Title page

Diagnostic markers of acute encephalitis syndrome and COVID-associated multisystem inflammatory syndrome in children from Southern India

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Author Contribution

Tina Damodar contributed to funding acquisition, conceptualization, data curation, methodology, formal analysis, investigation, and writing the original draft. Cordelia Dunai participated in formal analysis and contributed to writing the original draft. Namratha Prabhu, Maria Jose, Srilatha Marate, and Akhila L were involved in investigation, methodology, and project administration. Uddhava V Kinhal, Anusha Raj K, A V Lalitha, Fulton Sebastian Dsouza, Sushma Veeranna Sajjan, Vykuntaraju K Gowda, and G V Basavaraj were responsible for patient recruitment for the study. Prathyusha P V, Ruwanthi Kolamunnage-Dona and Kukatharmini Tharmaratnam conducted formal analysis. Tom Solomon, Lance Turtle, Vasanthapuram Ravi, and Ravi Yadav provided supervision and reviewed the manuscript. Benedict D Michael contributed to conceptualization, supervision, writing the original draft, and review & editing. Reeta Mani was involved in conceptualization, methodology, writing the original draft, review & editing, funding acquisition, and supervision. All authors revised the manuscript, approved it for publication, and agreed to be accountable for all aspects of the work.

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Abstract

Background: Acute encephalitis syndrome (AES) in children poses a significant public health challenge in India. This study aims to explore the utility of host inflammatory mediators and neurofilament (NfL) levels in distinguishing aetiologies, assessing disease severity, and predicting outcomes in AES.

Method: We assessed 12 mediators in serum (n=58) and 11 in cerebrospinal fluid (CSF) (n=42) from 62 children with AES due to scrub typhus, viral aetiologies, and COVID-associated multisystem inflammatory syndrome (MIS-C) in southern India. Additionally, NfL levels in serum (n=20) and CSF (n=18) were examined. Clinical data, including Glasgow coma scale (GCS) and Liverpool outcome scores, were recorded.

Result: Examining serum and CSF markers in the three AES aetiology groups revealed notable distinctions, with scrub typhus differing significantly from viral and MIS-C causes. Viral causes had elevated serum CCL11 and CCL2 compared with scrub typhus, while MIS-C cases showed higher HGF levels than scrub typhus. However, CSF analysis showed a distinct pattern with the scrub typhus group exhibiting elevated levels of IL-1RA, IL-1β, and TNF compared with MIS-C, and lower CCL2 levels compared with the viral group. Modelling the characteristic features, we identified that age ≥ 3 years with serum CCL11 <180 pg/ml effectively distinguished scrub

typhus from other AES causes. Elevated serum CCL11, HGF, and IL-6:IL-10 ratio were associated with poor outcomes (p=0.038, 0.005, 0.02). Positive CSF and serum NfL correlation, and negative GCS and serum NfL correlation were observed. Median NfL levels were higher in children with abnormal admission GCS and poor outcomes.

Discussion: Measuring immune mediators and brain injury markers in AES provide valuable diagnostic insights, with potential to facilitate rapid diagnosis and prognosis. The correlation between CSF and serum NfL, along with distinctive serum cytokine profiles across various aetiologies, indicates the adequacy of blood samples alone for assessment and monitoring. The association of elevated levels of CCL11, HGF, and an increased IL-6:IL-10 ratio with adverse outcomes suggests promising avenues for therapeutic exploration, warranting further investigation.

Keywords: Inflammatory markers, cytokines, chemokines, neurofilament, acute encephalitis syndrome, COVID-associated multisystem inflammatory syndrome

Text

Introduction:

Acute encephalitis syndrome (AES) is a significant public health concern in India, particularly affecting children (1). While primarily associated with infectious aetiologies, the broad definition of AES includes patients with acute fever and altered mental state, covering diverse causes such as systemic infections, metabolic derangements, or post-infectious neurological complications. Prompt identification

of the aetiology is crucial for initiating timely and targeted treatment, leading to positive outcomes. However, diagnosis is hindered by overlapping clinical features and limitations in common methods like IgM ELISA and pathogen-based PCRs, which yield inconclusive or negative results in about 50% of cases (1,2). IgM antibodies can persist for an extended period post-acute illness and exhibit cross-reactivity with other circulating pathogens. Delays in presentation and sampling, typical in developing countries, impede critical cerebrospinal fluid (CSF) examination, leading to unreliable PCR test results (1–4).

During the 2020-21 COVID-19 outbreak, patients with scrub typhus and dengue, common contributors to AES in India, were occasionally misdiagnosed as COVID-19 (5,6). Simultaneously, hospitals in India noted a rise in paediatric cases with AES-like features, later identified as multisystem inflammatory syndrome associated with COVID-19 (MIS-C) (7,8). The unclear neurological spectrum in children with MIS-C (9,10) complicates the ongoing challenge of identifying AES causes.

While viral central nervous system (CNS) infections involve direct invasion of resident cells leading to inflammatory responses, neurological complications in scrub typhus primarily occur through vasculitis triggered by endothelial cell invasion of bacterium *Orientia tsutsugamushi* (11,12). In contrast, MIS-C results from an exaggerated immune response following prior SARS-CoV-2 exposure, characterized by a cytokine storm affecting multiple organ systems, including the CNS (13). Given the differing underlying pathophysiology but similar clinical presentations, there is an urgent need for tests to distinguish significant aetiologies in childhood AES presentations.

Global literature highlights the significance of cytokines and chemokines in encephalitis, suggesting their potential use as biomarkers for identifying specific causes, assessing disease severity, and predicting outcome (14,15). However, available data on inflammatory markers in the Indian population is mostly derived from studies focusing on adults infected with the Japanese encephalitis virus (JEV) (14). Recent surveillance data indicates a shift in prevalent causes of AES in India, with infections like scrub typhus, dengue, and chikungunya emerging as the predominant causes (1,2,16). This shift highlights the need to investigate host inflammatory responses and potential biomarkers in cases caused by these emerging infections. Additionally, while it is known that infections such as scrub typhus and dengue, similar to MIS-C, can trigger a cytokine storm (5,6,17), the specific differences in cytokine and chemokine profiles among these various causes remain unclear.

Another potential biomarker, neurofilament light chain (NfL), is a neuron-specific protein, found in the neuronal cytoplasm. It is consistently released at low levels from axons into both CSF and blood. Increased NfL levels are associated with neurodegenerative diseases and traumatic brain injury(18). Recent literature indicates that NfL is linked to encephalopathy and unfavourable outcomes in infectious conditions like malaria, meningitis, pneumonia, and COVID-19 (19-22). While the ability of elevated NfL levels to differentiate between various causes of CNS infections remains unclear (23), its diagnostic and prognostic potential in Indian children with AES is unexplored. Additionally, since these proteins are consistently released into the bloodstream and can withstand freezing and thawing (24), there is a need to explore serum NfL as a potential biomarker for different causes of AES.

