Serum Levels of Markers of Endothelial Activation Are Not Associated with a Positive Blood Culture in Surinamese Children with Suspected Severe Infection

Juliana, Amadu; Jongman, Rianne; van Meurs, Matijs; Plotz, Frans B.; Zonneveld, Rens

Published in:
Journal of Tropical Pediatrics

DOI:
10.1093/tropej/fmaa091

Publication date:
2021

Journal of Tropical Pediatrics, 2020, 00, 1–7
doi: 10.1093/tropej/fmaa091
Original Paper

Serum Levels of Markers of Endothelial Activation Are Not Associated with a Positive Blood Culture in Surinamese Children with Suspected Severe Infection

Amadu Juliana MD,[1] Rianne Jongman, PhD[2,3] Matijs van Meurs, MD, PhD[3,4] Frans B. Plötz, MD, PhD[5,6] *and* Rens Zonneveld MD, PhD[1,2]

[1]Academic Pediatric Center Suriname, Academic Hospital Paramaribo, Paramaribo, Suriname
[2]Department of Pathology and Medical Biology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
[3]Anesthesiology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
[4]Department of Critical Care and, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
[5]Department of Pediatrics, Tergooi Hospitals, Blaricum, The Netherlands
[6]Department of Pediatrics, Amsterdam UMC, University of Amsterdam, Emma Children's Hospital, Amsterdam, The Netherlands
Correspondence: Amadu Juliana, MD, Academic Pediatric Center Suriname, Academic Hospital Paramaribo, Flustraat 1, Paramaribo, Suriname. E-mail: <amadujuliana@gmail.com>.

ABSTRACT

Background: Systemic serum levels of markers of endothelial activation are associated with infection. We hypothesize that levels of markers of endothelial activation are associated with the presence of a positive blood culture as a manifestation of a systemic infection in children with a suspected severe infection in Suriname.

Methods: In this prospective observational cohort study, children between 1 month and 18 years of age suspected of severe infection as assessed by the threating physician, and in whom laboratory testing and blood culturing was performed before start of intravenous antibiotic treatment, were recruited at the emergency department of the Academic Hospital Paramaribo, Suriname. Serum was collected at blood culturing and after 48–72 h of admission. Serum was stored for measurement of levels of Angiopoietin (Ang)-1, Ang-2, soluble (s)P-selectin, sE-selectin, vascular cell adhesion molecule-1, intercellular adhesion molecule-1 and platelet and endothelial cell adhesion molecule-1.

Results: Fifty-one children were included of whom 10 had a positive blood culture. Baseline characteristics were similar between children with and without a positive blood culture. No significant differences in serum levels of the Angiopoietins or soluble cellular adhesion molecules between groups were observed at start of antibiotic treatment nor after 48–72 h.

Conclusions: The data from this study indicate that in children with severe infection, serum levels of markers of endothelial cell activation are not associated with a positive blood culture. Thus, having a positive bacterial blood culture may not be the only factor driving endothelial activation in this patient population.

KEYWORDS: children, endothelium, severe infection, Suriname

INTRODUCTION

Angiopoietin-(Ang)-1 and Ang-2 play fundamental roles in the maintenance of vessel integrity in health and disease [1–5]. Binding of Ang-2 to the tyrosine kinase receptor 2 (Tie-2) receptor during infection antagonizes Tie-2 signaling and disrupts vascular endothelial barrier function. This is followed by expression of endothelial cell adhesion molecules (CAMs), in particular, P-selectin, E-selectin, vascular CAM-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), to facilitate leukocyte recruitment [1, 6]. CAMs then orchestrate leukocyte rolling on, adhesion to, and diapedesis across the vascular endothelium [7]. Soluble isoforms of CAMs (sCAMs) become measurable in the systemic circulation after ectodomain shedding by shedding enzymes, such as matrix metalloproteinase-9 [7, 8]. These mechanisms are keys in vessel homeostasis in local and systemic infection. However, systemic infection can lead to endothelial dysfunction, which is associated with edema, hypotension and multi-organ failure [2–4, 9].

From clinical studies, it has become clear that lower Ang-1 and higher Ang-2 and higher sCAM levels are associated with presence of systemic infection and sepsis [4–7, 9–12]. However, the role of the Angiopoietins and sCAMs in non-systemic severe infections, like pneumonia or meningitis, is less clear. For example, data on meningitis and pneumonia in children and adults show that their levels could predict presence of these infections and worse outcome [13–17]. However, data on whether these markers could differentiate non-systemic from systemic infection is limited, especially from non-Western countries.

