pim22.html) for the predicted mortality rate to be calculated. Refer to annexure 8

Study implementation:

Clinical Sepsis or septic shock patients who fit the consensus definitions admitted to PICU or PHDU from June 2020 to September 2021 satisfying the inclusion criteria were recruited after obtaining informed consent or assent based on the age of the child.

Clinical Data collection

The comprehensive clinical data during admission was obtained through our clinical workstation through individual chart review. After admission to Paediatric intensive care unit (PICU) patients were categorised as suspected sepsis if they fulfilled the SIRS criteria, probable sepsis if additionally, CRP or procalcitonin were positive and confirmed sepsis if blood culture turned positive for any micro-organism. Septic shock patients were those meeting 3 or more of the 8 age appropriate clinical criteria as in Annexure 2. The Paediatric mortality index scoring system (PIM-2) was also used to calculate the predicted death rate on admission as detailed in annexure 5. It consists of 10 variables and calculation was done after entering the data into the system(www.sfar.org/scores2/pim22html). International Society on Thrombosis and Haemostasis scoring system as in Annexure 3 which is based on readily available global coagulation tests (platelet count, FDPs, PT, and fibrinogen) was used to aid in the

diagnosis of DIC. And the diagnosis of multi-organ dysfunction was done using paediatric organ dysfunction criteria based on 2002 international paediatric sepsis consensus conference as listed in annexure 4. The number of transfusions that happened were also noted.

38 non-sepsis children admitted under paediatric surgery were included as controls to obtain a baseline histone H3 level.

Lab data collection

On admission, after obtaining consent 2.5ml of blood was collected into a citrated tube. The sample was then centrifuged for 20 min at 2000×g. The plasma sample was then frozen at −80 °C until further analysis. At time of analysis, samples were thawed and diluted 1:2 in PBS.

Our study algorithm is detailed in Fig 8.

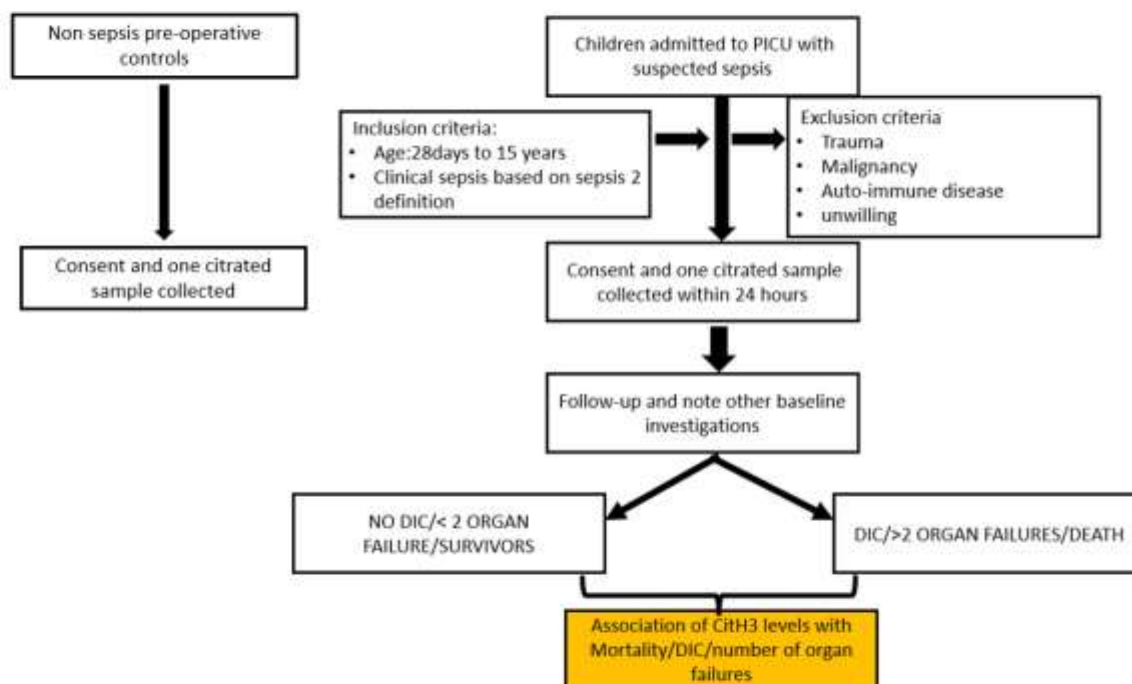


Figure 7: Detailed diagrammatic Algorithm of the study

Citrullinated Histone H3 (clone 11D3) by ELISA

Quantification of Histone H3 was performed using **sandwich ELISA Citrullinated Histone H3 (clone 11D3) ELISA kit (Cayman chemical, catalog 501620)** as a batch test.

Test Principle

This immunometric assay is based on a double antibody 'sandwich' technique. Each well of the microwell plate supplied in the kit has been coated with a monoclonal antibody specific for histone H3 (citrullinated at R2, R8, and R17). This antibody will bind any CitH3 introduced into the well. A second monoclonal antibody which recognizes the H3 core (Antibody/HRP Conjugate) is added to the well, forming a

"sandwich". The 'sandwiches' are immobilized on the plate so the excess reagents may be washed away. The Antibody/HRP Conjugate is labelled with horseradish peroxidase (HRP), allowing quantitation of the CitH3. Addition of HRP Substrate TMB, followed by Stop Solution produces a yellow-coloured product which can be measured spectrophotometrically. The intensity of the colour is directly proportional to the amount of bound Antibody/HRP Conjugate, which is proportional to the concentration of citrullinated histone H3.

Absorbance ∝ [Anti-CitH3/HRP] ∝ [CitH3]

ASSAY PROTOCOL

Procedure:

Step 1: 100ul of the standards or diluted sample were added to the appropriate wells on the plate.

Step 2: The samples were covered and incubated for 2 hours on an orbital shaker

Step 3: The wells were emptied and rinsed four times with Wash Buffer followed by gently tap the inverted plate on absorbent paper to remove the residual Wash Buffer.

Step 4: 100 μl of the HRP Conjugate working solution was added to each well of the plate.

Step 5: The plate was covered with the 96-Well Cover Sheet and incubated for one hour at room temperature on an orbital shaker.

Step 6: This was followed by washing and addition of 100 ul of TMB substrate solution and incubation in the dark.

Step 7: 100ul of HRP stop solution was added and the plate was read at 450nm on the Thermofischer Multiskan FC ELISA reader.

Analysis

Raw O.D. values were obtained. Concentrations of citH3 in the samples were derived by plotting a Standard curve using 4 parameter logistic equation with computer reduction software in the plate reader. Assay range obtained was 0.15 – 10 ng/ml.

The fit model used for the four-parameter logistic method is as follows:

$$y = b + \frac{a - b}{1 + (xc)^d}$$

https://tools.thermofisher.com/content/sfs/manuals/D01520~.pdf

Where, y is the signal, x the concentration, a is the response at high asymptote, b is the response at low asymptote, c is the 1/concentration corresponding to 50% specific binding, and d is the slope factor. Following is the standard curve obtained.

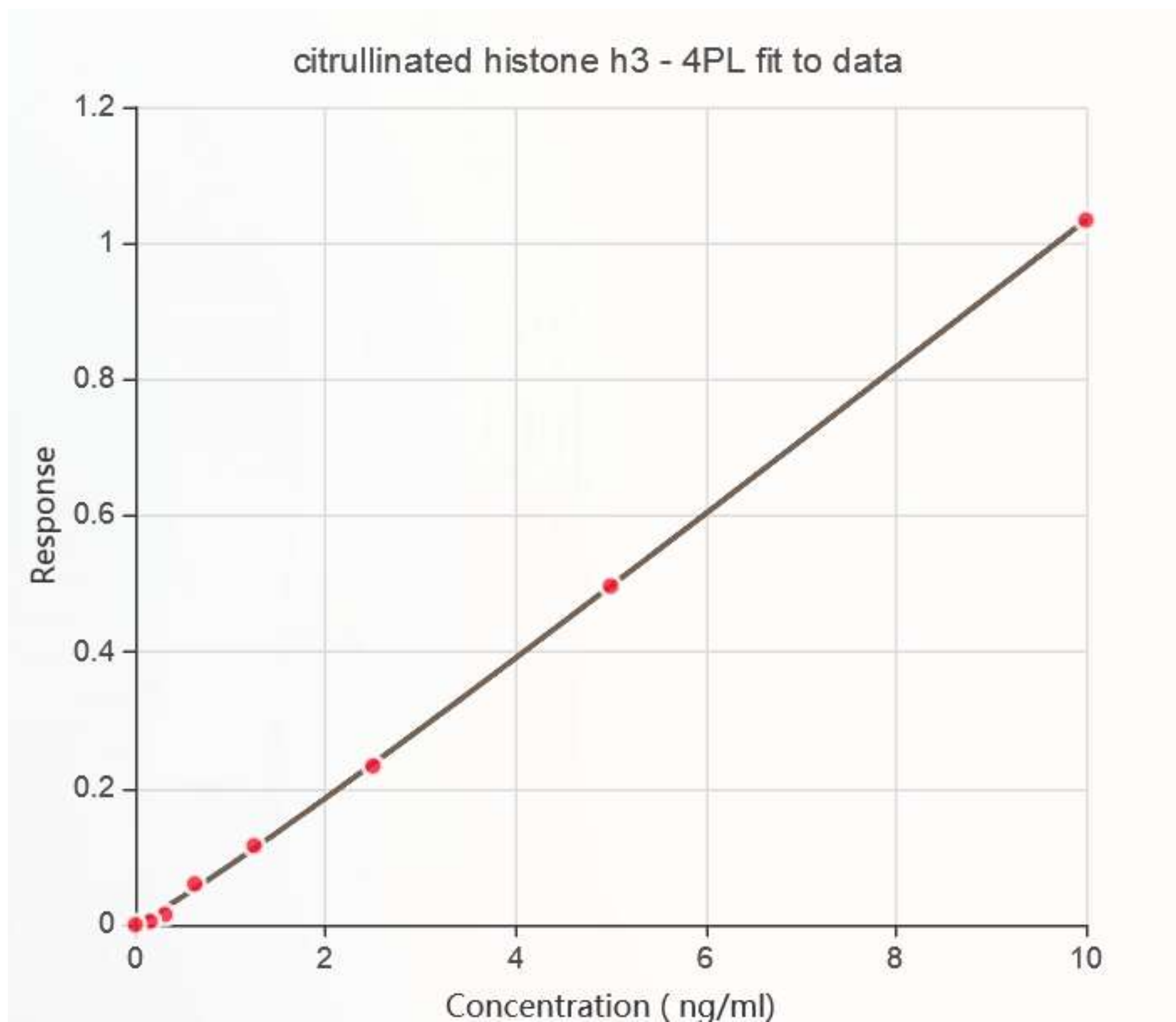


Figure 8: Typical standard curve obtained in our test for analysis

https://www.arigobio.com/elisa-analysis

The additional lab investigations such as CBC profile including Haemoglobin, platelet count, Total count, coagulation profile PT INR, APTT, fibrinogen, D-dimer, creatinine, lactate, liver function tests and inflammatory markers such as CRP, procalcitonin were obtained from online database wherever available.

Retrospective analysis of the patients was done using the prospectively collected clinical and laboratory data from hospital records.

Clinical outcome of the patients i.e., Recovered/DIC/MODS/death were documented. CitH3 levels were correlated with survival and transfusion requirement to analyse any association and predictability.

Statistical analysis:

Data entry was done by using EPIDATA software and converted to Microsoft excel sheet for analysis. Data was analysed using Microsoft excel and IBM SPSS Statistics 21 software.

For continuous data, the descriptive statistics mean, SD, and for non-normally distributed data median, IQR was done. All categorical variables were represented as numbers and percentages. Based on the normality of data, the parametric t-test or non-parametric Mann Whitney test was used to find the difference between groups. The chi-square and Fisher's exact test were used to find the association between categorical variables. The Receiver operating characteristics (ROC) curves were performed and the Youden index was calculated to determine cut-off values. The logistics regression analysis was used to predict the categorical dependent variable using a given set of independent variables. All tests were two-sided at alpha (α) =0.05 level of significance. All the statistical analysis was performed using Statistical Package for Social Sciences (SPSS) version 21.0.

RESULTS

Our study algorithm during the 1 year study period is depicted in Fig 10.

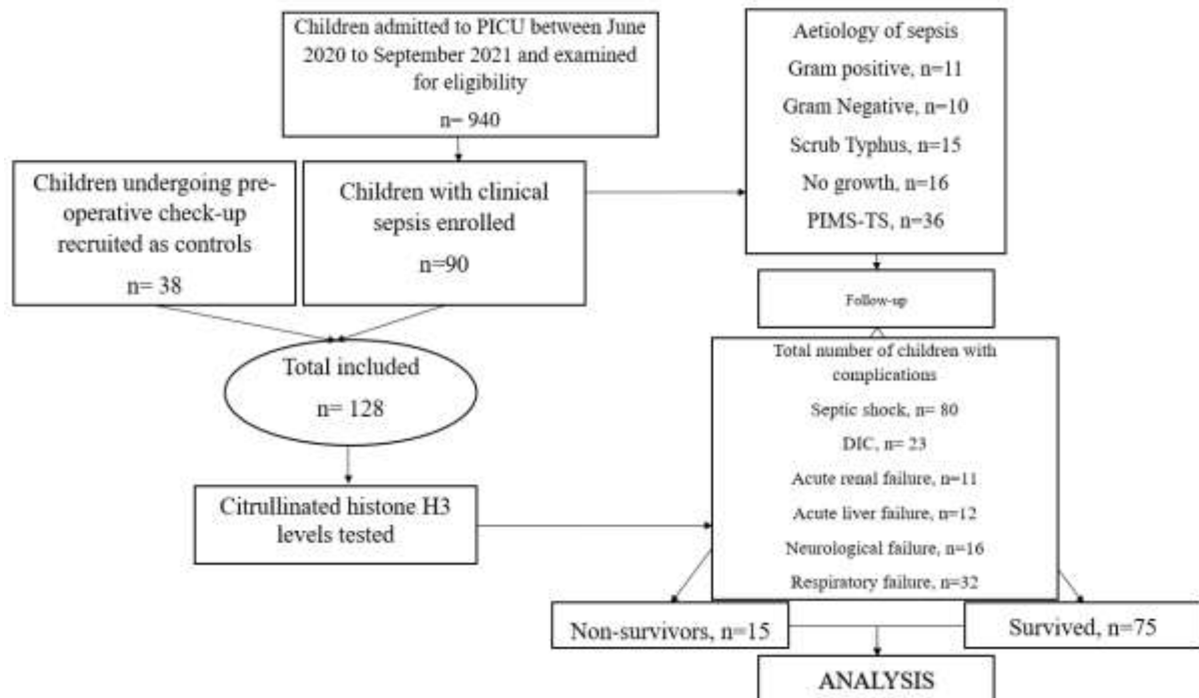


Figure 9: Flow diagram of our study

Baseline characteristics among cases and controls

Total number of children with clinical sepsis were 90. Median age among the clinical sepsis group was 72 months (IQR 24-120). Males were predominant in the sepsis group (55%, n=49). 38 non-sepsis pre-op patients posted for minor surgeries (e.g., circumcision, orchidopexy, herniotomy, laparoscopic cholecystectomy, pyeloplasty for pelvi-ureteric junctional obstruction, cystoscopy, Lymph-node excision biopsy,

colostomy) served as controls. Median age was among controls was 42 months (IQR 12-63). Males constituted 74% (n=28) of the control group.