This study aimed to compare clinical and laboratory characteristics, investigate serum and CSF inflammatory markers and NfL profiles among children with AES in southern India, focusing on scrub typhus, viral aetiologies and MIS-C. It also sought to identify markers indicating disease severity and predicting patient outcomes.

Methods

Children aged 1 month to 18 years meeting the Indian National Vector Borne Disease Control Programme and WHO case definition for AES (25), with illness duration of less than 30 days at presentation, were prospectively enrolled at three tertiary care hospitals in Bangalore, Karnataka, India – Indira Gandhi Institute of Child Health, St. John's Medical College and Hospital, Vani Vilas Hospital, from March 2020 to December 2022. In brief, the case definition included children with fever and altered mental status (altered behaviour/ personality, irritability, lethargy, drowsiness, altered speech) with or without new-onset seizures. The study received approval from the institutional ethics and review boards of the hospitals and the coordinating centre, National Institute of Mental Health and Neurosciences (NIMHANS). The study team, trained in obtaining consent from parents/ guardians and assent from older children, followed approved procedures and forms for the process. A subset of few patients with different aetiologies, as mentioned below, were selected for this study.

Microbiological tests

Utilising a previously outlined laboratory algorithm (2) (**Supplementary Figure 1**), blood and CSF samples underwent extensive testing for infectious causes of AES. Briefly, initial assays included serological tests for JEV, dengue, chikungunya, *Leptospira* sp, and scrub typhus. Subsequent tests involved real-time PCR for bacterial pathogens, herpes simplex virus (HSV)-1⁄2, enterovirus, varicella-zoster virus (VZV), mumps virus, and parechovirus. Samples were further stored at −80°C for additional tests mentioned below, with a focus on minimising freeze-thaw cycles.

Children with viral aetiologies were categorised as "AES-Viral", while those with *Orientia tsutsugamushi* as the causal pathogen were categorised as "AES-Scrub typhus". Patients meeting the 2020 CDC MIS-C case definition without an alternative diagnosis were grouped as "AES-MIS-C" (26). The patient distribution in each aetiological group was as follows: AES-Viral (n=21), AES-Scrub typhus (n=29), and AES-MIS-C (n=12).

SARS-CoV-2 anti-nucleocapsid antibody and RNA detection

Quantitative determination of anti-nucleocapsid antibodies was performed in all serum samples using the Elecsys Anti-SARS-CoV-2 N electrochemiluminescence immunoassay (Roche Diagnostics International Ltd, Rotkreuz, Switzerland). A cutoff index ≥1.0, as recommended by the manufacturer, indicated a reactive/positive result for anti-SARS-CoV-2 antibodies. Additionally, serum and CSF specimens underwent real-time PCR for SARS-CoV-2 RNA detection. RNA extraction utilised the QIAamp Viral RNA Mini Kit, and real-time PCR was conducted using the NIV-ICMR Single Tube Four Target Assay Kit (27).

Clinical findings and patient outcome

Clinical characteristics and routine laboratory findings were entered into an online platform (28). Clinical findings of children with AES-MIS-C were described based on 2020 CDC MIS-C case definition (26), which includes fever, laboratory evidence of inflammation, clinically severe illness requiring hospitalisation, multisystem organ involvement (>2), and positive current or recent SARS-CoV-2 infection by RT-PCR, serology, or antigen test; or exposure to a suspected or confirmed COVID-19 case within 4 weeks prior to symptom onset, and absence of an alternative diagnosis. Admission Glasgow Coma Scale (GCS) scores were recorded, with a score of 15 considered normal and ≤14 deemed abnormal. Patient outcomes, recorded by telephone consultation 3 months after discharge using the Liverpool Outcome Score (LOS) (29), were classified good at a score of 5 or 4 (indicating no or minor disability) and poor as scores of ≤3 (indicating severe or moderate disability or death).

Cytokine and chemokine profiling

Serum and CSF cytokines/chemokines were measured using a Bio-Plex Pro Human Cytokine Screening Panel (Bio-Rad, Cat No. 12007283). Seventeen human cytokines/chemokines were assessed, with Panel 1 including IL-1RA, IL-12(p40), CCL11 (Eotaxin), GM-CSF, CCL2 (MCP-1), GROα (CXCL1), HGF, and M-CSF, while Panel 2 included IL-1β, IL-2, IL-6, IL-10, IFN-γ, TNF, IL-8, IL-17A,

and IL-1α. Briefly, samples and standards were incubated with antibody-conjugated beads, biotinylated detection antibodies, and streptavidin–phycoerythrin. The plate was read using a Bio-Plex MAGPIX multiplex reader (Bio-Rad), and data were analysed using Bio-Plex Manager Software version 6.0. Only mediators detected in >80% of samples were included in analyses to avoid bias due to undetectable levels or missing data (30). The balance between proinflammatory and anti-inflammatory cytokines was assessed by calculating the IL-1β to IL-1RA ratio in CSF and the IL-6 to IL-10 and IL-17A to IL-10 ratios in serum.

NfL levels

NfL in CSF and serum was analysed using a SIMOA® immunoassay (Quanterix Corporation, Billerica, MA, USA) following a two-step procedure as described previously (31). Serum samples were added without dilution, while CSF samples underwent a 1:25 dilution on-site, followed by a 4x instrument dilution for both CSF and serum specimens, resulting in a final dilution of 1:100 for CSF and 1:25 for serum. A calibration curve with provided calibrators covered the expected concentration range, and NfL concentrations were interpolated using a 1/y^2 weighted four-parameter logistic curve fit, following the manufacturer's instructions.

Statistical Analysis

Statistical analyses and data visualisation utilised R version 3.6.3 (The R Project for Statistical Computing) and Prism 8 version 10.1.0 (GraphPad Software). Descriptive statistics for categorical variables were presented as frequencies and percentages, while continuous variables were summarized using median and interquartile range (IQR) after assessing normality through visual inspection of histograms. Categorical data were compared using the Chi-square or Fisher's exact test, depending on sample size. Continuous skewed variables among the three aetiological groups were compared using the Kruskal-Wallis test with Bonferroni correction. Additionally, differences between poor and good outcomes were assessed using the Mann-Whitney U test. A p-value < 0.05 was considered statistically significant. Explanatory variables identified following initial descriptive analysis were fitted into logistic regression models to evaluate their predictive value for determining aetiology. Final model selection was performed using a "criterion-based approach" to minimise the Akaike information criterion (AIC) and maximise the concordance statistic (C-statistic). We further controlled for the potential confounding effects of age and duration of illness by including them as covariates in the logistic regression model.