The purpose of this study was to investigate endothelial activation in children suspected of severe infection. We hypothesized that lower Ang-1, higher Ang-2 and higher sCAM levels at presentation, and sustained after 48–72 h of admission, predicted a positive blood culture as a manifestation of a systemic infection. Ultimately, this may help in initiating prompt treatment of appropriate broad-spectrum antibiotics and supportive therapy.

MATERIALS AND METHODS

Study design and subjects

A prospective observational cohort study was performed at the Academic Pediatric Center Suriname at the Academic Hospital Paramaribo in Paramaribo, Suriname. In a 14-month period between 1 April 2015 and 31 May 2016, pediatric patients with an age between 1 month and 18 years old with suspected severe infection at the emergency department were recruited. Suspicion of severe infection was based upon the assessment of the treating physician, and included at least one of the following symptoms: temperature $<36^{\circ}C$ and $>38^{\circ}C$, tachypnea, chest retractions, grunting, apnea, hypoxia ($SpO_2 < 90\%$), tachycardia, capillary refill time > 2 s, vomiting, severe diarrhea, altered state of consciousness and seizures. For all patients, the standard local protocol for the management of suspected severe infection was followed. This included the start of broad-spectrum intravenous antibiotics after blood collection for culture and laboratory testing. After the inclusion period, patients were excluded if informed consent was not obtained, if blood culture results were absent, if infection was not the primary diagnosis, if not sufficient information was available after the study period to determine the primary diagnosis, and if there was a confirmed HIV infection. The patients included in the final database were divided into two groups based on blood culture results, namely a negative blood culture group and positive blood culture group.

The study protocol was approved by the Surinamese Medical-Ethical Board (VG-021-14A) and was made available on clinicaltrials.gov (NCT02486783). Written informed consent was obtained from at least one parent for the use of residual serum and clinical information.

Data collection

For all patients the following demographic and clinical variables were recorded after the inclusion period: age, weight, gender, oxygen supplementation, length of stay (LOS), need for intensive care admission, clinical discharge diagnosis and mortality. Clinical discharge diagnosis was based upon the treating physicians final discharge diagnosis in the patient chart and/or discharge letter. Levels of C-reactive protein (CRP), white blood cell (WBC) count, neutrophil count and blood culture result were recorded from the paper charts or the laboratory identification system.

Sample collection, preparation and analysis

Blood samples were collected in serum microtainers using standard blood collection during the insertion of a venous cannula. This time point was labeled $t = 0$. If needed and only upon clinical indication, a second blood sample was obtained after 48–72 h using capillary collection. Blood was allowed to clot at room temperature and serum was separated by centrifugation at $2300 \times g$ for 8 min, the serum was harvested and used for routine determination of CRP at the clinical laboratory and residual sample was stored at -80°C until further analysis. Frozen samples were transported on dry ice from Suriname to the Netherlands. For analysis, the samples were thawed on ice and immediately analyzed. Levels of Ang-1 and Ang-2 were measured using enzyme-linked immunosorbent assay (ELISA; DANG10 and DANG20 Quantikine ELISAs, Minneapolis, MN, USA, R&D Systems), according to the manufacturers' instructions. Levels of sP-selectin, sE-selectin, sVCAM-1 and sICAM-1, were measured using Luminex (Human Adhesion 6-plex, Waltham, MA, USA, Thermo-Fischer Scientific), also according to the manufacturers' instructions. A standard curve of each molecule was used to determine if levels were in the linear part of the curve. For the Angiopoietins and sCAMs, levels under the lowest (Ang = 1 $n = 3$ and Ang-2 $n = 4$) and above the highest (E-selectin $n = 1$ and VCAM-1 $n = 4$) quantitative cutoff we reported as the lowest and the highest standard curve values, respectively. Due to limited amounts of serum available, not all markers could be measured in all samples. Numbers of samples analyzed per marker and time point are given in Table 2.

Statistical analysis

The outcome variable was a positive bacterial blood culture, and the predictor variables were Ang-1, Ang-2 and sCAMs, at $t = 0$ and $t = 48$–72 h. Categorical variables were presented as numbers and percentages and continuous variables were presented as median and interquartile range (IQR). Chi-square or Fisher exact test was used for nominal or categorical data. Due to the non-parametric nature of the data, a Mann–Whitney test with was used for analysis of continuous variables. p-values <0.05 were considered statistically significant. Multiple regression analysis was performed for LOS as a dependent variable. Analyses were performed using JASP version 0.13.1 (University of Amsterdam, the Netherlands) and Prism version 8.4.2 (Graphpad Software Inc., San Diego, CA, USA).