Aetiology of clinical sepsis in cases

Out of 90 cases, 15 had scrub typhus, 10 were positive for other gram-negative infections, 11 with gram positive organisms. 16 children had no growth in culture and 36 children were diagnosed with Paediatric multi-system inflammatory syndrome temporally associated with COVID-19(PIMS-TS) as shown in Fig.11

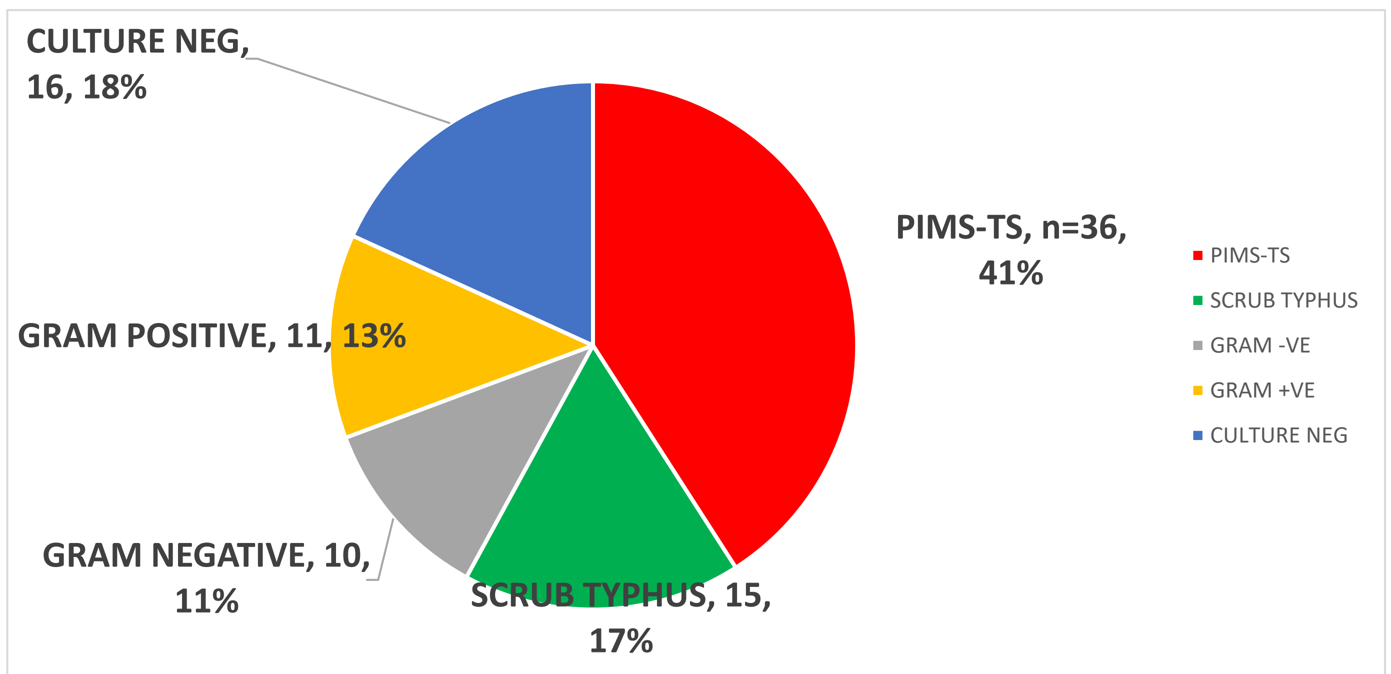
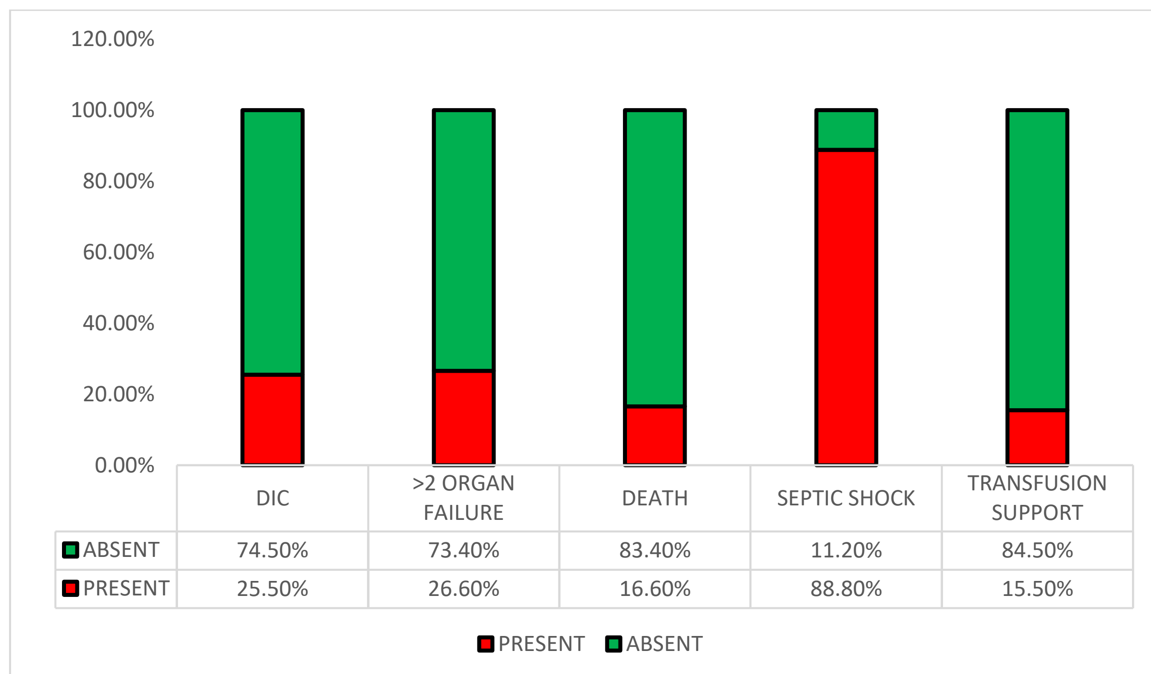


Figure 10: Distribution of cases according to aetiology

PIMS-TS Paediatric multi-system inflammatory syndrome temporally associated with COVID-19

SEPSIS RELATED COMPLICATIONS

As shown in Fig 12, among 90 patients, 23 (25.5%) children had Disseminated intravascular coagulation (DIC), and 14(15.5%) needed transfusion support, 80(88.8%) children had septic shock, 24(26.6%) children had more than 2 organ involvements and 15 (16.6%) children succumbed to illness.



	DIC	>2 ORGAN FAILURE	DEATH	SEPTIC SHOCK	TRANSFUSION SUPPORT
ABSENT	74.50%	73.40%	83.40%	11.20%	84.50%
PRESENT	25.50%	26.60%	16.60%	88.80%	15.50%

Figure 11: Number of children with sepsis related complications

DIC Disseminated Intravascular Coagulation

Levels of Citrullinated histone H3 in plasma of cases and controls

The median (IQR) Citrullinated histone H3(CitH3) levels were significantly elevated in the sepsis group, 1.275ng/ml (0.01-23.10)) when compared to the non-sepsis, 0.35ng/ml (0.15-0.94) controls at enrolment ($p<0.0002$) as shown in Fig. 13.

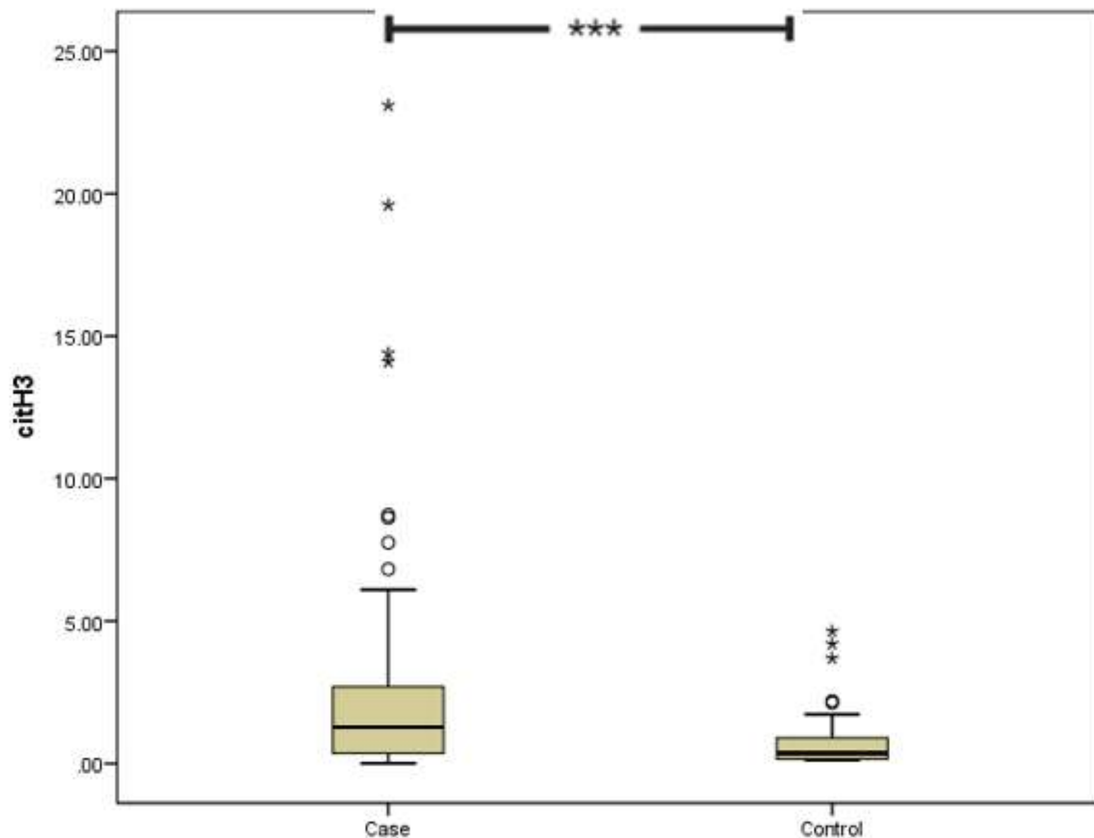


Figure 12: Box and whisker plot comparing Citrullinated histone H3(Cit H3) levels in Cases and Controls

CitH3 and diagnosis of sepsis versus controls

CitH3 levels as a predictor of sepsis in clinical sepsis patients had a discrimination value of 70.6 percent (95 percent CI: 61.0 percent -80.2 percent; p <0.0001). Through Youden index, the best cut off was obtained for CitH3 levels at > 0.899 ng/ml which gave a sensitivity and specificity of 61.11% and 76.32% respectively for a diagnosis of sepsis. A positive likelihood ratio (LR+) of 2.58 and a negative likelihood ratio (LR-) of 0.51 were achieved using CitH3 levels > 0.899ng/ml as a sepsis marker. The Receiver Operator Curve (ROC) curve is shown in Fig 14.

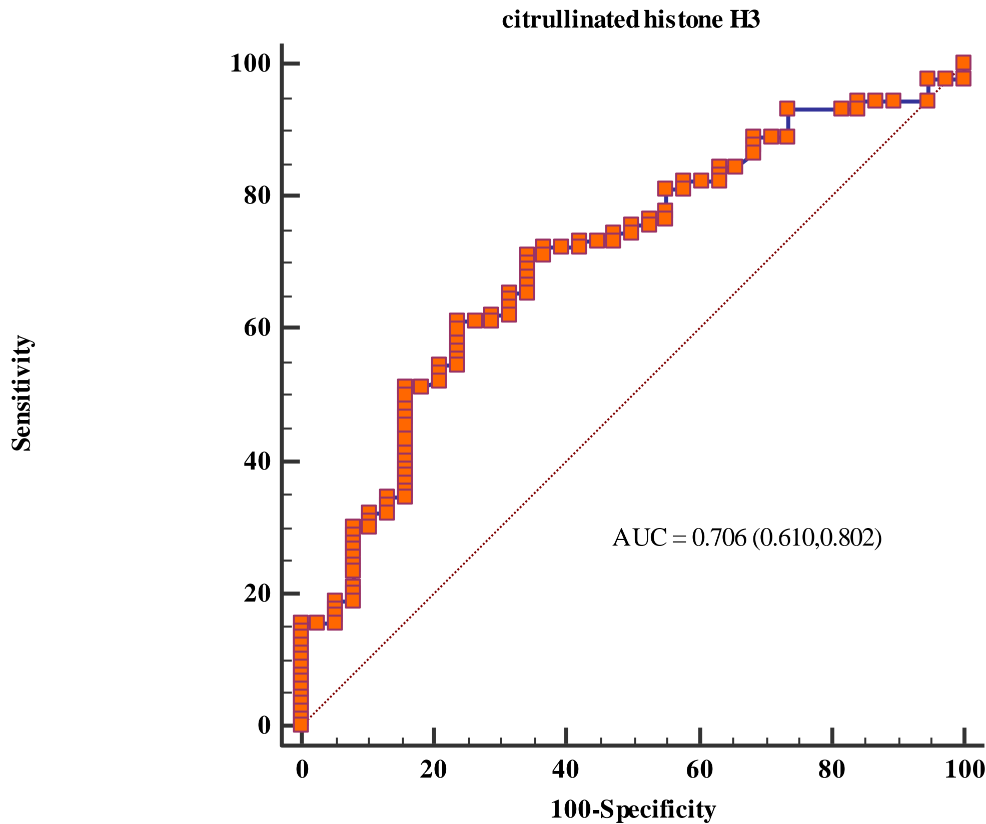


Figure 13: Receiver Operating Characteristics curve of plasma CitH3 levels as sepsis predictor in paediatric sepsis

Baseline characteristics among survivors and non-survivors

As shown in Table 7, among children with sepsis, 75 survived, 15 succumbed to the illness. Among survivors, the median age was 84 months (IQR 1-180) compared to the median age in non-survivors which was 12 months (IQR 1-180) (p=0.132). There was no statistically significant difference in sex distribution among survivors and non-survivors (p=0.508).