Exploratory correlations were examined using Spearman's correlation test, for examining relationships between CSF and serum cytokine/chemokine levels, CSF and serum NfL levels, NfL and cytokine/chemokine levels, GCS and cytokines/chemokines levels, and GCS and NfL levels. Heatmaps were generated from the correlation matrices to visually represent the mediators and show those which have a strong positive (dark red) or a strong negative (dark blue) correlation, as previously described (15). A subtraction matrix heatmap was created by subtracting CSF cytokine correlations from serum cytokine correlations. The 2D cytokine network analyses were created using the Spearman's rank correlation coefficients of greater than 0.6 to generate lines between the cytokines using the qgraph package in R software and matrix differences were assessed by the Steiger's test (32)

Results:

Out of the 62 children in the study, 12 (19%) were classified as AES-MIS-C, 29 (47%) as AES-Scrub typhus, and 21 (34%) as AES-Viral, determined by the laboratory algorithm. The median duration between illness onset and the day of sample collection after hospitalisation varied across groups, with 7 (IQR 5-9) days for AES-Scrub typhus, 5 (IQR 4-8) days for AES-Viral, and 9 (IQR 6.5-17) days for AES-MIS-C (p=0.023). Serum samples from all 62 children underwent testing for anti-SARS-CoV-2 nucleocapsid antibodies and SARS-CoV-2 PCR. For cytokine/chemokine assays, 58 serum and 42 CSF samples from 62 children were available, while for determining NfL levels, 20 serum and 18 CSF samples from 21 children were available.

SARS-CoV-2 anti-nucleocapsid antibody and RNA detection

Anti-nucleocapsid antibodies were detected in all AES-MIS-C cases, 83% of AES-Scrub typhus cases (24/29), and 62% of AES-Viral cases (13/21). No samples tested positive for SARS-CoV-2 RNA by real-time PCR.

Clinical and routine laboratory parameters

Supplementary table 1 outlines the characteristics of children with AES-MIS-C. All 12 had fever, positive anti-nucleocapsid antibodies, and evidence of inflammation. Haematological involvement was observed in all 12 children, cardiac involvement and features of shock in 8 (67%). Gastrointestinal, respiratory, and mucocutaneous involvement were observed in 9 (75%), 6 (50%), and 5 (42%) children, respectively. Neurological findings were non-specific and included altered sensorium in the form of lethargy, inconsolable cry, drowsiness, abnormal speech, refusal of feed, decreased activity, irrelevant talk or irritability; seizures in 9 (75%), hypertonia, signs of meningeal irritation in 2 (16.7%) each, headache and limb weakness in one child each.

Tables 1 details variations in clinical and laboratory variables among the three groups. Significant differences include age, illness and hospitalisation duration, personality/behavioural changes, respiratory abnormalities, hepatomegaly, blood neutrophil and lymphocyte percentages, platelet count, direct bilirubin levels, serum albumin levels, CSF white cell count, CSF lymphocyte count, and CSF protein concentrations.

Table 1: Differences in clinical and laboratory findings between the three aetiological groups

Clinical/ laboratory variables		n	AES-Viral (N=21)	n	AES-Scrub typhus (N=29)	n	AES-MIS-C (N=12)	p value	Multiple comparison
Age (years)		21	1.0 (0.7, 7)	29	9 (6, 12)	12	2.5 (1.4, 7.5)	**<0.001**	Scrub typhus vs MISC (p=0.033) Scrub typhus vs Viral (p=<0.001)
Gender	Male	21	12 (57.1)	29	14 (48.3)	12	9 (75)	0.29	
	Female		9 (42.9)		15 (51.7)		3 (25)		
Glasgow coma scale score (at		15	11 (8, 15)	23	13 (11, 15)	10	8.5 (8, 12.3)	**0.047**	-

admission)									
Duration of illness (days)		21	3.0 (2, 4)	29	5 (4, 7)	12	7 (3.8, 10.5)	**0.001**	Viral vs. Scrub typhus p=0.001; Viral vs. MISC p=0.0331
Admission in ICU required	No	21	8 (38.1)	29	13 (44.8)	12	3 (25)	0.49	
	Yes		13 (61.9)		16 (55.2)		9 (75)		
Change in personality/ behaviour	No	21	13 (65)	29	29 (100)	12	12 (100)	**<0.001**	
	Yes		7 (35)		0 (0)		0 (0)		
Abnormal speech	No	21	15 (71.4)	29	25 (86.2)	12	11 (91.7)	0.31	
	Yes		6 (28.6)		4 (13.8)		1 (8.3)		
Irrelevant talk	No	21	19 (90.5)	29	27 (93.1)	12	11 (91.7)	1	
	Yes		2 (9.5)		2 (6.9)		1 (8.3)		
Seizures	No	21	5 (23.8)	29	15 (51.7)	12	3 (25)	0.08	
	Yes		16 (76.2)		14 (48.3)		9 (75)		
Respiratory symptoms (cough/ shortness of breath/ sore throat)	No	21	16 (76.2)	29	27 (93.1)	12	4 (33.3)	**<0.001**	
	Yes		5 (23.8)		2 (6.9)		8 (66.7)		
Musculoskeletal symptoms (joint/ muscle pain)	No	21	18 (85.7)	29	28 (96.6)	12	12 (100)	0.24	
	Yes		3 (14.3)		1 (3.4)		0 (0)		
Gastrointestinal symptoms (diarrhoea/ pain abdomen/ abdominal distension)	No	21	13 (61.9)	29	15 (51.7)	12	5 (41.7)	0.52	
	Yes		8 (38.1)		14 (48.3)		7 (58.3)		
Lymphadenopathy	No	21	21 (100)	29	24 (82.8)	12	12 (100)	0.07	
	Yes		0 (0)		5 (17.2)		0 (0)		

Oedema	No	21	19 (90.5)	29	22 (75.9)	12	11 (91.7)	0.35	
	Yes		2 (9.5)		7 (24.1)		1 (8.3)		
Mucocutaneous findings (Conjunctival congestion/ Skin rash/ Eschar)	No	21	17 (81)	29	22 (75.9)	12	7 (58.3)	0.35	
	Yes		4 (19)		7 (24.1)		5 (41.7)		
Hepatomegaly	No	21	17 (81)	29	13 (44.8)	12	6 (50)	**0.03**	
	Yes		4 (19)		16 (55.2)		6 (50)		
Splenomegaly	No	21	21 (100)	29	24 (82.8)	12	12 (100)	0.07	
	Yes		0 (0)		5 (17.2)		0 (0)		
Cranial nerve abnormality	No	21	21 (100)	29	28 (96.6)	12	12 (100)	1.000	
	Yes		0 (0.0)		1 (3.4)		0 (0.0)		
Cerebellar signs	No	21	20 (95.2)	29	25 (86.2)	12	12 (100)	0.411	
	Yes		1 (4.8)		4 (13.8)		0 (0.0)		
Meningeal irritation	No	21	15 (71.4)	29	14 (48.3)	12	10 (83.3)	0.088	
	Yes		6 (28.6)		15 (51.7)		2 (16.7)		
Total leukocyte count ($\times 10^9$/l)		21	12.1 (7.7, 16.1)	29	12.8 (8.3, 15.5)	12	14.3 (9.2, 17.5)	0.756	-
Neutrophils %		21	63.8 (42.2, 73.7)	29	54.8 (43.8, 69)	12	74.3 (55.4, 83.4)	**0.022**	Scrub typhus vs MISC (p=0.01)
Lymphocytes %		21	27.5 (19.1, 46.7)	29	38.4 (27.4, 51.9)	12	21.1 (12.1, 37.1)	**0.010**	Scrub typhus vs MISC (p=0.011)
Platelets ($\times 10^9$/l)		21	196 (79, 376.5)	29	108 (36, 156.5)	12	159 (51.3, 267.5)	**0.019**	Scrub typhus vs Viral (p=0.016)
Direct bilirubin ((mg/dL)		17	0.2 (0.1, 0.4)	25	0.6 (0.2, 2.1)	10	0.2 (0.1, 0.6)	**0.021**	Scrub typhus vs Viral

									(p=0.023)
Aspartate transaminase (AST) (IU/L)		17	61.6 (24.9, 286.4)	28	144.4 (79.7, 397.1)	10	127.1 (36.6, 265.5)	0.170	-
Alanine transaminase (ALT) (IU/L)		17	44.5 (18.3, 148.1)	28	79.6 (54.2, 145.6)	9	80 (24.5, 274.2)	0.304	-
Serum albumin (g/dl)		16	3.6 (3.1, 4.2)	27	2.7 (2.3, 3.2)	10	3.1 (2.7, 4.1)	**0.006**	Scrub typhus vs Viral (p=0.005)
Urea (mg/dl)		17	28.5 (20, 37.2)	26	27.2 (22.1, 31.7)	11	37.7 (20.9, 67.7)	0.199	-
Creatinine (mg/dl)		21	0.3 (0.3, 0.5)	29	0.4 (0.3, 0.5)	12	0.5 (0.4, 0.7)	0.165	-
CSF total white cell count (cells/µL)		14	3 (0, 88.3)	20	36.5 (10.8, 82.5)	10	2 (0, 6.8)	**0.006**	Scrub typhus vs MISC (p=0.006)
CSF lymphocyte count (cells/µL)		14	3 (0, 83.3)	20	27 (10, 56)	9	2 (0, 8.5)	**0.017**	Scrub typhus vs MISC (p=0.019)
CSF neutrophil count (cells/µL)		14	0 (0, 4.5)	20	1.5 (0, 19)	8	0 (0, 0)	0.104	-
CSF protein concentration (mg/dl)		14	23.4 (18, 52.9)	20	64.8 (48.3, 97.8)	10	21 (10.5, 39.3)	**0.001**	Scrub typhus vs MISC (p=0.002) Scrub typhus vs Viral (p=0.009)
Duration of hospitalisation (days)		15	9 (7, 9.5)	25	8 (5, 9)	12	17 (11.8, 28.5)	**0.002**	Viral vs. MISC p=0.049; Scrub typhus vs. MISC p=0.002

Categorical variables are represented as no. patients (%) and continuous variables as Median (Q1, Q3); N= Total no. of patients in each aetiology, n= no. of patients in whom variable was analysed

The median (IQR) age of the AES-Scrub typhus group was 9 (6,12) years, while it was 2.5 (1.4, 7.5) years for AES-MIS-C and 1.0 (0.7, 7) years for AES-Viral. Multivariable logistic regression revealed that higher age predicted AES-Scrub typhus over AES-Viral [Odds Ratio (CI, p value): 1.52 (1.18-2.23, p=0.006)] and AES-MIS-C [Odds Ratio (CI): 1.37 (1.13-1.7, p=0.005)]. Longer duration of illness was an independent predictor for AES-MIS-C over AES-Viral [Odds Ratio (CI): 1.26 (1.05-1.61, p=0.028)]. A substantial majority (93%) of AES-Scrub typhus cases were aged ≥3 years (27/29) compared with 45% in AES-Viral/MIS-C (15/33) (p <0.005). Assessing the diagnostic performance of age ≥3 years in distinguishing AES-Scrub typhus from AES-Viral/MIS-C, indicated a sensitivity (95% CI) of 93% (77.2-99.1%), specificity of 54.5% (36.3-71.9%), positive predictive value of 64.3% (55.-72.6%), negative predictive value of 90% (69.5-97.3%), and an overall accuracy of 62.6% (59.8-83.1%). Therefore, while age ≥3 years may provide an initial indication, it is essential to note that further diagnostic laboratory confirmation is necessary for a definitive diagnosis.

Inflammatory mediator profile

Analysing cytokine/chemokine profiles, we examined 9 serum and 10 CSF samples from AES-MIS-C, 28 serum and 17 CSF samples from AES-Scrub typhus, and 21 serum and 15 CSF samples from AES-Viral. Mediators not identified in >80% samples (CSF & Serum IL-12p40, GM-CSF, IL-2, IL-1α; CSF IL-10, IL-17A; and Serum IL-1β) were excluded from analysis. The cytokine/chemokine profiles of children in the three aetiological groups are shown in **Tables 2 & 3 and represented in Figure 1**. Serum CCL11, CCL-2, and HGF

concentrations significantly differed between aetiological. Logistic regression was fitted to predict AES-Viral considering serum CCL11 and CCL2 as predictors. The results indicated higher serum CCL11 as predictive of AES of viral aetiology over scrub typhus [AES Viral vs. AES-Scrub typhus: 1.013 (1.003-1.023, p=0.010)]. In CSF, AES-Scrub typhus exhibited significantly higher levels of IL-1RA (p=0.012), IL-1 β (p=0.001), and TNF (p=0.001) than AES-MIS-C (**Table 3**), while higher CCL-2 levels were predictive of AES-Viral over AES-Scrub typhus [AES-Viral vs AES-Scrub typhus: Odds Ratio (CI, p value): 1.02 (1.004-1.036, p=0.012)] using logistic regression. After accounting for age and duration of illness as potential confounders, the observed relationships between cytokine levels aetiological groups were consistent with the initial findings.

Table 2: Serum inflammatory mediator profiles of patients in the study

Mediator (pg/ml)	AES-Viral (n=21)	AES-Scrub typhus (n=28)	AES-MISC(n=9)	p-value	Multiple comparisons
	Median (Q1, Q 3)				
IL-1RA	2601.3 (796.1, 5323.2)	3049 (748.7, 9104.3)	3569 (731, 9783.5)	0.898	-
CCL11	166 (113.6, 261)	77.1 (43, 114.1)	185.1 (71.1, 239.5)	**0.001**	Scrub typhus vs Viral (p=0.001)
CCL2	181.8 (123.1, 463.7)	60 (31.1, 135.4)	132.2 (96.9, 312.1)	**0.004**	Scrub typhus vs Viral (p=0.003)
GROα	546.5 (339, 948.5)	230.2 (53.8, 765.9)	281.1 (53.8, 557.7)	0.080	-