Mean Heart rate (HR) was 138 bpm among survivors compared to non-survivors 149 bpm which was not statistically significant (p=0.170). Mean Respiratory rate (RR) was

40bpm among survivors compared to non-survivors 46bpm with no statistical significance (p=0.102). Mean Systolic Blood Pressure was 90mmHg among survivors compared to non-survivors 84mmHg which was not statistically significant (p=0.09). Mean Temperature was 100.9*c among survivors compared to non-survivors 101.1*c which was not statistically significant (p=0.701).

Among the survivors majority were diagnosed with PIMS-TS (45%) when compared to non-survivors (13%) (p=0.0215) and followed by scrub typhus infection (20%) while none of the non-survivor group had scrub typhus infection. There was no significant difference between the infection with other pathogens (Gram-positive, Gram-negative). The others group for whom culture was negative included severe dengue, meningoencephalitis, severe pneumonia, intestinal obstruction and no specific diagnosis, were higher in the non-survivor group (53%) when compared to survivors (14%) p=0.0007).

The median (IQR) DIC score among survivors was 3 (0-7) which was significantly lower than in non-survivors, 5.5 (4.25-6) (p = 0.003). The median (IQR) PDR score was also higher in non-survivors [9.0 (3.0 – 58)] when compared to survivors, [3.0 (1 – 35), p = 0.010].

57.1% in the non-survivor group required transfusions as compared to 42.9% of the Patients in the survivor group (p 0.000). The need for mechanical ventilation was also more in non-survivors (39.1% vs 60.9%, p 0.000). There was no difference in PICU stay (p 0.08) and hospital stay (0.776) among both the groups.

Table 7: *SD Standard deviation, min minimum, max maximum, DIC Disseminated intravascular coagulation, ISTH International society of thrombosis and haemostasis, PDR predicted death rate, PICU Paediatric intensive care unit, PIMS-TS Paediatric Inflammatory multisystem Syndrome Temporally Associated with SARS-CoV-2, *Indicates a significant value, p < 0.05.*

Table 7: Baseline characteristics among survivors and non-survivors

	SURVIVORS	NON-SURVIVORS	p-value
Number	75	15	
Demographics			
Age (months), median (min/max)	84(1-180)	12(1-180)	0.132
Male (n=49)	56% (n=42)	47% (n=7)	0.508
Female (n=41)	44% (n=33)	53% (n=8)	0.508
Vitals at admission			
Heart rate (bpm)	138(+/-28.6)	149.5(+/-19.5)	0.170
Respiratory rate (bpm)	40.6(+/-10.9)	46(+/-13.8)	0.102
Systolic blood pressure (mmhg), mean SD	90.9(+/-12.5)	84.7(+/-14.6)	0.095

Temperature (*c), mean SD	100.9(+/-1.5)	101.1(+/-2.09)	0.701
	SURVIVORS	**NON-SURVIVORS**	**p-value**
Sepsis aetiology			0.001*
gram positive (n=11)	12% (n=9)	13% (n=2)	0.9144
gram negative (n=10)	9% (n=7)	21% (n=3)	0.1775
scrub typhus (n=15)	20% (n=15)	0	0.0592*
PIMS-TS (n=36)	45% (n=34)	13% (n=2)	0.0215*
Others (n=18)	14% (n=10)	53% (n=8)	0.0007*
Clinical scoring			
DIC (ISTH score), median(min/max)	3(0-7)	5.5(4.25-6.00)	0.003*
PDR score, median(min/max)	3(1-35)	9(3-58)	0.010*
Treatment			
Mechanical ventilation(n=23)	39.1% (n=9)	60.9% (n=14)	0.000*
Transfusion support(n=14)	42.9% (n=6)	57.1% (n=8)	0.000*
PICU stay (days), (median(min/max))	3(2-20)	7(1-17)	0.089

Hospital stays (days), (median(min/max))	7(4-26)	7(2-17)	0.776

Frequency of individual organ failures among survivors and non-survivors

Fig. 15 shows that 66 percent (n=10) of non-survivors had DIC, whereas only 17 percent (n=13) of survivors had DIC (p=<0.0001). Acute renal failure occurred in 3 patients (4%) among the 75 survivors compared to 8 (53%) among 15 non-survivors (p=<0.0001). Acute liver failure was found in 6% (n=5) of survivors compared to 46 percent (n=7) of non-survivors (p=<0.0001). Respiratory failure was in 22 percent of survivors (n=17) compared to 100 percent of non-survivors (n=15) (p=<0.0001). Neurological dysfunction was found in 9 children (12%) among survivors compared to 7 (46%) of non-survivors (p=0.001). Septic shock affected 65 (86%) of survivors compared to all children (100%) in the non-survivor group (p=0.683).

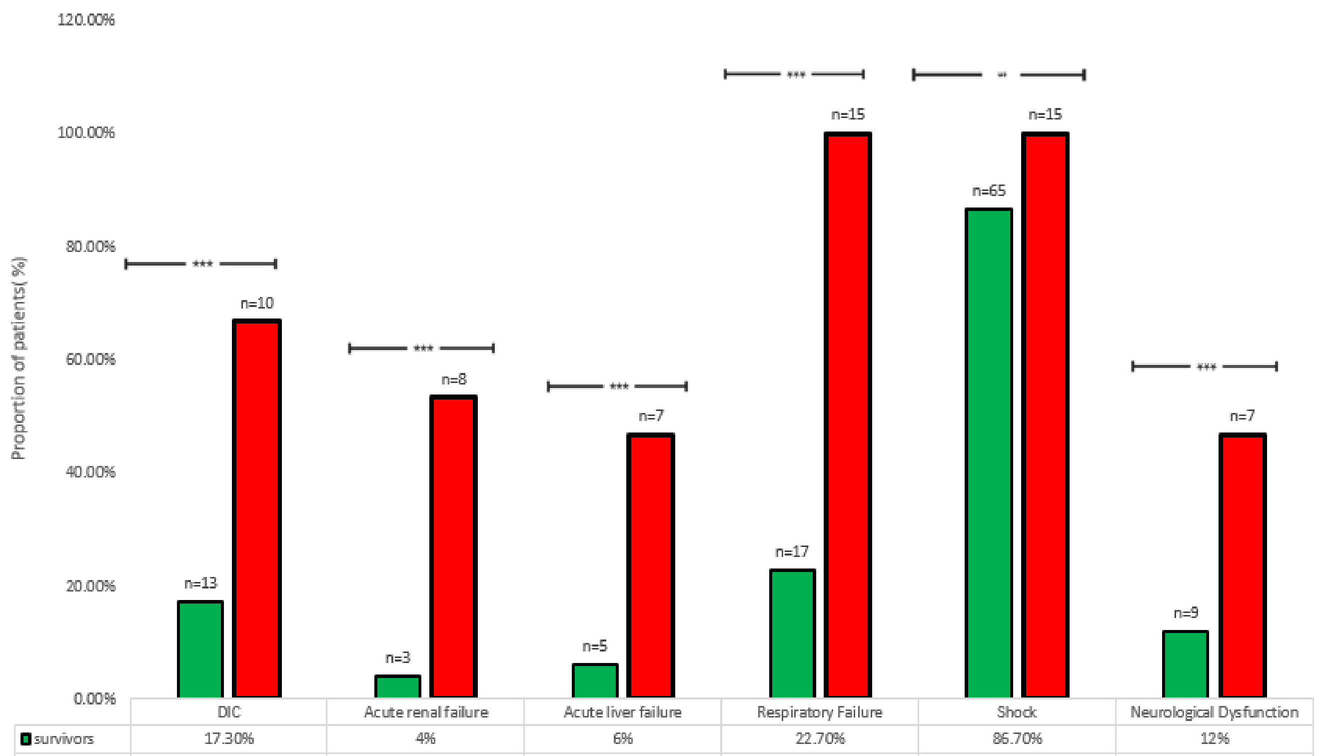


Figure 14: Proportion of patients with each organ failure in survivors versus non-survivors

DIC Disseminated Intravascular Coagulation

LABORATORY PARAMETERS IN SEPSIS

Table 8 shows that there was no significant difference in mean haemoglobin levels and median platelet counts between the survivors and non-survivors (p=0.406). The median total counts were significantly elevated in the non-survivors' group (13,200/cumm (9100-26,000) (p=0.03) compared to survivors (10,100/cumm (2800-31,400). Prothrombin Time (PT) in non-survivors [25.2 (+/-9.5)] was significantly higher than in survivors[18.8 secs (+/-5.9) p=0.001], Activated Partial Thromboplastin Time (APTT) in non-survivors [44 secs (29.3-154)] was significantly higher than survivors [38.9 secs (23-73 secs) p=0.019] and fibrinogen was significantly reduced in the non-survivors' group [202 mg/dl (83-654)] compared to survivors [356.5mg/dl (86-695), p

= 0.01]. There was no difference in the liver and renal function among the two groups. Admission lactate was also similar. There was no significant difference in inflammatory markers levels like CRP, Procalcitonin.

Table 8: *SD Standard deviation, min minimum, max maximum, Hb Haemoglobin, TC Total count, PT Prothrombin time, INR International normalised ratio, APTT activated partial thromboplastin time, CRP c-reactive protein, *Indicates a significant value, p< 0.05.*

Table 8: Comparison of levels of blood biomarkers between survivors and non-survivors.

Blood biomarker	**Survivor**	**Non-survivor**	**P value**
	75	15	
Hb (g/dl), mean/SD	10.61(+/-1.88)	10.14(+/-2.44)	0.406
TC(/cumm), median(min/max)	10,100(2800-31,400)	13,200(9100-26,000)	0.035*
COAGULATION FUNCTION			
Platelet(/cumm), (median-min/max)	1,54,000 (4000-5,12,000)	1,60,000 (91,000-4,34,000)	0.439
PT (secs), (mean/SD)	18.8(+/-5.9)	25.2(+/-9.5)	0.001*
INR, (mean/SD)	1.37+-0.45	1.85+-0.75	0.002*
APTT (secs), (median-min/max)	38.9(23-73)	44(29.3-154)	0.019*

Fibrinogen(mg/dl), (median(min/max))	356.5(86-695)	202(83-654)	0.031*
RENAL FUNCTION			
Creatinine(mg/dl), mean SD	0.48(0.20-1.41)	0.38(0.28-1.13)	0.914
LIVER FUNCTION			
SGOT(I.U.), (median(min/max))	50.5(8-3460)	95(18-3502)	0.078
SGPT(I.U.), (median(min/max))	40(8-4132)	48(5-1683)	0.812
CIRCULATORY			
Lactate(mmol/L), (median(min/max))	2(0.6-7.1)	2.9(0.9-15)	0.101
INFLAMMATORY MARKERS			
CRP (mg/dl), (median(min/max))	107(3-406)	86(3-342)	0.290
Procalcitonin(ng/ml), (median(min/max))	12(0.13-588)	11.59(2-57)	0.919

Association between CitH3 levels and mortality

As depicted in Figure 16, there was no significant difference between the median CitH3 levels in survivors' [1.16ng/ml (0.01-19.60)] versus the non-survivors' [1.35ng/ml (0.14-23.1), (p=0.669)] among the paediatric clinical sepsis patients.

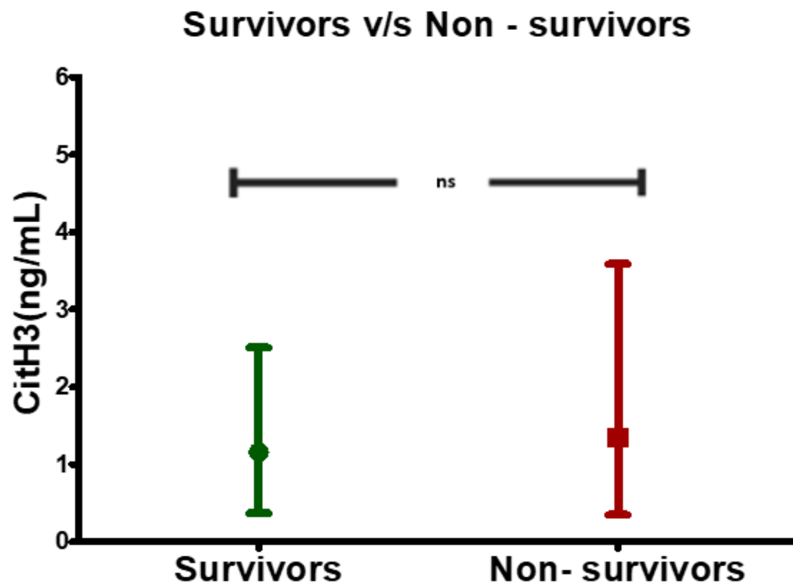


Figure 15: Association between Citrullinated histone H3(CitH3) and Mortality

Association between CitH3 levels and disseminated intravascular coagulation (DIC)

Figure 17 shows that the median Cit H3 levels was not significantly different between patients with DIC (1.44ng/ml (0.01-23.1) and without DIC (1.12ng/ml (0.01-14.4)), (p=0.614).

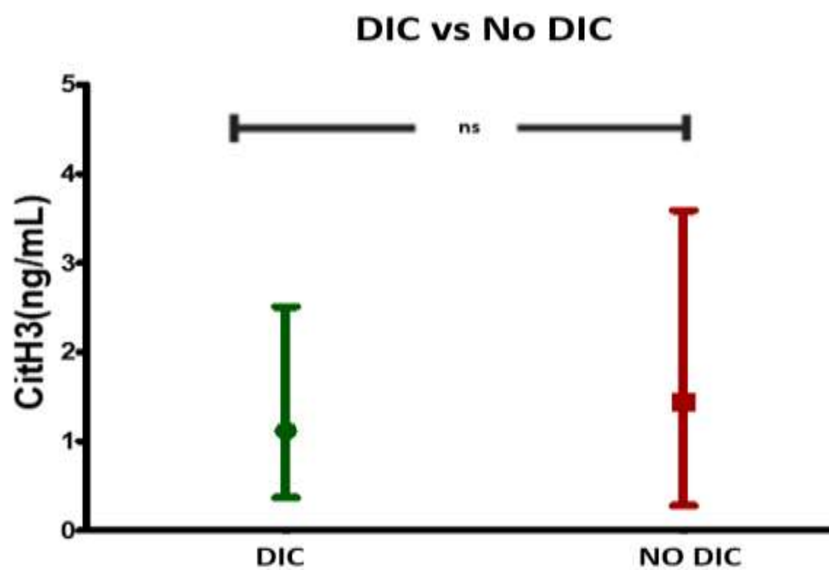


Figure 17: Association between CitH3 levels and disseminated intravascular coagulation (DIC)

Association between the number of organ failures and CitH3 levels

As shown in Fig 18, the median Circulating histone H3 levels among patients with ≥ 2 organ failures [1.65 (0.55-4.7)] was not significantly different than in those with less than 2 organ failures [1.09 (0.28-2.19)], ($p = 0.06$).