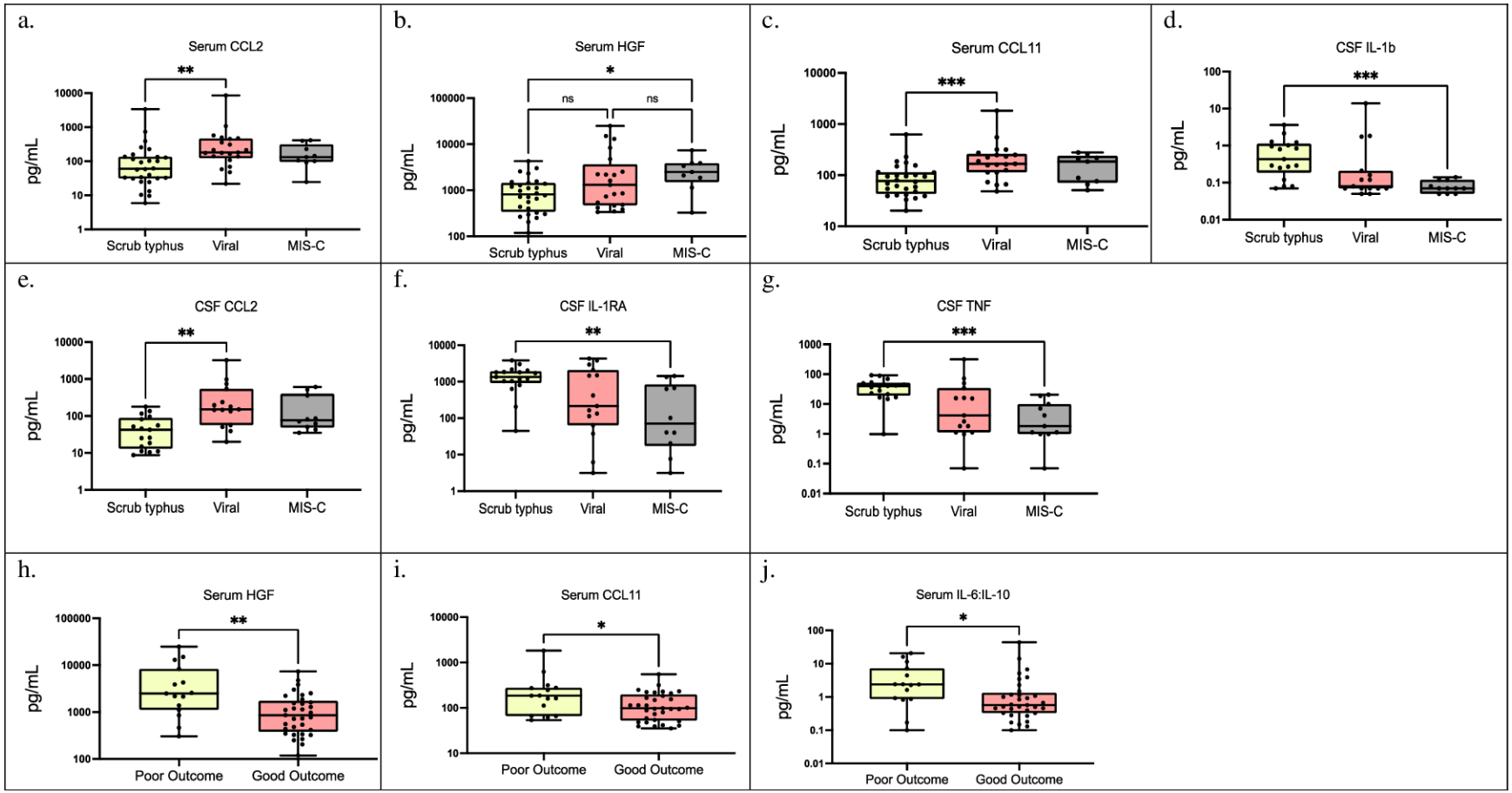
HGF	1304 (471.5, 3635.6)	817.1 (342.6, 1430)	2489.4 (1503.1, 3858.6)	**0.018**	Scrub typhus vs MISC (p=0.025)
M-CSF	44 (19.9, 99.5)	38.8 (15.4, 57.3)	19.8 (16, 36.6)	0.223	-
IL-6	8.8 (4.1, 24.5)	8.9 (2.1, 21.1)	12.2 (1.4, 44.1)	0.955	-
IL-10	9.6 (1.7, 31.6)	10.2 (3.5, 27.3)	3.8 (1.9, 15.7)	0.526	-
IFN-γ	1 (0.4, 6)	1 (0.4, 7.8)	0.6 (0.4, 1.4)	0.489	-
TNF	10.8 (6.1, 35.9)	10.5 (4.6, 27.8)	12.9 (5.8, 20.2)	0.902	-
IL-8	31.5 (11.8, 223.5)	36.8 (9.5, 122.9)	19.4 (11.8, 157.9)	0.952	-
IL-17A	0.7 (0.5, 4.9)	0.7 (0.3, 7.5)	1.8 (0.3, 6.3)	0.983	-
IL-6/ IL-10	0.9 (0.5, 6)	0.7 (0.3, 1.2)	2.4 (0.3, 3.8)	0.396	-
IL-17A/ IL10	0.2 (0.1, 0.5)	0.2 (0.1, 0.4)	0.1 (0.1, 0.7)	0.754	-

Table 3: CSF inflammatory mediator profiles of patients in the study

Mediator (pg/ml)	AES-Viral		AES-Scrub typhus		AES-MISC		p-value	Multiple comparisons
	n	Median (Q1, Q3)	n	Median (Q1, Q3)	n	Median (Q1, Q3)		
IL-1RA	15	214.9 (63.8, 2085.2)	17	1345.5 (919.9, 1925.7)	10	70.9 (17.2, 838.2)	**0.012**	Scrub typhus vs MISC (p=0.01)
CCL11	15	1.6 (0.7, 2.6)	17	1.7 (1.1, 3.7)	10	1.8 (0.2, 4.1)	0.691	-
CCL2	15	151 (56.6, 544.1)	17	42.4 (13, 88.5)	10	76.6 (48.5, 399.2)	**0.002**	Scrub typhus vs Viral (p=0.01)
GROα	15	13.5 (13.5, 13.5)	16	13.5 (13.5, 106.7)	10	13.5 (13.5, 78.2)	0.866	-

HGF	15	224.5 (106, 860.9)	17	540 (191.7, 737.6)	10	203.6 (88.3, 1399)	0.560	-
M-CSF	15	6.5 (1.8, 8.6)	17	4 (3.5, 6.4)	10	3.5 (2.1, 5.6)	0.545	-
IL-1β	15	0.1 (0.1, 0.2)	17	0.4 (0.2, 1.1)	11	0.1 (0.1, 0.1)	**0.001**	Scrub typhus vs MISC (p=0.001)
IL-6	15	17.3 (0.9, 105.2)	17	11.4 (3.4, 27.9)	11	4.9 (2.2, 45.7)	0.838	-
IFN-γ	15	1.1 (1.1, 2.9)	17	1.6 (0.2, 2)	11	1.1 (0.2, 1.1)	0.076	-
TNF	15	4.2 (1.1, 34.6)	17	40.2 (19.2, 51.1)	11	1.8 (1, 9.9)	**0.001**	Scrub typhus vs MISC (p=0.001)
IL-8	15	21.3 (11, 42.3)	17	71.3 (21.5, 118)	11	29 (8.3, 63)	0.145	-

Figure 1: Graphical representation of cytokines/ chemokines significantly associated with aetiologies and patient outcomes