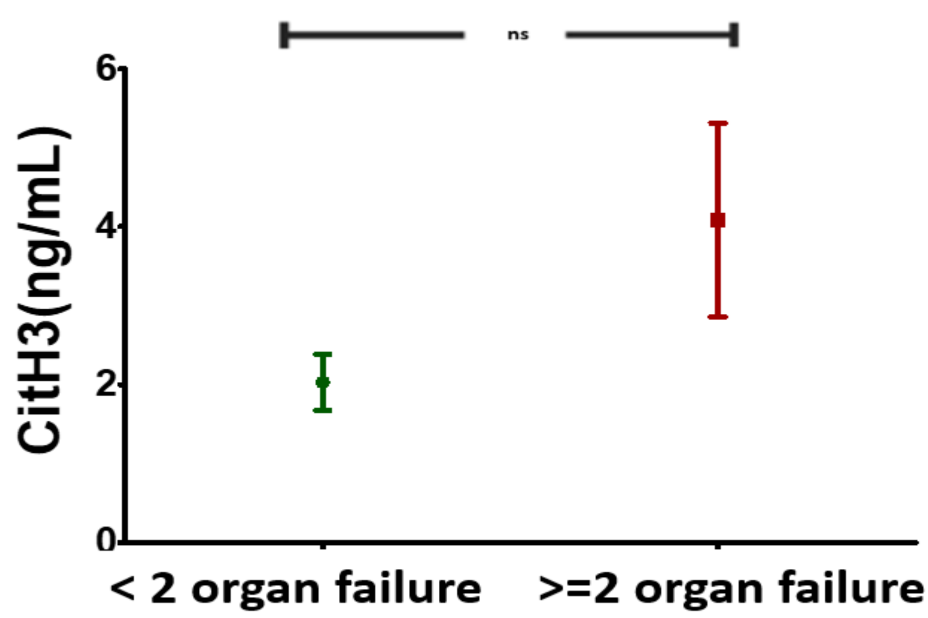


Figure 18: Association between Cit H3 and number of organ failures

Association of CitH3 with aetiology of sepsis

The median Cit-H3 values in each of the aetiologies are shown in Figure 20. Highest values were seen in Scrub typhus, followed by PIMS-S, Statistical analysis demonstrated that median (IQR) Cit H3 values in children positive for Scrub typhus (2.480ng/ml (0.53-8.62)) and those diagnosed with PIMS-TS (1.36ng/ml (0.16-19.6))

were significantly higher than those with gram-negative infections (0.24ng/ml (0.14-2.50) (p 0.0042) and from the control population 0.35ng/ml (0.15-0.94). There was no significant difference in CitH3 between scrub typhus, PIM-TS, gram positive [0.520ng/ml (0.01-14.10)] and culture negative sepsis patients [(0.775ng/ml (0.140 - 23.10), (p=>0.05)]. The median Cit H3 levels in children with PIMS-TS (1.36ng/ml (0.16-19.6) were significantly higher than controls who's median Cit H3 levels were 0.35 ng/ml (0.12-4.64) with p-value <0.0001. The median CitH3 levels in scrub typhus cases (2.480 ng/ml) were also significantly higher than controls(p<0.0001). However, those with gram-positive, gram-negative infections and culture negative reports did not have significantly different levels from the controls.

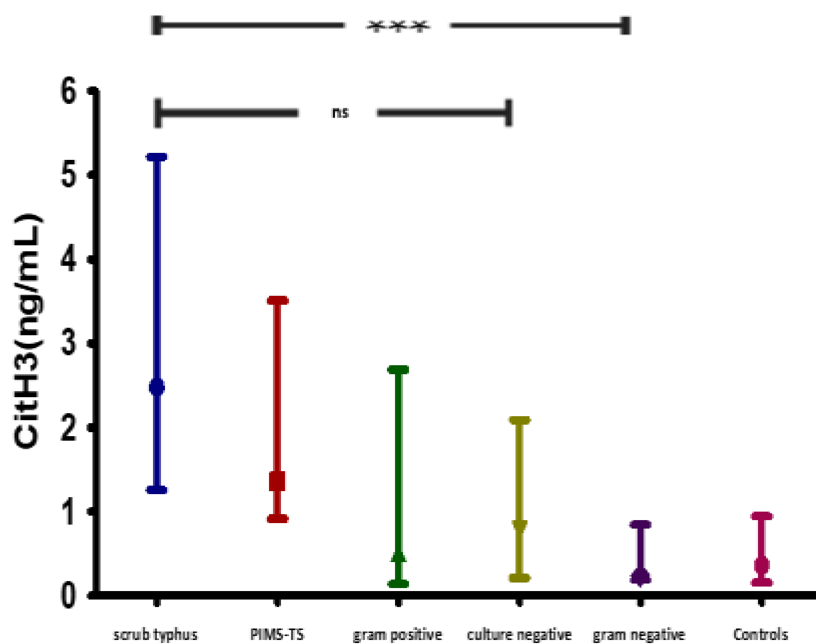


Figure 19: Association of Cit-H3 with the aetiology of sepsis

Association between CitH3 and PDR score

The median Cit H3 levels in patients with PDR score 0-10 was 1.16ng/ml (0.01-19.6), PDR score 10-20 was 1.37ng/ml (0.16-14.4) and for PDR score >20 was 0.68 (0.16-23.1). There was no statistical significance between these three groups (p=0.44) as shown in Figure 21.

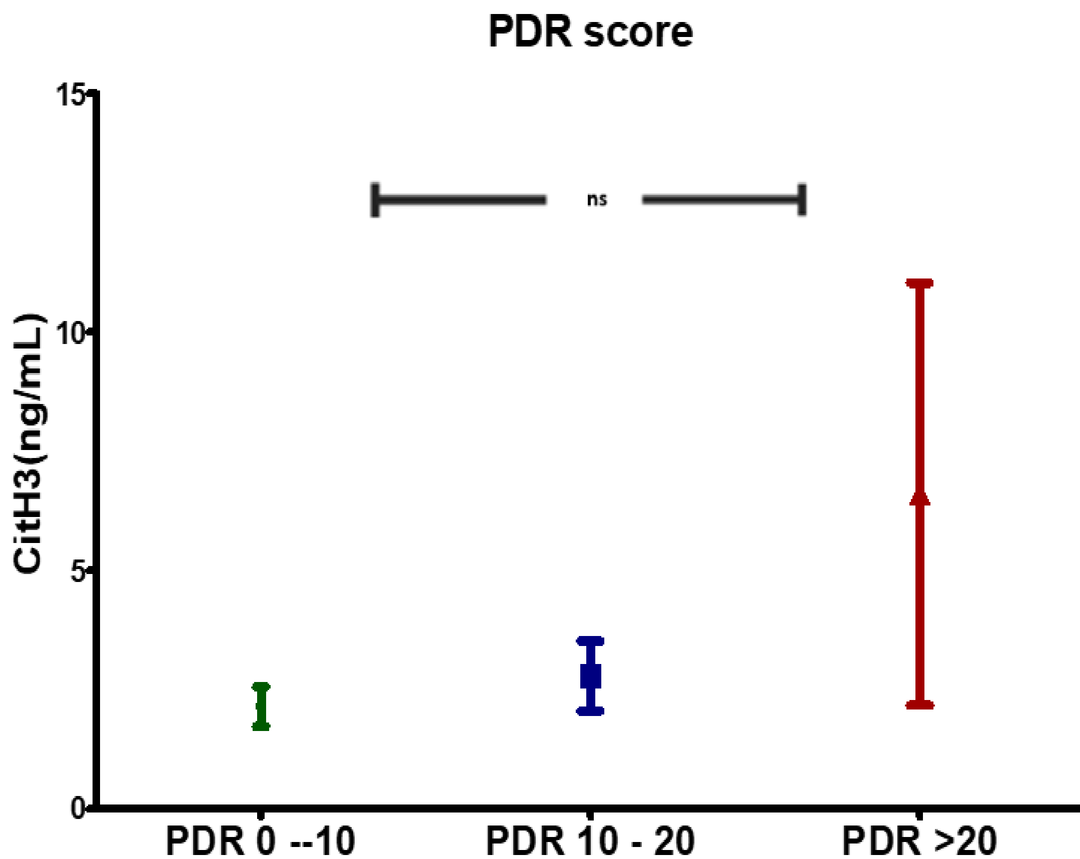


Figure 20: Association between PDR score and CitH3 levels

PDR scoring system and mortality prediction

In clinical sepsis, the area under the curve (AUC) for PDR score derived at admission as a mortality predictor was 70.7 percent (95 percent CI: 55.6-85.9%; p=0.007). 0.378 was the Youden index. With 100 percent sensitivity and 37.84 percent specificity, the best cut-off point for hour 0 PDR score to predict early mortality in clinical sepsis paediatric patients was >2. The ROC curve is depicted in Figure 22.

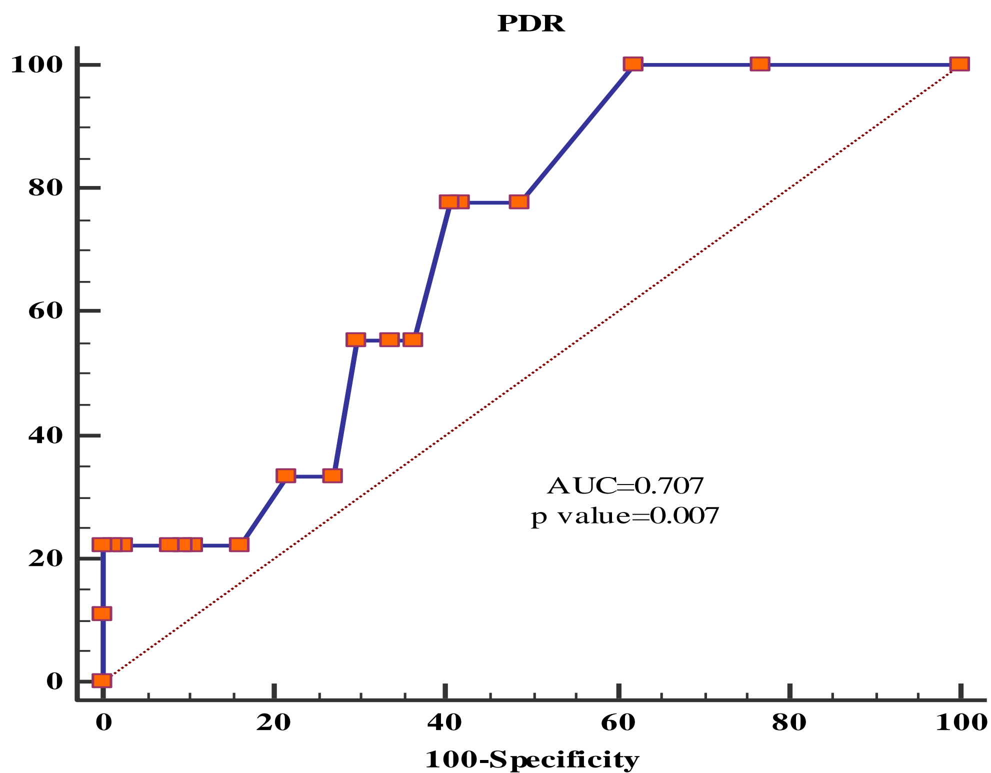


Figure 21: Receiver Operating Characteristics curve of initial PDR score at admission as a mortality predictor in children admitted to PICU

ROC curve of blood markers and clinical scores for predicting mortality in paediatric patients with clinical sepsis

The ability of selected biomarkers, PDR, and DIC scores to predict mortality in children with clinical sepsis according to ROC curve analysis is shown in Table 9 and Fig. 22. The optimal cut-off value for total count was 20,300/cumm, with sensitivity of 40.0%, and specificity of 94.67% (AUC 0.673, 95% CI 0.515–0.831, P = 0.0320); 2.6 mmol/l for lactate with 60% sensitivity and 69.86% specificity (AUC 0.635, 95% CI 0.460–0.810, P = 0.131); 2.62 ng/ml for CitH3 with 40% sensitivity and 77.33% specificity (AUC 0.535, 95% CI 0.355–0.715, P=0.701); 18.3 s for PT with 93.33% sensitivity and 65.22% specificity (AUC 0.794, 95% CI 0.687–0.901, P <0.0001); 48.60 s for APTT with 46.67% sensitivity and 86.96% specificity (AUC 0.693, 95% CI 0.536–0.850, P = 0.0157); 1.43 for INR with 86.67% sensitivity and 71.01% specificity (AUC 0.795, 95% CI 0.689–0.901, P <0.0001), 242 mg/dl for fibrinogen, with sensitivity of 81.82%, and specificity of 72.58% (AUC 0.705, 95% CI 0.496–0.913, P = 0.0546); a score of 2 for predicted death rate(PDR) with 100% sensitivity and 37.84% specificity (AUC 0.711, 95% CI 0.585–0.837, P = 0.0010); a score of 4 for DIC with 66.67% sensitivity and 82.67% specificity (AUC 0.740, 95% CI 0.568–0.912, P=0.0062). The ROC curve is detailed in Fig 23.