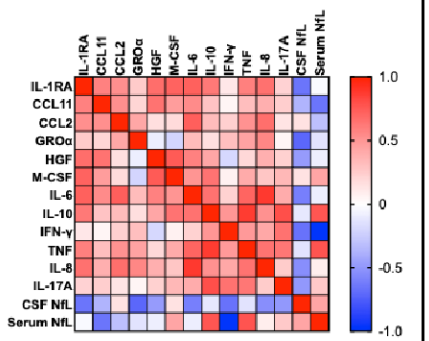
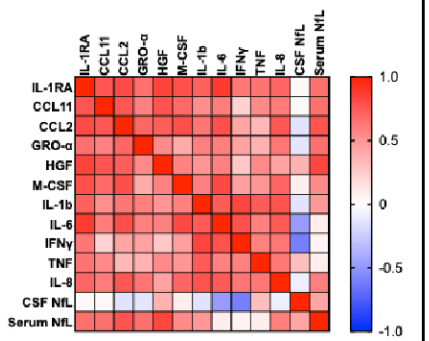
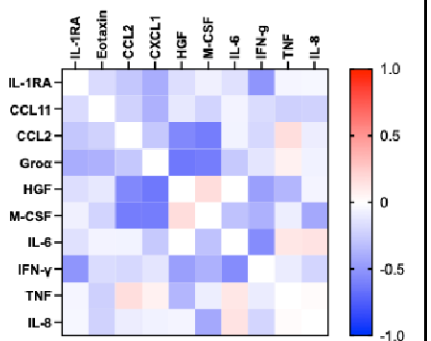
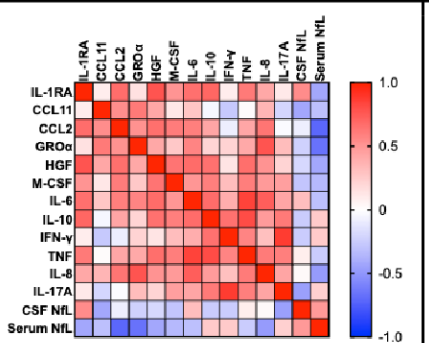
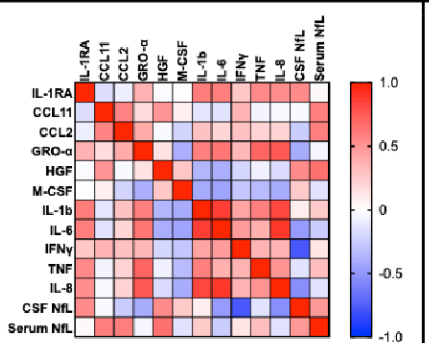
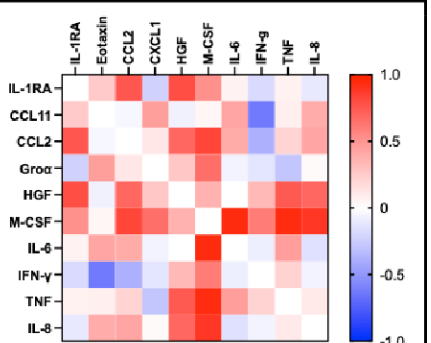
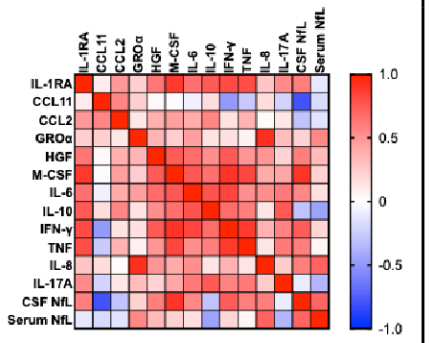
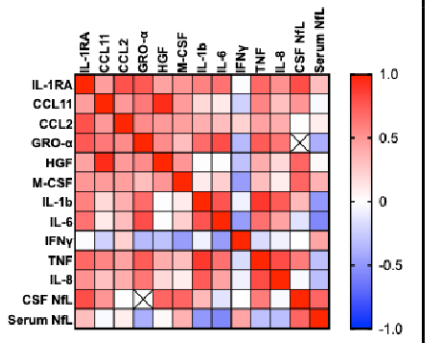
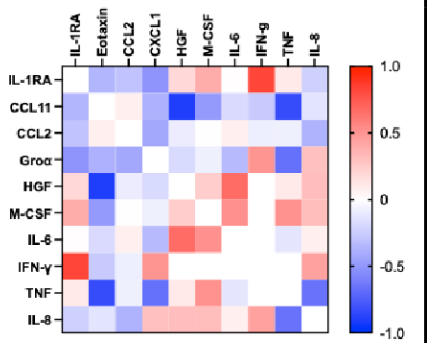
1(a)- 1(g): Concentrations of mediators associated with aetiological groups of AES in serum and CSF

1(h)- 1(j): Serum concentrations of mediators associated with outcomes

Around 89% (25/28) of AES-Scrub typhus cases had serum CCL11 levels <180 pg/ml compared with 50% in AES-Viral/MIS-C (15/30) ($p<0.001$). A combined criteria of age ≥3 years and serum CCL11 levels <180 pg/ml was observed in 24/28 AES-Scrub typhus cases and 5/30 AES-Viral/MIS-C cases. The combined criteria led to an improved discrimination, achieving a sensitivity (95% CI) of 85.7% (67.3-95.9%), specificity of 83.3% (65.3- 94.4%), positive predictive value of 82.8% (68-91.5%), negative predictive value of 86.2% (71.3- 94%), and an overall accuracy of 84.5% (72.6-92.7%) for a diagnosis of scrub typhus. Similarly, a combined criteria of age ≥3 years with serum CCL2 levels <140 for a diagnosis of scrub typhus, demonstrated sensitivity and specificity of 75% and 77%, respectively and a combined criteria of age ≥3 years with CSF CCL2 <100 demonstrated sensitivity and specificity of 82% and 56%, respectively.

Heat-map (**Figure 2**) and 2D network analyses (**Supplementary Figure 2**) revealed distinct patterns among different aetiologies and between CSF and serum within each aetiology. **Supplementary Figure 3** shows subtraction heatmaps comparing the three aetiologies**.** AES-Viral group demonstrated a coordinated upregulation of most mediators primarily in CSF, AES-Scrub typhus exhibited this phenomenon mainly in serum, and AES-MIS-C displayed a mixed pattern. The results of Steiger's test indicated a statistically significant difference between the 2D network analyses of cytokines/ chemokines in CSF of patients with AES-Scrub typhus and AES-Viral groups (**Supplementary table 2).**

368 **Figure 2**: Heatmaps of correlations between different mediators in serum and CSF of aetiological groups

Aetiology	Serum	CSF	Subtraction heatmaps*
AES-Viral			
AES-Scrub typhus			
AES-MIS-C			

*Subtraction matrix shows CSF cytokine correlations subtracted from the serum cytokine correlations with white showing the correlations which are similar between the two samples, while red and blue show the many distinct relationships (Figure 2 far-right column)

NfL levels

NfL testing was conducted on a total of 38 samples (CSF=18, serum =20) obtained from 21 patients. Paired CSF and serum samples were analysed for 17 patients (AES-Viral, n=6; AES-Scrub typhus, n=6; AES-MIS-C, n=5), while additional four AES-MIS-C patients were specifically examined for either serum NFL (n=3) or CSF NfL (n=1). The median (IQR) serum and CSF NfL values were 60.5 (23.2-153.0) and 22.0 (13.0-40.8) pg/ml, respectively.