Table 9: *AUC Area under the curve, CI confidence interval, INR international normalized ratio, PT prothrombin time, APTT activated partial thrombin time, Fib fibrinogen. CitH3 Citrullinated histone H3, PDR score predicted death rate, DIC score disseminated intravascular coagulation score, *Indicates a significant value, P < 0.05.*

Table 9: Prognostic value of clinical parameters to predict mortality

Variable	AUC	95% CI	Cut-off	specificity	sensitivity	p-value
Total count(/cumm)	0.673	0.515-0.831	>20,300	94.67%	40.0%	0.0320*
PT(secs)	0.794	0.687 to 0.901	>18.3	65.22%	93.33%	<0.0001*
INR	0.795	0.689 to 0.901	>1.43	71.01%	86.67%	<0.0001*
APTT (secs)	0.693	0.536 to 0.850	>48.6	86.96%	46.67%	0.0157*
Fibrinogen (mg/dl)	0.705	0.496 to 0.913	≤242	72.58%	81.82%	0.0546*
Lactate (mmol/L)	0.635	0.460 to 0.810	>2.6	69.86%	60%	0.1312
CIT H3(ng/ml)	0.535	0.355 to 0.715	>2.62	77.33%	40.0%	0.7018

PDR score	0.711	0.585 to 0.837	>2	37.84%	100%	0.0010*
DIC score	0.740	0.568 to 0.912	>4	82.67%	66.67%	0.0062*

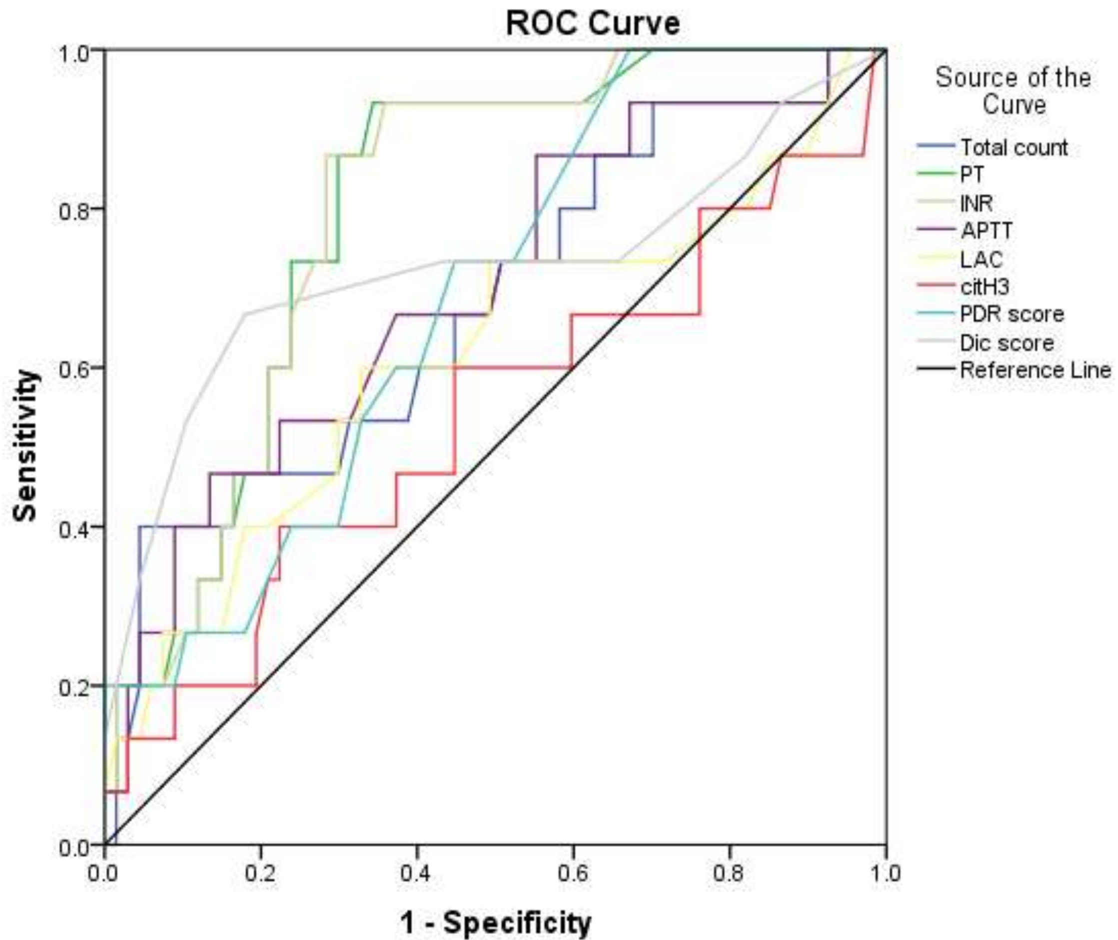


Figure 22: The receiver operating characteristic (ROC) curves of the selected variables for predicting mortality of paediatric with clinical sepsis

The ROC curves for variables had the following areas: total count, 0.673; Prothrombin time, 0.794, international normalized ratio (INR), 0.795; Activated partial thromboplastin time, 0.693; lactate, 0.635; citrullinate histone H3, 0.535, predicted death rate, 0.711 and disseminated intravascular coagulation score, 0.740.

Logistic regression analysis of blood biomarkers and clinical scores as risk factors for mortality.

In this study, the variables which were significantly different ($p < 0.05$) between survivors and non survivors in the initial analysis, like Total count, PT, INR, APTT, fibrinogen and DIC score were included in the univariate analysis. Cit H3 and lactate were also included. In the univariate analysis by logistic regression method, the reduced levels of fibrinogen <242mg/dl, Prolonged PT > 18.3 secs, Increased INR >1.43, Prolonged APTT > 48.6 secs, increased lactate > 2.6 mmol/L and a DIC score > 4 were associated with mortality in sepsis patients. In the multivariate analysis, the following parameters were included - total count, INR, APTT, lactate, fibrinogen and DIC score. However, multivariate analysis, showed that only reduced Fibrinogen <242mg/dl [odds ratio (OR) 12.388; 95% confidence interval (CI) 1.335-114.980; p = 0.027] and increased INR > 1.43 (OR 15.740; 95% CI 1.236–200.426; p= 0.034), within the first 24 h of admission were independently associated with mortality of sepsis (Table 10). Other variables like total count, lactate, APTT and DIC score were not significant predictors of sepsis mortality in multivariate analysis.

Table 10:. *OR odds ratio, CI confidence interval, INR international normalized ratio, PT prothrombin time, APTT activated partial thrombin time, Fib fibrinogen. CitH3 Citrullinated histone H3, PDR score predicted death rate, DIC score disseminated intravascular coagulation score, *Indicates a significant value, P < 0.05.*

Table 10: The logistic regression analysis predicting mortality in paediatric patients with sepsis admitted to PICU

Variable	Univariate analysis			Multivariate analysis		
	OR	95% CI	p value	OR	95% CI	p value
Total count	11.833	2.796-50.083	0.001*			
PT	26.250	3.252-211.883	0.002*			
INR	15.925	3.290-77.078	0.001*	15.740	1.236-200.426	0.034*
APTT	5.833	1.700-20.013	0.005*			
Fibrinogen	11.912	2.332-60.833	0.003*	12.388	1.335-114.981	0.027*
Lactate	3.477	1.104-10.956	0.033*			

CIT H3	2.275	0.709-7.298	0.167
DIC score	9.538	2.791-32.593	0.000*

DISCUSSION

In sepsis patients, early diagnosis and commencement of effective antimicrobial therapy are critical in improving clinical outcomes (23). Even with advent of effective antibiotics and diagnostic tools, it is still one of the major causes of death in individuals of all ages around the world. Sepsis entails not only the complicated impacts of systemic inflammation and immunological dysfunction, but also the acute failure of several organ systems across the body.

As a result, we need to understand the molecular pathways and processes involved in sepsis pathophysiology in order to intervene at the biomolecular level in the hopes of achieving a better outcome and developing novel diagnostic and therapeutic approaches to improve outcome.

CitH3 levels have been examined as a possible predictive biomarker and therapeutic target in a number of researches till date. The purpose of our research was to see if there is an association between Cit H3 levels in sepsis children in terms of complications of sepsis such as DIC, MODS, and to see if greater CitH3 levels suggest more severe disease also whether this biomarker can be used as a mortality predictor when done at admission in children with clinical sepsis as it was shown in septic shock murine models (1) and other preceding studies.

Our control group consisted of a greater number of males and younger age group because orchidopexy and circumcision were the most frequent operations for which the children were scheduled.

We employed an anti-CitH3 antibody ELISA Kit (Citrullinated Histone H3 (Clone 11D3 by Cayman chemical) that selectively recognises regions of peptidyl deiminase (PAD) 4 citrullination (R2 + R8 + R17) on the Cit H3 protein in our study (28). The utility of ELISA to assess CitH3 levels in humans has been validated previously (12). PADs, or peptidyl arginine deiminase enzymes, catalyse citrullination. Only nuclear located PAD2 and PAD4 can citrullinate histone H3 and produce NETosis. (18, 26-27). Tian et al used an in-house prepared ELISA kit which could detect citrullination histones by both PAD 2 and PAD 4 enzymes stating that current commercial ELISA kits can only recognise Peptidyl arginine deiminase (PAD) 4 citrullination (R2 + R8 + R17) on the CitH3 protein (23)(46). From this we hypothesize that in our study PAD2 catalysis could have gone undetected. Therefore, we deduce that the use of an ELISA technique which can detect citrullination by both PAD2 and PAD4 enzymes may prevent underestimation.

In our study, we identified that children having clinical sepsis showed significantly higher levels of citrullinated Histone H3(CitH3) compared to controls and our study. This was consistent with both Murine and human models which showed that CitH3 levels were high in case of septic shock (2),(26),(27). However, CitH3 values were very heterogeneously distributed with many overlapping with the controls.

In our study, Cit H3 performed fairly as a diagnostic test for sepsis and had a AUC value of 0.70. The cut off for detecting sepsis was 0.899 ng/ml with a sensitivity of 61.11% and specificity of 76.32%. Some prior studies in the paediatric population showed that the discrimination value of Cit-H3 levels as a predictor of sepsis in clinical sepsis patients was 77.2% (95% CI: 64.6-89.7%; $p<0.001$) and a cut off of Cit-H3 >1,200

ng/mL was able to predict sepsis event in children with 83.3% sensitivity and 75.7% specificity. (29). Another study in adults found Cit H3 showed an excellent diagnostic power for CitH3 to differentiate septic shock from a healthy state with an AUC (95% CI) 0.91 (0.82–0.99) ($p < 0.0001$) and the cut-off value of CitH3 level above 39 pg/mL was highly predictive of patients with septic shock compared to healthy volunteers.(46)

In comparison to our investigation, Table 11 summarises the studies that have shown CitH3 as a marker for sepsis that we are aware of.

Table 11: **Indicates a significant value, P < 0.05*

Table 11: Summary of studies showing CitH3 as a diagnostic marker of clinical sepsis,

Studies	**AUC**	**95% CI**	**Cut-off (ng/ml)**	**Specificity**	**Sensitivity**	**p-value**
Our study	0.706	0.610-0.802	>0.899	76.32%	61.11%	<0.0001*
Chandra et al	0.772	0.646- 0.897	>1200	75.70%	83.30%	<0.001*
Tian et al	0.910	0.820- 0.990	>0.039	89.47%	87.25%	<0.0001*

Research has shown that histones are toxic and pro-inflammatory as well. Studies have theorized that circulating histone H3 is pro-coagulant in nature. They have the propensity to damage endothelial cells, activate platelets, and impede fibrinolysis cumulatively leading to immune-thrombosis and DIC (19).They are attributed to dysregulated inflammatory response, impaired endothelial barrier, which can lead to circulatory failure, ARDS, renal failure, and liver failure, altogether causing Multiple

organ dysfunction (MODS) leading to death in the most severe form of sepsis(4). However, in our study, we were not able to identify a significant association between Cit H3 levels with complications of sepsis such as DIC, multi-organ dysfunction, and non-survival($p>0.05$). However, previous research from other investigators has shown that Cit H3 is a potential prognostic biomarker in sepsis in both adults and children. (24-25). One study showed that Serum histone H3 levels in patients with coagulation failure (11.1 [2.2–48.4] ng/mL) were significantly higher than those in patients without coagulation failure. Serum histone H3 levels in patients without organ failure were 1.0 (0.2–2.7) ng/mL, while those in patients with one, two, and three or more organ failures were 3.0 (1.1–5.3), 4.9 (2.5–9.2), and 5.5 (2.2–36.1) ng/mL, respectively. They were significantly higher in those with multi-organ failure as compared to those without organ failure ($p<0.05$). Serum histone H3 levels in non-survivors (9.1 [5.1–13.0] ng/mL) were significantly higher than those in survivors (3.4 [1.5–6.1] ng/mL) at day 1 (28). Table 12 summarises studies to our knowledge which have looked at CitH3 as a prognostic marker.

Table 12 *DIC Disseminated intravascular coagulation, *Indicates a significant value,* $P < 0.05$

Table 12: Summary of available studies showing CitH3 as a prognostic marker,

	Yokoyama et al(n=85)		**Ito et al (n=81)**		**Chandra et al(n=66)**		**Our study(n=90)**	
Prognosis	**Median**	**p value**	**Median**	**p value**	**Median**	**p value**	**Median**	**p value**
Survivor	3.4 (1.5-6.1)	<0.05*	-	-	1100ng/ml (800-25900)	0.016*	1.16 (0.01-19.6)	0.669

Non-survivors	9.1 (5.1-13)		4.75	-	1175 (950-32160)	1.35 (0.14-23.1)	
DIC	11.1(2.2-48.4)	<0.05*	3.55	-	-	1.44 (0.01-23.1)	0.614
No-DIC	3.5 (1.5-5.9)		-	-	-	1.12 (0.01-14.4)	
Organ failure	4.9 (2.5-9.2)	<0.05*	-	-	-	1.65 (0.55-4.7)	0.06
No organ failure	1 (0.2-2.7)		-	-	-	1.09 (0.28-2.19)	

A study by Tian et al has shown that, while CitH3 levels at the time of initial presentation were not significantly different between those who survived and those who did not, CitH3 concentrations at 24 h (p 0.01) and 48 h (p 0.05) were significantly higher in Septic shock patients who died within 90 days compared to those who survived and the AUCs (95 percent CI) for mortality were statistically significant at 24 h [0.72 (0.60–0.83)] and 48 h [0.66 (0.51–0.81)], but not at enrolment 0 h [0.52 (0.39–0.64), according to the ROC analysis. We infer from these findings that initial Day 1 measurements of CitH3 may not act as a prognostication factor unless performing serial monitoring of values. Furthermore, a CitH3 level of more than 307 pg/mL at 48 hours was 96.72 percent accurate in predicting death within 90 days.

(46). Another study in children (n=60) with meningococcal sepsis found that another marker of NETs i.e., MPO-DNA levels (on admission and 24-hour) was neither associated with disease severity nor mortality. They concluded that a smaller sample size and difference in the sepsis model and balanced charge between NETosis and cell-free-DNA affected the quantification and measuring instruments (47).

Among the cases recruited a fair number of children were detected to have scrub typhus infection in our study and we have detected that Cit H3 levels in these were also significantly elevated in children compared to gram-positive, other gram -negative infections and non-sepsis controls. These findings were similar to a study done by Paris et al (48) where they investigated the relationship of markers of neutrophil activation and cell death with disease severity in patients with acute scrub typhus. They identified strong correlations between circulating markers of cell death and neutrophil activation (like nucleosome releasing factor(FSAP) and Neutrophil elastase complex(ELA)) in patients with scrub typhus, providing indirect evidence that neutrophil extracellular traps could contribute to the vascular damage and pro-coagulant state leading to exacerbation of disease in scrub typhus, thus indicating the detrimental role of neutrophil activation. (48)

As our study was conducted during the COVID-19 pandemic, majority of the children who presented with features of septic shock at admission were diagnosed with PIMS-TS eventually. Paediatric inflammatory multisystem syndrome temporally associated with severe acute respiratory syndrome coronavirus 2(SARS-CoV-2)-PIMS-TS currently known as Multisystem inflammatory syndrome of children (MIS-C) is a

recent, rare phenomenon which is a post-infectious complication of SARS-CoV-2 infection in children, where patients present with a septic shock-like picture and multiorgan dysfunction. Therefore, we were able to analyse Cit H3 levels in these children. In our study we also found that the Cit H3 levels in the PIMS-TS group were significantly elevated when compared to controls. We also detected that the Cit H3 levels were significantly elevated in PIMS-TS when compared to bacterial causes of sepsis i.e., gram-negative infections**.**

A study by Zuo et.al (49) revealed that serum levels of CitH3 were increased in coronavirus disease 2019 (COVID-19) patients. Several other studies have shown that Cit H3 levels were raised in COVID 19 infection(50), (51). One recent study by Seery et al has revealed that Citrullinated Histone H3 levels were high in children with MIS-C compared to those with asymptomatic, mild, and moderate COVID-19 disease(52). We deduce that these higher levels in these cases could be due to heightened inflammatory response and cytokine storm seen in COVID-19 compared to other infections. Because this is a new occurrence, the implications of many biomarkers will be highly beneficial in future research for elucidating pathophysiology in COVID-19 disease.

Also, the median CitH3 levels in scrub typhus infections were significantly higher than in non-sepsis controls but the median Cit H3 levels in gram positive, gram negative infections were not significantly different from those in non-sepsis controls for reasons unknown for which further investigation in the future is required to elucidate this finding.

Cit H3 levels have been known to be elevated in other diseases associated with an exacerbated inflammatory response such as Auto-immune diseases, trauma, malignancy (24) which is why we excluded these cohorts of children in our study.

Our study involved the use of Paediatric index of mortality 2 score (PIM2) score for Predicted death rate (PDR), which one of the severities scoring system assigned to all children at admission to PICU. PIM2 score in other studies has discriminated well between survival and death at PICU when done on children at admission in many tertiary paediatric care hospitals (53).

Our study showed that the criterion for hour 0 PDR score to predict early mortality in clinical sepsis paediatric patients was >2 with a 100 percent sensitivity and 37.84 percent specificity. We tried to correlate Cit H3 levels with the PDR PIM-2 score to see whether any association between higher scores and raised CitH3. But there was no significance noted between the levels of Cit H3 levels and PDR score.

We performed a logistic regression analysis using variables which were significantly different between the survivors and non-survivors to evaluate them as predictors of death. We have found that an elevated INR and reduced fibrinogen were independently valuable for predicting mortality in clinical sepsis patients. INR performed fairly as a standalone outcome predictor with an 86.67% sensitivity and 71.01% specificity and an AUC of 0.795 in our study. It is said that in sepsis, due to the inflammatory response associated procoagulant pathways are upregulated while natural anticoagulants are downregulated, resulting in coagulopathy. Despite the fact that sepsis produces significant changes in the overall coagulation profile, the variations in the amounts of

specific coagulation components in individuals with sepsis-induced coagulopathy are less well characterised. Because sepsis induced coagulopathy results in a thrombotic phenotype it has been linked to sepsis-related multiorgan dysfunction, failure and subsequent mortality in a number of studies. In a study by Liu et al multivariate analysis demonstrated that INR level was an independent factor for critically ill patients with sepsis, and high INR (> 1.47) was significantly associated with increased mortality risk(54) which are similar to the findings in our study. Similar results were demonstrated in another study (55). The PT/INR was developed to monitor the anticoagulation status of warfarin-treated patients and is commonly used for this reason in clinical practise. This suggests that the information provided by this common laboratory test may be significantly different in these two patient populations. In liver disease, INR is connected to protein synthesis impairment (56). The prognosis of liver disease is intrinsically connected to the advancement of protein synthesis dysfunction. We believe that a high INR in sepsis is linked to the presence and degree of organ failure, and so can be used to predict poor prognosis and mortality. (56)

Fibrinogen is a biomarker that is used to identify coagulopathy in critically sick individuals. As an acute phase reactant, increased fibrinogen levels (hyper-fibrinogenaemia) can be detected in the setting of inflammation or tissue injury, whereas low levels of fibrinogen can signal a systemic activation of the clotting system, with clotting factor consumption outpacing synthesis. A recent study indicated that in adult patients with severe sepsis, a fibrinogen level of less than 200 mg/dL is connected to a considerably greater mortality risk which is similar to the results of our study. In

paediatric investigations, high levels of fibrinogen were linked to sepsis diagnosis, while low levels were linked to sequelae such septic shock and DIC. (57) (58)

Logistic regression analysis to show that PDR score as a risk factor for death could not be done as there were no children belonging to death group who had a score less than 2.

Though other studies have suggested that Cit H3 is a novel parameter which could be used as a new biomarker molecule that could lead to new diagnostic and prognostic methods to recognize disease severity and develop new strategies involving histone targeted therapies for better outcomes. (25), (27), (28),(29) Our study did not show results as anticipated. Though the levels were significantly different in sepsis versus the control population, the occurrence rate of CitH3 levels was not significantly associated with DIC, MODS, or death.

We currently presume that Cit H3 measured at a single time point might not be a good prognostic marker in sepsis. Nevertheless, we hypothesize that the strategy of serial measurement of Cit H3 levels may add substantially to the assessment of risk in this patient population. Future studies involving serial measurement at different time points as done with other biomarkers of sepsis are recommended for a better understanding of the role of histones as mediators of complications of sepsis and sepsis-related death.

LIMITATIONS

1) The sample size could not be reached as the number of admissions were low owing to the pandemic.

2) The control group was not age or gender matched.

3) The number of non-survivors were less for statistical analysis (rough expected mortality in PICU is 18-20%)

4) Our kit can only detect PAD4 citrullination on histone protein, due to which PAD 2 catalysation which is also involved in sepsis, might not have been recognised.

5) In our study we performed measurement of CitH3 only at one time point, i.e. within 24 hours of admission and we did not perform serial measurements. A study by Tian et al showed that CitH3 levels at 48 hours of admission were predictive of 90 days mortality(27).

6) Our patients included a heterogenous group with sepsis due to multiple causes like scrub typhus, PIMS-TS which could have been a confounding factor.

7) Since, ours is a tertiary centre, cases which could have received prior treatment with antibiotics from outside may also have influenced the results.

8) The comparison of CitH3 levels were done only between apparently healthy controls and children admitted to PICU. A comparison between children with fever and no sepsis would have been helpful as well.

9) We did not look at number of mortality days

CONCLUSIONS

1. Citrullinated histone H3 levels measured at admission within 24 hours were higher in children with clinical sepsis than controls.
2. Paediatric inflammatory multisystem syndrome temporally associated with severe acute respiratory syndrome coronavirus 2(SARS-CoV-2)-PIMS-TS currently known as Multisystem inflammatory syndrome of children (MIS-C) is associated with raised circulating citrullinated Histone H3 levels when compared to controls. This suggests a role of NETosis in the pathophysiology of this novel entity.
3. Citrullinated histone H3 levels were highest in scrub typhus, followed by PIMS-TS. The levels in scrub typhus were significantly higher than controls and other gram-negative infections.
4. In our study, circulating histone levels were not associated with coagulopathy, Multi-Organ Dysfunction and did not predict mortality. Therefore, it cannot be used as a prognostication marker in paediatric sepsis patients.
5. Baseline raised INR > 1.43 and low fibrinogen levels < 242mg/dl in paediatric clinical sepsis patients were independent predictors of mortality in a multivariate analysis.

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APPENDIX

ANNEXURES

Annexure 1: Scanned copy of IRB approval

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

Dr. B.J. Prashantham, M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Anna Benjamin Pulimood, MD., Ph.D.,
Chairperson,
Research Committee & Principal

Dr. Suceena Alexander, MD., DM., FASN.,
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

April 18, 2020

Dr. Akshata Pandiri
PG registrar,
Department of Transfusion Medicine,
Christian Medical College,
Vellore – 632 002.

₹ 2,12,500/-

Sub: **Fluid Research Grant New Proposal:**
Single Centre, prospective observational study to evaluate association between circulating citrullinated Histone H3 and sepsis induced complications in paediatric patients with clinical sepsis.
Dr. Akshata Pandiri, PG Registrar, Transfusion Medicine, Dr. Sukesh Nair, Dr. Joy Mammen, Dr Dolly Daniel, Transfusion Medicine, Dr Tulasi Geevar, Transfusion Medicine, Dr Ebor Jacob, Dr Jolly Chandran, Dr Siva Vyasam, Pediatric Intensive care Unit. Mrs.K.Reka, Lecturer, Biostatistics.

Ref: IRB Min. No. 12407 [OBSERVE] dated 02.12.2019

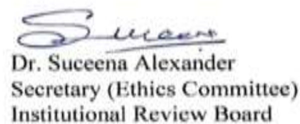
Dear Dr. Akshata Pandiri,

I enclose the following documents:-

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Suceena Alexander, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,



Dr. Suceena Alexander
Secretary (Ethics Committee)
Institutional Review Board

Dr. Suceena Alexander, MD.,DM.,FASN.
Secretary - (Ethics Committee)
Institutional Review Board
Christian Medical College,
Vellore - 632 002, Tamil Nadu, India.

Cc: Dr. Sukesh Nair, Transfusion Medicine, CMC, Vellore

1 of 4

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788, 2284481 E-mail: research@cmcvellore.ac.in

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

Dr. B.J. Prashantham, M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Anna Benjamin Pulimood, MD., Ph.D.,
Chairperson,
Research Committee & Principal

Dr. Suceena Alexander, MD., DM., FASN.,
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

April 18, 2020

Dr. Akshata Pandiri
PG registrar,
Department of Transfusion Medicine,
Christian Medical College,
Vellore – 632 002.

Sub: **Fluid Research Grant New Proposal:**
Single Centre, prospective observational study to evaluate association between circulating citrullinated Histone H3 and sepsis induced complications in paediatric patients with clinical sepsis.
Dr. Akshata Pandiri, PG Registrar, Transfusion Medicine, Dr. Sukesh Nair, Dr. Joy Mammen, Dr Dolly Daniel, Transfusion Medicine, Dr Tulasi Geevar, Transfusion Medicine, Dr Ebor Jacob, Dr Jolly Chandran, Dr Siva Vyasam, Pediatric Intensive care Unit. Mrs.K.Reka, Lecturer, Biostatistics.

Ref: IRB Min. No. 12407 [OBSERVE] dated 02.12.2019

Dear Dr. Akshata Pandiri,

The Institutional Review Board (**Blue**, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "Single Centre, prospective observational study to evaluate association between circulating citrullinated Histone H3 and sepsis induced complications in paediatric patients with clinical sepsis" on December 02nd 2019.

The Committee reviewed the following documents:

1. IRB application format
2. Proforma
3. Patient information sheet Consent and Assent forms (Tamil, English, Hindi and Telugu)
4. Cvs of Drs. Tulasi Geevar, Dolly, Ebor, Siva Vyasam, Joy Mammen, Sukesh Chanda Nair, Reka.
5. Proforma
6. No. of documents 1- 5

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on December 02nd 2019 in the New IRB Room, Christian Medical College, Vellore 632 004.

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788, 2284481 E-mail: research@cmcvellore.ac.in

Annexure 2: Funding letter

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

Dr. B.J. Prashantham, M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Anna Benjamin Pulimood, MD., Ph.D.,
Chairperson,
Research Committee & Principal

Dr. Suceena Alexander, MD., DM., FASN.,
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Dr. Anuradha Rose	MBBS, MD, MHSC (Bioethics)	Associate Professor, Community Health, CMC, Vellore	Internal, Clinician
Mrs. Sophia V	M.Sc Nursing	Addl. Deputy Dean CMC, Vellore	Internal, Nurse
Mrs. Nirmala Margaret	MSc Nursing	Addl. Deputy Nursing Superintendent, College of Nursing, CMC, Vellore	Internal, Nurse
Mrs. Sheela Durai	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Rev. Joseph Devaraj	BSc, BD	Chaplaincy Department, CMC, Vellore	Internal, Social Scientist

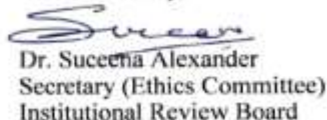
We approve the project to be conducted as presented.

Kindly provide the total number of patients enrolled in your study and the total number of Withdrawals for the study entitled: "Single Centre, prospective observational study to evaluate association between circulating citrullinated Histone H3 and sepsis induced complications in paediatric patients with clinical sepsis" on a monthly basis. Please send copies of this to the Research Office (research@cmcvellore.ac.in).

The Institutional Ethics Committee expects to be informed about the progress of the project. Any **adverse events** occurring in the course of the project, any **amendments in the protocol and the patient information / informed consent**. On completion of the study you are expected to submit a copy of the **final report**. Respective forms can be downloaded from the following link: http://172.16.11.136/Research/IRB_Polices.html in the CMC Intranet and in the CMC website link address: http://www.cmch-vellore.edu/static/research/Index.html.

Fluid Grant Allocation:
A sum of 2,12,500/- INR (Rupees Two Lakh Twenlve Thousand and Five Hundred Only) will be granted for 2 years. 1,50,000/- INR (Rupees One Lakh fifty Thousand only) will be granted for 12 months as an Ist Installment. The rest of the 62,500/- INR (Rupees Sixty Two Thousand and Five Hundred only) will be released at the end of the first year as 2 nd Installment.

Yours sincerely,



Dr. Suceena Alexander
Secretary (Ethics Committee)
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Vellore - 632 002, Tamil Nadu, India.