Overall, a significant correlation was present between CSF & serum NfL (r=0.49, p=0.04). However, no significant differences were found between serum and CSF NfL values of different groups (**Supplementary table 3**). A significant negative correlation was observed between CSF Nfl and serum CCL11 concentrations (r=-0.6, p=0.01) and a weak correlation was observed between serum NfL and HGF concentrations (r=0.5, p=0.03).

Glasgow coma scale score

GCS scores at admission were available for 48 (77%) children, and information on abnormal (<15) or normal (15) GCS, based on clinical presentation at admission, were available for 61 (98%) children. AES-MIS-C children had significantly lower GCS scores (p=0.04) (Table 2), with 83% having a abnormal admission GCS, compared with 67% and 62% in AES-Viral and AES-Scrub typhus, respectively. Median CSF and serum NfL levels were higher in children with abnormal GCS, but the difference was not significant (**Supplementary table 4**). A weak correlation existed between GCS score and serum NfL (r=-0.57, p=0.022). In children with AES-Viral, GCS significantly correlated negatively with CSF IL-8 (r=-0.76, p=0.01); whereas in children with AES-MIS-C, GCS significantly correlated negatively with Serum IL-6/ IL-10 ratio (r= -0.88, p= 0.006).

Patient outcomes

Fifty-four children survived to hospital discharge. LOS could be assessed at three months after discharge in 51/ 62 (82%) children, with 16 (31.4%) experiencing poor outcomes and 35 (68.6%) achieving good outcomes. Full recovery (LOS=5/5) occurred in 33 (65%) children, mild sequelae (LOS=4/5) in 2 (4%), moderate sequelae (LOS=3/5) in 7 (14%), and severe sequelae (LOS=2/5) in 1 (2%). Eight children succumbed to their illness (LOS=1/5). AES-Viral exhibited a higher percentage of poor outcomes (38%) compared to AES-Scrub typhus (17%) and AES-MIS-C (25%) (p=0.5). Significantly higher concentrations of serum CCL11, HGF, and IL-6/IL-10 ratio were associated with poor outcomes (p=0.038, 0.005, and 0.02, respectively) (**Table 4**). No significant associations were observed

between CSF cytokine levels and patient outcomes. Median CSF and serum NfL values were higher in patients with poor outcomes, but these differences were not significant.

Table 4: **Serum mediator profiles and NfL levels in patients with poor and good outcomes**

Mediators & NfL (pg/ml)	N (total)	Poor outcome N (%)= 16 (31.4)	Good outcome N (%)= 35 (68.6)	Total N=51	p
IL-1RA	48 (94.1)	4419.9 (749.4 to 14088.5)	2601.2 (798.0 to 6699.1)	3341.2 (758.3 to 7131.7)	0.470
CCL11	48 (94.1)	185.4 (89.3 to 275.4)	98.7 (54.2 to 185.1)	113.6 (64.9 to 219.1)	**0.038**
CCL2	48 (94.1)	181.8 (119.9 to 427.3)	115.6 (61.4 to 213.1)	134.9 (67.1 to 269.2)	0.122
HGF	48 (94.1)	2489.4 (1253.0 to 6317.7)	858.7 (415.4 to 1610.2)	1168.3 (475.1 to 2497.0)	**0.005**
M-CSF	48 (94.1)	52.5 (21.8 to 81.6)	40.0 (18.1 to 56.7)	40.1 (19.4 to 61.5)	0.281
IL-6	48 (94.1)	12.4 (5.4 to 44.8)	8.8 (2.3 to 16.9)	9.2 (3.3 to 22.7)	0.164
IL-10	48 (94.1)	9.1 (2.4 to 28.9)	9.9 (2.8 to 18.6)	9.4 (2.6 to 27.2)	0.991
IFN-γ	45 (88.2)	0.6 (0.5 to 2.1)	1.1 (0.4 to 6.3)	0.9 (0.5 to 5.9)	0.455
TNF	47 (92.2)	10.0 (5.6 to 31.4)	14.2 (6.6 to 25.6)	11.0 (6.5 to 27.2)	0.825
IL-8	48 (94.1)	38.7 (15.4 to 146.9)	26.3 (11.6 to 137.3)	33.7 (12.9 to 139.7)	0.664

IL-17A	48 (94.1)	0.5 (0.3 to 0.9)	1.1 (0.5 to 6.8)	0.7 (0.5 to 4.4)	0.139
IL-6/ IL-10	48 (94.1)	2.4 (0.9 to 5.9)	0.6 (0.3 to 1.2)	0.9 (0.4 to 2.4)	**0.020**
IL-17a/ IL-10	48 (94.1)	0.1 (0.0 to 0.6)	0.2 (0.1 to 0.4)	0.1 (0.1 to 0.4)	0.311
NfL	18 (35.3)	176.5 (113.5 to 799.5)	54.0 (22.8 to 150.5)	79.0 (22.8 to 155.0)	0.339

Discussion

This study identified distinct clinical and laboratory features, along with inflammatory mediator and NfL profiles in children with AES due to scrub typhus, viral aetiologies, and MIS-C in southern India.

A significant proportion of children with AES-Scrub typhus and AES-Viral in our study exhibited positive results for SARS-CoV-2 anti-nucleocapsid antibodies, despite no prior vaccination against SARS-CoV-2. This observation implies potential prior exposure or asymptomatic infection, which presents a diagnostic challenge, as noted in previous studies (33,34). Therefore to ensure appropriate treatment strategies, an exclusion of endemic causes of AES is paramount before making a diagnosis of MIS-C in patients with neurological manifestations, especially in regions with a high prevalence of AES (6).

As shown in Table 1, there were a few significant differences in the clinical and routine laboratory parameters of children with AES-MISC compared to other groups. Notably, the AES-MIS-C group had highest anti-nucleocapsid antibody cut-off [median (Q1-Q3): 68.6 (46.9-137.4)] compared with AES-Scrub typhus [35.2 (9.1-126.1)] and AES-Viral [59 (17.6-121.7)]. No AES-MIS-C case tested PCR positive for SARS-CoV-2, most likely due to delayed hospital presentation and sampling [Median (Q1, Q3): 9 (6.5, 17) days].