IRB Min. No. 12407 [OBSERVE] dated 02.12.2019 4 of 4

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788, 2284481 E-mail: research@cmcvellore.ac.in

Annexure 3A: DATA set from Excel sheet

Annexure 3B: CASE REPORT FORM

CHIPS STUDY PROFORMA
CMC VELLORE

1. Name: 2. Age:

3. Gender: M/F 4. Place:

5. Child Health Unit: 6. Hospital Number:

7. Date of Admission in PICU:

8. PIM 2 Score (At Admission):

9. PDR

10. Symptoms:

A. Fever: YES/ NO,
If Yes Duration:
B. Cough
C. Loose stools
D. Altered sensorium
E. Seizures
F. Decreased urine output
G. Rash

CHIPS STUDY PROFORMA
CMC VELLORE

11. AT PRESENTATION

	YES/NO	Cit H3 level
SUSPECTED SEPSIS		
PROBABLE SEPSIS		
CONFIRMED SEPSIS		

12. SUSPECTED SEPSIS

a) Fever:
b) Features of SHOCK (3/8 Criteria):
a. HR:
b. RR:
c. BP:
d. CRT:
e. Temperature:
f. Sensorium:
g. Peripheries:
h. Skin colour:

13. PROBABLE SEPSIS: (Suspected sepsis with elevated biomarkers)

a) CRP: __________ (OR) Procalcitonin Level __________

14. CONFIRMED SEPSIS: (Culture positivity)

a) Blood culture: ______________

15. IS IT A NON SEPSIS CONTROL: YES/ NO

16. CITRULLINATED H3 LEVEL: ____________

CHIPS STUDY PROFORMA
CMC VELLORE

17. SEVERITY OF ILLNESS-YES/NO

	AT PRESENTATION	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 28	DAY OF ONSET/NMR
SEPTIC SHOCK								
DIC								
CARDIAC								
RESP								
HEPATIC								
NEURO								
FEVER								

18. SEPTIC SHOCK CHECKLIST (3/8 CRITERIA)

1. Temperature abnormality: ......°C
2. Hypotension: ....mmHg
3. Tachycardia: ....bpm
4. Tachypnoea: ....bpm
5. Capillary refill abnormality: ......
6. Mental status abnormality: ......
7. Pulse abnormality: ....
8. Skin abnormality: ....

19. DISSEMINATED INTRAVASCULAR COAGULATION:

a) PLATELET COUNT
b) PT/INR
c) % pt +1/2 control
d) APTT
e) % pt +1/2 control

CHIPS STUDY PROFORMA
CMC VELLORE

f) FIBRINOGEN
g) ***D-Dimer***

20. MULTIORGAN DYSFUNCTION: YES/NO

A.ACUTE RENAL FAILURE: YES/NO
Day of onset
Creatinine
Est creat clearance decrease by 75% or less than 35 ml/kg/min
u/o less than 0.5ml/kg/hr x 24 hours or anuric 12 hours

B.ACUTE LIVER FAILURE: YES/NO
Day of onset
TB
DB
SGOT
SGPT
TP
ALB
PT
APTT
HEPATIC ENCEPHALOPATHY

C.Cardiac: YES/NO
Day of onset:
Hypotension/inotropic support
Met acidosis
Elevate abg lactate
Prolonged capillary refill time

CHIPS STUDY PROFORMA
CMC VELLORE

D. Respiratory: YES/NO
Day of onset
PF ratio< 300
Paco2>65
>50% [illegible] to maintain spo2> 92%
Mechanical Ventilation
E.Neurological
Day of onset
GCS<11 acute change in mental status

21.Duration of PICU stay:

A.<5 days B.5-10 days C.>10 days

22.Duration of Hospital stay:

A.<5 days B.5-10 days C.>10 days

23. OUTCOME :

A. SURVIVED
B. DIED
If Yes Cause of Death!
C. LAMA-***yes/no***
On what day

QUANTIFICATION OF CITRULLINATED HISTONE H3 using SANDWICH ELISA

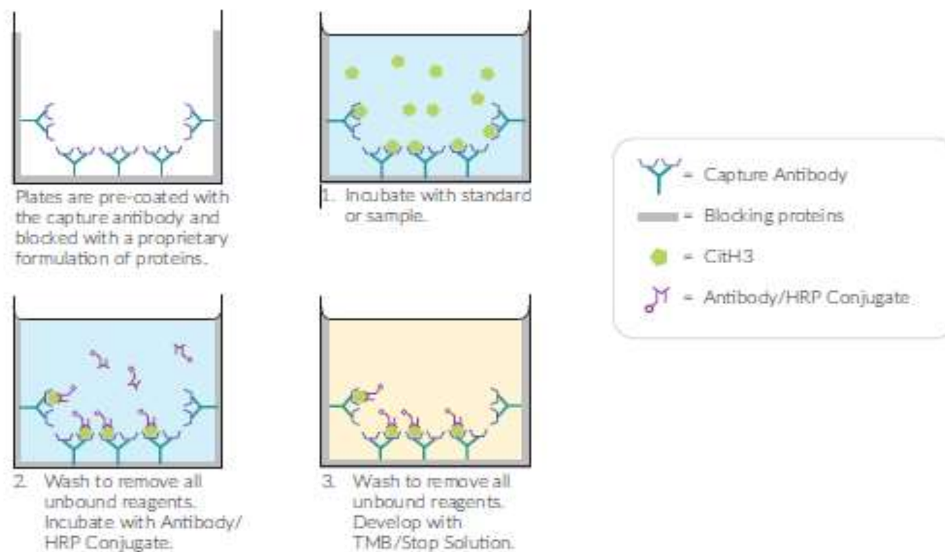
Purpose:

ELISA assay for the quantitative determination of citrullinated Histone H3 levels in human citrated plasma .

Principle:

This immunometric assay is based on a double-antibody ‘sandwich’ technique. Each well of the micro-well plate is coated with a monoclonal antibody specific for histone H3 (citrullinated at R2, R8, and R17). This antibody will bind any CitH3 introduced into the well. A second monoclonal antibody which recognizes the H3 core (Antibody/HRP Conjugate) is added to the well, forming a “sandwich”. The ‘sandwiches’ are immobilized on the plate so the excess reagents may be washed away. The Antibody/HRP Conjugate is labelled with horseradish peroxidase (HRP), allowing quantization of the CitH3. Addition of HRP Substrate TMB, followed by Stop Solution produces a yellow colour product which can be measured spectrophotometrically. The intensity of the colour is directly proportional to the amount of bound Antibody/HRP Conjugate, which is proportional to the concentration of citrullinated histone H3.

Absorbance ∝ [Anti-CitH3/HRP] ∝ [CitH3]



Performance specifications:

Limits of detection – 0.15ng/ml to 10ng/ml

Primary sample

Citrated blood.

Type of container

1. Light blue top vacutainer.

Materials :

1. Anti Histone H3 HRP conjugate-1 vial/1.5ml…………………………………………. Item number-401620
2. Anti-Citrullinated Histone H3 ELISA Strip Plate-1 plate…………………………… Item number-401621
3. Citrullinated Histone ELISA Standard-2 vials………………………………………. Item number-401444
4. Immunoassay Buffer B Concentrate (10X)- 1 vial/10 ml…………………………… Item number-400054
5. Wash Buffer Concentrate (400X)- 1 vial/5 ml………………………………………. Item number-400062
6. Polysorbate 201 vial/3 ml……………………………………………………………. Item number-400035
7. TMB Substrate Solution-1 vial/12 ml……………………………………………….. Item number-401620
8. HRP Stop Solution-1 vial/12 ml…………………………………………………….. Item number-10011355
9. 96-Well Cover Sheet-3 covers……………………………………………………… Item number-400012
10. A plate reader with the ability to measure absorbance at 450 nm
11. An orbital shaker.
12. Adjustable pipettes and a repeating pipettor
13. A source of pure water; glass distilled water or HPLC-grade water is acceptable. *NOTE: UltraPure water is available for purchase from Cayman*
14. Assay buffer
15. Patient citrated plasma

PRE-ASSAY PREPARATION

A. **Buffer Preparation**

Store all diluted buffers at 4°C; they should be stable for two months.

NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with water. Polysorbate 20 is a viscous liquid and cannot be measured by a regular pipette. A positive displacement pipette or a syringe should be used to deliver small quantities accurately.

1. **Assay Buffer Preparation**
   Dilute the contents of one vial of Immunoassay Buffer B Concentrate (10X) (Item No. 400054) with 90 ml of water and add 100 µl of Polysorbate 20 (Item No. 400035). Be certain to rinse the vial to remove any salts that may have precipitated.
2. **Wash Buffer Preparation**

5 ml vial Wash Buffer Concentrate (400X) (Item No. 400062): Dilute to a total volume of 2 L with water and add 1 ml of Polysorbate 20.

B. **Sample Preparation**

1. **Sample Collection and Storage**

Citrated plasma (prepared using citrate as the anticoagulant) can be used without a purification step in the assay if they are first diluted a minimum of 1:2 in Assay Buffer.

2. **Sample Dilution**

All human plasma, human serum and cell culture supernatant or lysate samples MUST be diluted at least 1:2 with Assay Buffer prior to use in this assay. A minimum volume of 200 µl of each diluted sample is needed to run the samples in duplicate in the assay; for convenience, preparing 250 µl of each diluted sample is recommended by the kit manufacturer.

C. Reagent preparation:

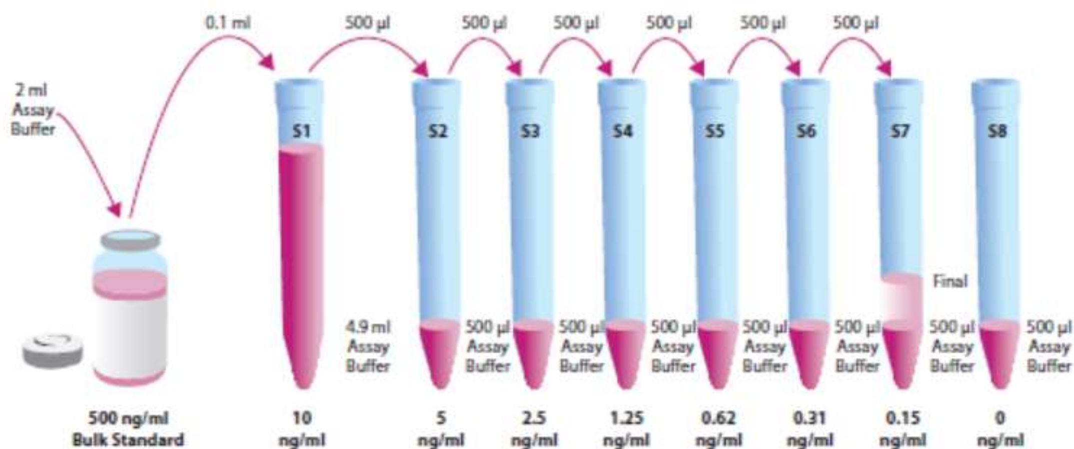
1. **Citrullinated Histone H3 ELISA Standard**

Reconstitute the lyophilized CitH3 ELISA Standard (Item No. 401444) with 2 ml of Assay Buffer and Mix gently. The concentration of this solution (the bulk standard) is 500 ng/ml.

(The reconstituted standard is relatively unstable at 4°C and should be used within three hours of reconstitution. A second lyophilized standard has been supplied should the assay need to be repeated).

To prepare the standard for use in the ELISA: steps

- Obtain eight clean test tubes and label them, #1 through #8.
- Aliquot 4.9 ml of Assay Buffer into tube #1.
- Aliquot 500 µl of Assay Buffer into tubes #2-8.
- Transfer 0.1 ml of freshly prepared stock standard (500 ng/ml) to tube #1. Mix gently.
- Serially dilute the standard by removing 500 µl from tube #1 and placing into tube #2. Mix gently.
- Next, remove 500 µl from tube #2 and place into tube #3; mix gently.
- Repeat this process for tubes #4-7.
- Do not add any CitH3 ELISA Standard to tube #8.
- This tube is the zero-point vial, the lowest point on the standard curve.



2. **Anti-Histone H3 HRP Conjugate**

This reagent is supplied as a concentrated (10X) stock solution of a mouse anti-histone H3 monoclonal antibody conjugated to HRP.

- On the day of the assay, thaw the reagent (Item No. 401620) at room temperature.
- For a full plate, dilute 1.2 ml of HRP Conjugate into 10.8 ml of Assay Buffer; for a half plate, dilute 0.6 ml of HRP Conjugate into 5.4 ml of Assay Buffer.
- Do not prepare diluted HRP Conjugate until immediately before use.
- Discard any unused 1X Anti-Histone H3 Conjugate.
- Store unused 10X stock anti-histone H3 HRP Conjugate at -20°C and minimize freeze thaw cycles.

3. Plate set up:

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	1	1	1	9	9	9	17	17	17	25
B	S2	S2	2	2	2	10	10	10	18	18	18	25
C	S3	S3	3	3	3	11	11	11	19	19	19	25
D	S4	S4	4	4	4	12	12	12	20	20	20	26
E	S5	S5	5	5	5	13	13	13	21	21	21	26
F	S6	S6	6	6	6	14	14	14	22	22	22	26
G	S7	S7	7	7	7	15	15	15	23	23	23	27
H	S8	S8	8	8	8	16	16	16	24	24	24	27

The 96-well plate(s) included with this kit is supplied ready to use.

(It is not necessary to rinse the plate(s) prior to adding the reagents. *NOTE: If you do not need to use all of the strips at once, place the unused strips back in the plate packet and store according to the plate insert at 4°C. Be sure the packet is sealed with the desiccant inside.)*

Each plate or set of strips must contain an eight-point standard curve run in duplicate. *NOTE: Each assay must contain this minimum configuration in order to ensure accurate and reproducible results.*

Each sample should be assayed at a minimum of two dilutions and each dilution should be assayed in duplicate. For statistical purposes, assaying samples in triplicate is recommended.

The contents of each well are to be recorded on the template sheet provided

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Assay protocol:

Pipetting hints
• Use different tips to pipette each reagent. • Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times). • Do not expose the pipette tip to the reagent(s) already in the well(s).

STEPS:

Addition of Standards and Samples and First Incubation

i. Add 100 µl of the standards or diluted sample to the appropriate wells on the plate.

(Each sample should be assayed in duplicate, triplicate recommended)

ii. Cover the plate with 96-Well Cover Sheet (Item No. 400012). Incubate for two hours at room temperature on an orbital shaker.

Addition of HRP Conjugate and Second Incubation

iii. Empty the wells and rinse four times with Wash Buffer. Each well should be completely filled with Wash Buffer during each wash. Invert the plate between wash steps to empty the fluid from the wells. After the last wash, gently tap the inverted plate on absorbent paper to remove the residual Wash Buffer.

iv. Add 100 µl of the HRP Conjugate working solution to each well of the plate.

v. Cover the plate with the 96-Well Cover Sheet (Item No 400012) and incubate for one hour at room temperature on an orbital shaker.

Development of the Plate

vi. Empty the wells and rinse four times with Wash Buffer. Each well should be completely filled with Wash Buffer during each wash.

vii. Invert the plate between wash steps to empty the fluid from the wells.

viii. After the last wash, gently tap the inverted plate on absorbent paper to remove the residual Wash Buffer.

ix. Add 100 µl of TMB Substrate Solution (Item No. 400074) to each well of the plate.

x. Cover the plate with the 96-Well Cover Sheet (Item No 400012) and incubate for 30 minutes at room temperature in the dark on an orbital shaker.

DO NOT WASH THE PLATE.

xi. Add 100 µl of HRP Stop Solution (Item No. 10011355) to each well of the plate.

Blue wells should turn yellow and colourless wells should remain colourless.

NOTE: The Stop Solution in this kit contains an acid. Wear appropriate protection and use caution when handling this solution.

Reading the Plate

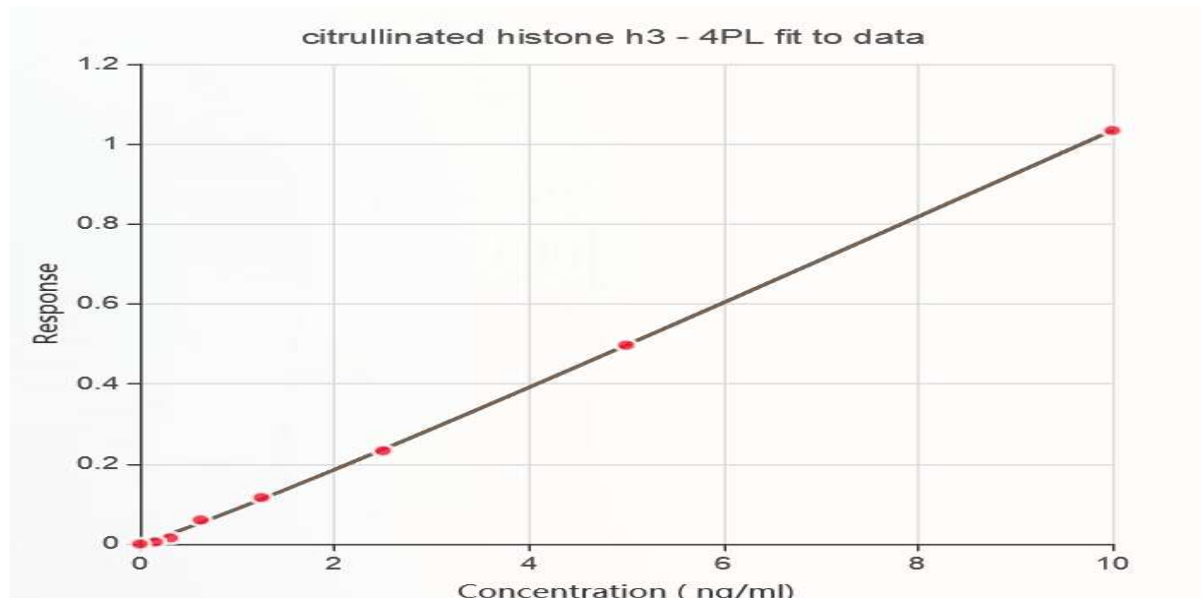
xii. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.

xiii. Read the plate at a wavelength of 450 nm.

xiv. Calculations

Plotting the Standard Curve and Determining the Sample Concentration

Using computer reduction software, plot absorbance (linear y-axis) *versus* concentration (linear x-axis) for standards (S1-S7) and fit the data with a four-parameter logistic equation, or alternatively a linear curve fit.



https://www.arigobio.com/elisa-analysis

SUMMARY OF ASSAY PROCEDURE

Procedure	Blank	Standards/Samples
Mix all reagents gently	--	--
Add standards/samples to plate	--	100 µl
Seal the plate and tap gently to mix	→	→
Incubate plate for 2 hours at RT, shaking and sealed	→	→
Aspirate wells and wash 5 x well volume (~300 µl) with 1X Wash Buffer	→	→
Apply 1X HRP Conjugate Solution	--	100 µl
Incubate for 1 hour at RT, shaking and sealed	→	→
Aspirate wells and wash 5 x well volume (~300 µl) with 1X Wash Buffer	→	→
Apply TMB Substrate Solution	100 µl	100 µl
Incubate 30 min at RT, shaking, sealed, and *protected from light*	→	→
Do Not Wash, apply HRP Stop Solution	100 µl	100 µl
Read absorbance at 450 nm	→	→

QC protocol:
QC reagents are provided for each kit and tested for quality

Possible interference
1. Change in the PH of various buffers.
2. Incubation time was not followed properly.
3. Deterioration of reagents.
4. Pipetting errors.
5. Errors in calculation.
6. Serum and plasma contain nucleases and other substances which can interfere
7. Plasma samples prepared with heparin or EDTA as the anticoagulant have been shown to cause a slight interference in the assay with higher than expected recoveries.

Result interpretation:

Results are reported as ng/ml and the normal level has not been universally established

Safety protocols

1. Always use the necessary PPE for all procedures done in the laboratory.

2. Consider every biological sample as a potential bio hazard.

3. Ensure that all laboratory work benches are mopped with 70% ethanol before and after work.
4. In case of any needle stick injury, take a patient sample & hospital number, wash the wound with soap and water, inform the any department staff/ DSA and rush to the SSHS.

5. The Stop Solution provided with this kit is an acid solution. Please wear appropriate personal protection equipment (*e.g.*, safety glasses, gloves, and lab-coat) when using this material.

Trouble shooting:

PROBLEM	POSSIBLE CAUSES	RECOMMENDED SOLUTIONS
Poor development of test sample	CitH3 is a part of a complex extracellular trap and unavailable for binding the capture antibody	Treat the sample with DNAse to disrupt the extracellular trap, thereby releasing the free CitH3
Poor development (low signal) of standard curve	• Standard was diluted incorrectly • The standard is degraded	

Annexure 5A: Sepsis screening tool:TARGET HAEMODYNAMIC PARAMETRES

- Capillary refill ≤ 2 seconds
- Threshold HRs or heart rate less than 20% baseline
- Normal pulses with no differential between the quality of the peripheral and central pulses,
- Warm extremities
- Normal mental status(GCS)
- Normal blood pressure for age (>5th Centile for age and sex)

Annexure 5B: Heart rates normal for age

(Paediatric Crit Care Med 2007; 8:138 –144)

Age group

Heart

Rate (bpm)

Term newborn 120–180

Up to 1 yr 120–180

Up to 2 yrs 120–160

Up to 7 yrs 100–140

Up to 15 yrs 90–140

Annexure 5C. Reference ranges for Systolic Blood pressure in paediatric age group

(Paediatric Crit Care Med 2007; 8:138 –144)

. Calculated 5th percentile systolic blood pressure (mm Hg) according to height percentiles among boys (M) and girls (F) 1–18 yrs old

Fifth Percentile Systolic Blood Pressure, Percentile for Height

Fifth Percentile Systolic Blood Pressure, Percentile for Height

	5th	25th	50th	75th	95th					
Age, Yrs	M	F	M	F	M	F	M	F	M	F
1	62	66	65	68	67	68	70	71	72	73
2	67	68	70	70	70	71	72	71	74	73
3	68	68	71	71	73	71	76	74	77	76
4	70	71	73	73	75	74	78	74	79	76
5	72	71	76	74	78	76	78	77	80	79
6	73	74	76	76	78	77	81	79	83	81
7	74	76	77	78	79	79	81	79	83	82
8	77	78	80	78	82	81	82	82	84	84
9	77	78	80	81	82	83	85	84	87	86
10	79	80	83	83	85	85	85	86	89	88
11	81	82	85	85	87	85	87	88	89	90
12	83	85	86	87	89	87	91	90	93	92
13	87	87	88	89	90	90	92	92	94	92
14	88	89	91	89	94	92	96	93	98	95
15	92	90	95	92	95	93	97	93	99	95
16	93	91	96	93	98	93	101	96	103	98
17	97	91	98	93	100	93	102	96	104	98

Annexure 5D. Reference ranges for Mean Blood pressure in pediatric age group

(Paediatric Crit Care Med 2007; 8:138 –144)

Calculated mean arterial blood pressure (mm Hg) according to height percentiles among boys (M) and girls (F) 1–18 yrs old

Mean Arterial Blood Pressure for Boys and Girls, Percentile for Height

Age, Yrs	Percentile for Blood Pressure	5th		25th		50th		75th		95th	
		M	F	M	F	M	F	M	F	M	F
1	5	30	35	33	37	34	37	36	39	37	40
	50	49	53	52	54	53	55	54	57	56	58
	95	69	71	70	72	72	73	73	74	74	76
2	5	35	39	38	41	39	42	40	42	41	44
	50	54	57	56	58	57	59	59	60	60	62
	95	73	75	75	76	76	77	77	78	79	80
3	5	39	42	41	44	42	44	44	46	45	47
	50	58	60	60	61	61	62	62	64	64	65
	95	77	78	78	79	80	80	81	81	82	83
4	5	42	45	43	46	46	47	47	47	48	49
	50	61	63	63	64	64	65	66	65	67	67
	95	79	80	82	82	83	83	84	84	86	85
5	5	45	46	47	48	49	49	49	50	51	52
	50	63	64	66	66	67	67	68	68	69	69
	95	82	82	84	83	85	85	87	86	88	87
6	5	47	49	49	50	50	51	52	52	53	54
	50	66	66	67	68	69	69	70	69	71	71
	95	84	84	86	85	87	86	88	87	90	89
7	5	51	50	50	51	52	52	53	53	54	55
	50	67	68	69	69	70	70	72	71	73	72
	95	83	85	88	87	89	88	90	89	92	90
8	5	50	52	53	52	54	54	55	55	56	56
	50	69	70	71	70	72	71	73	72	75	74
	95	87	87	89	88	91	89	92	90	93	91
9	5	51	53	53	54	55	55	56	56	58	57
	50	70	71	72	71	73	73	75	74	76	75
	95	88	89	91	89	92	90	93	91	94	93
10	5	52	54	55	55	56	56	56	57	59	59
	50	71	72	73	73	75	74	75	75	77	76
	95	90	90	92	90	93	92	94	93	96	94
11	5	54	55	56	56	57	57	58	59	59	60
	50	72	73	74	74	75	75	76	76	78	78
	95	91	91	92	92	94	93	95	94	96	95
12	5	54	57	57	58	58	58	60	60	61	61
	50	73	75	75	75	77	76	78	78	79	79
	95	92	92	94	93	95	94	96	95	98	97
13	5	56	58	57	59	59	60	60	61	61	62
	50	75	76	76	77	77	78	79	79	80	80
	95	93	94	95	94	96	95	97	97	99	98
14	5	59	60	59	60	61	61	62	62	63	64
	50	75	77	78	78	79	79	80	80	82	81
	95	91	95	96	96	97	97	99	98	100	99
15	5	58	61	61	61	62	62	63	63	64	64
	50	77	78	79	79	80	80	82	81	83	82
	95	96	96	98	97	99	98	100	99	102	100
16	5	60	61	62	62	63	63	65	63	66	66
	50	79	79	81	80	82	81	83	82	85	84
	95	98	96	99	98	101	99	102	100	104	101

ANNEXURE 6: DIC ISTH SCORING SYSTEM

Platelet Count		
○	>100 x 10^9/L	0 Points
○	>50 - <100 x 10^9/L	1 Point
○	<50 x 10^9/L	2 Points
Increase in Fibrin-related Markers [D Dimers]		
○	No change	0 Points
○	Moderate rise	2 Points
○	Strong rise	3 Points
Prothrombin Time [PT] Prolongation		
○	3 s or less	0 Points
○	>3 s but <6 s	1 Point
○	>6 s	2 Points
Fibrinogen [Clauss] Level		
○	>1.0 g/L	0 Points
○	<1.0 g/L	1 Point

ANNEXURE 7: Paediatric organ dysfunction criteria: the diagnostic criteria for paediatric mods based on 2002 international paediatric sepsis consensus conference

MODS is defined as the simultaneous occurrence of at least two organ dysfunctions.

Cardiovascular Dysfunction: Despite administration of isotonic intravenous fluid bolus ≥40 mL/kg in 1 h:

▪ Decrease in BP (hypotension) <5 percentile for age or systolic BP <2 SD below normal for age

OR

▪ Need for vasoactive drug to maintain BP in normal range (dopamine ≥5 µg/kg/min or dobutamine, epi or norepi at any dose)

OR

▪ Two of the following

▪ Unexplained metabolic acidosis: base deficit >5.0 mEq/L

▪ Increased arterial lactate >2 times upper limit of normal

▪ Oliguria: urine output <0.5 mL/kg/h

▪ Prolonged capillary refill: >5 s

▪ Core to peripheral temperature gap >3°C

Respiratory System

▪ PaO2/FIO2 ratio <300 in the absence of cyanotic congenital heart disease or preexisting lung disease

OR

▪ PaCO2 >65 mmHg or 20 mm Hg above baseline PaCO2

OR

▪ Proven need or >50%FIO2 to maintain saturation ≥92%

OR

▪ Need for nonelective invasive or noninvasive mechanical ventilation

Central Nervous System

▪ Glasgow Coma Score ≤11

OR

▪ Acute change in mental status with a decrease in GCS ≥3 points from abnormal baseline

Hematologic System

▪ Platelet count <80,000/mm3 or a decline of 50% in platelet count from highest valued recorded over the past 3 d (for chronic heme-onc patients)

OR

▪ INR >2

Renal System

▪ Serum creatinine level >2 times upper limit for age or twofold increase in baseline creatinine

Hepatic System

▪ Total serum bilirubin ≥4 mg/dL (in the absence of hemolysis, hyperbilirubinemia of the newborn, or primary liver disease)

OR

▪ ALT 2 times upper limit of normal for age

ANNEXURE 8: Variables in PIM-2 scoring system

Variables (help)	Values (1 if Yes, 0 otherwise)	Beta
Elective admission		0
Recovery post procedure		0
Cardiac bypass		0
High risk diagnosis		0
Low risk diagnosis		0
No response of pupils to bright light (> 3 mm and both fixed)		0
Mechanical ventilation (at any time during first hour in ICU)		0
Systolic Blood Pressure (mmHg)	120	0.01395
Base Excess (mmHg) (arterial or capillary blood)	0	0.1040
FiO2*100/ PaO2 (mmHg)	0	0.2888

Predicted Death Rate : Compute

0 Clear

Logit = (-4.8841) + (values * Beta) + (0.01395 * (absolute(SBP-120))) + (0.1040 * (absolute base excess)) + (0.2888 * (100*FiO2/PaO2))

Predicted death rate = $e^{Logit}/(1+e^{Logit})$

ANNEXURE 9: CONSENT FORM, ASSENT FORM, INFORMATION SHEET