While reports, mostly based on studies from developed nations suggest a median age of 9 years for MIS-C, our study, alongside multicentre studies in Asia and Africa, observed a lower median age (35-37). We hypothesize that exposure to a diverse range of pathogens and antigens through natural infections and vaccination at a younger age in these regions may foster the development of a robust immunological memory. This enhanced immune memory could potentially lead to more prompt and potent immune responses upon subsequent encounters with SARS-CoV-2, thereby contributing to MIS-C at a younger age. However, further research is warranted to validate this hypothesis and elucidate the underlying mechanisms. In contrast to AES-MIS-C, children with AES- Scrub typhus had a higher median age, as reported in other studies (38). An older age [Median (IQR): 9 (6, 12) years] was identified as an independent predictor for children with AES-Scrub typhus, distinguishing them from AES-Viral [Median (IQR): 1.0 (0.7, 7) years] and AES-MIS-C [Median (IQR): 2.5(1.4, 7.5) years]. This association suggests that higher outdoor activities and increased exposure to vectors in older age may contribute to susceptibility. Given that scrub typhus is the primary cause of childhood AES in southern India (2), these insights provide valuable information on associated risk factors.

The cytokine profiles in our study provided distinct discriminatory information for each AES aetiology. Notably, serum and CSF patterns within each aetiology showed differences, highlighting the unique immune profile of the CNS compared to peripheral blood. Correlation among cytokines/chemokines within the CSF network differed significantly between scrub typhus and viral aetiologies, indicating distinct underlying pathophysiological mechanisms within CNS associated with these two causes of AES (**Figure 2** & **Supplementary Figure 2**)

AES-Viral group showed the highest concentrations of both serum and CSF CCL2, followed by AES-MIS-C and AES-Scrub typhus. Elevated CCL2 levels were identified as an independent predictor and potential biomarker for differentiating AES-Viral from AES-Scrub typhus. This aligns with established knowledge, that infected astrocytes, macrophages/microglia, and neurons produce elevated CCL2 levels during viral encephalitis and neuro-COVID, contributing to blood-brain barrier disruption and neuroinvasion (30,39,40) Elevated CCL11 levels, linked to diminished hippocampal neurogenesis and cognitive symptoms observed in long COVID ("COVID fog") (41,42), were observed in children with AES-MIS-C. Serum CCL11, also associated with neuroinflammation and neurodegeneration in chronic traumatic encephalopathy (43), was identified as an independent predictor and potential biomarker for distinguishing AES-Viral from AES-Scrub typhus.

A significant finding was that combining age ≥ 3 years with serum CCL11 <180 pg/ml in children with AES demonstrated robust diagnostic accuracy in distinguishing AES-Scrub typhus from AES-Viral and AES-MIS-C groups. However, given the limited number of patients in the study, these findings need validation on a larger population. Additionally, increased CSF TNF was linked to AES-

Scrub typhus, consistent with TNF's recognized role in enhancing permeability of infected brain endothelial cells in vitro and its active expression during scrub typhus infection in vivo (44,45).

Interestingly, serum CCL11 and HGF, as well as the ratio of pro-inflammatory IL-6 to anti-inflammatory IL-10, exhibited significant associations with adverse outcomes across all aetiologies. The ratio of CSF IL-1β to IL1RA, previously linked to poorer outcomes in HSV-1 encephalitis, underscores the essential role of balancing pro-inflammatory and anti-inflammatory signalling in pathogen control and inflammation regulation (15). Moreover, both HGF and IL-6 have been identified to correlate with severity in COVID-19 (46,47). HGF, recognized for its involvement in tissue repair and brain development, may suggest a reparative response with increased expression (48). IL-6, crucial in antiviral and antibacterial immune responses, has also shown negative effects on neurogenesis in both in vitro and in vivo settings (49,50).

The evaluation of NfL in CSF and sera was limited to a small number of children in each group. Consequently, while NfL levels were lower in AES-Viral compared with the other two groups, this difference did not reach statistical significance. These findings align with the results reported by van Zeggeren et al., where CSF NfL levels were lower in patients with viral CNS infections compared with those with bacterial infections, CNS inflammatory disease or systemic infection (23). Children with more severe neurological impairment (indicated by lower GCS scores) and poorer clinical outcomes had higher levels of NfL in their CSF and serum. Furthermore, as the GCS scores decreased, serum NfL levels tended to increase, though the strength of this correlation was relatively weak. These findings

462 indicate the utility of NfL as a potential marker for assessing brain injury and predicting outcomes in children with AES, consistent with
463 observations in other CNS conditions (23,39,51).

464

465 Another significant positive correlation, albeit weak, was observed between CSF and serum levels of NfL, similar to what has been
466 reported in patients with VZV CNS infections (52). This suggests the potential use of serum NfL as a surrogate marker for evaluating
467 neuronal damage without need for invasive CSF sapling. The weak negative correlation between CSF NfL and serum CCL11 may
468 indicate that the extent of neuroaxonal damage does not precisely align with the level of neuroinflammation marked by CCL11 alone.
469 in contrast, serum HGF, with known neuroprotective effects (48), exhibited a weak positive correlation with serum NfL. This observation
470 suggests a concurrent biological response involving the upregulation of HGF, potentially aimed at repair and regeneration, in response
471 to increased levels of serum NfL, a marker of neuronal damage. Interestingly, HGF was also significantly associated with poor outcomes
472 across all aetiological groups and were particularly elevated in children with MIS-C.

473

474 Our study is limited by the small sample size for testing mediator and NfL profiles within each aetiological group and the lack of
475 samples from healthy controls. By grouping viral causes of AES into a single group, potential differences among specific viral aetiologies
476 may have been overlooked.. Furthermore, since all participants were enrolled during the COVID-19 pandemic, most had positive SARS-
477 CoV-2 antibodies, which complicated definitive diagnose. Additionally, there were significant differences in sampling times in the

disease process and average age between these patient groups, which may have influenced the measured levels of inflammatory markers in the study. However, even after controlling for these two factors, results of the regression model remained consistent. These limitations highlight the importance of validating these findings in a larger, age-matched population study to enhance the generalizability and reliability of the results.

In summary, viral AES is associated with increased serum and CSF levels of CCL2 and CCL11, indicating their potential as biomarkers that warrant further investigation. The composite criterion—children aged ≥ 3 years with serum CCL11 <180 pg/ml—shows promise in differentiating scrub typhus from other causes, necessitating validation in a larger population. Elevated HGF levels, positively correlating with brain injury markers, were observed in children with poor outcomes and MIS-C. The association of elevated CCL11, HGF and IL-6:IL-10 ratio with poor outcome highlights potential therapeutic strategies that warrant further investigation. While serum NfL analysis holds promise as an alternative to CSF NfL for assessing neuronal injury in AES, its efficacy in distinguishing aetiologies, severity, and outcomes requires further exploration.

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Supplementary figures- Titles & Legends

Supplementary Figure 1: Laboratory algorithm used for serologic and molecular testing of samples from children with acute encephalitis syndrome, southern India.

*AES=Acute encephalitis syndrome

Supplementary Figure 2: 2D network analyses of cytokines/ chemokines in CSF & Serum of patients in the three groups

*CXC = GROα, M.C = M-CSF

Supplementary Figure 3: Subtraction heatmaps comparing the aetiologies

*White shows the correlations which are similar between the two groups, while red and blue show the many distinct relationships