RESULTS

Demographics

A total of 127 patients were deemed eligible at the emergency department, with informed consent obtained for 80 patients (Fig. 1). Twelve patients were excluded because of lack of serum. Further exclusion was due to absence of infection as the clinical discharge diagnosis ($n = 7$), unclear clinical discharge diagnosis ($n = 4$) and absence of blood culture results ($n = 6$). Four patients had a blood culture with a coagulase negative staphylococcus, which were considered contamination and therefore regarded as negative. A total number of 51 patients were included in the final database, of whom 41 with a negative, and 10 with a positive blood culture.

Demographic and clinical characteristics are shown in Table 1. Overall, the most common clinical discharge diagnoses were pneumonia (41.2%), gastroenteritis (15.7%), fever/infection of uncertain cause (13.7%) and bronchiolitis (7.8%). There were no significant differences between groups except for

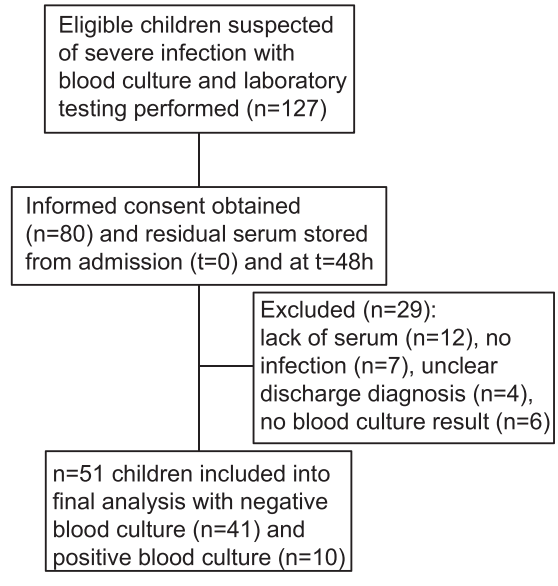

Fig. 1. Flowchart of the study.

Table 1. Demographic and clinical characteristics of the study cohort ($n = 51$).

		Negative blood culture ($n = 41$)	Positive blood culture ($n = 10$)
Age, median (IQR) (months)		12 (24)	15 (60.3)
Female, *n* (%)		19 (43.9)	4 (40)
Supplemental oxygen, *n* (%)		17 (45.9)[a]	1 (11)[b]
ICU admission, *n* (%)		3 (7.9)[c]	0[d]
LOS in days, median (IQR)[c]		3 (3)	7 (4.5)
Mortality, *n* (%)		1 (2.4)	0
Clinical discharge diagnoses, *n* (%)	Pneumonia	17 (41.5)	4 (40)
	Gastroenteritis	7 (17.1)	1 (10)
	Bronchiolitis	4 (9.8)	0
	Urinary tract infection	1 (2.4)	3 (30)
	Endocarditis	1 (2.4)	1 (10)
	Pericarditis	1 (2.4)	0
	Meningitis	2 (4.9)	0
	Subcutaneous infection	1 (2.4)	1 (10)
	Fever of uncertain cause	7 (17.1)	0

[a]Data missing in 4 cases;
[b]Data missing in one case;
[c]Data missing in 3 cases;
[d]Data missing in 1 case.

longer LOS in the positive blood culture group ($p = 0.012$) and for presence urinary tract infection, which was significantly more common in de blood culture positive group ($p = 0.021$).

Serum levels of the Angiopoietins and soluble endothelial CAMs

Serum levels of all markers at $t = 0$ and 48–72 h are given in Table 2. Figure 1 shows the distribution of levels of all markers at $t = 0$ and 48–72 h. CRP levels were significantly higher in children with a positive blood culture. At $t = 0$ serum levels of Ang-1, Ang-2 and sCAMs sP-selectin, sE-selectin, sICAM-1, sVCAM-1 and soluble platelet endothelial CAM-1 (sPECAM-1) were not significantly different between children with or without a positive blood culture. At $t = 48$–72 h serum levels of all markers were sustained and not different between groups (Table 2 and Fig. 2). Also, the Ang-2/Ang-1 ratios at $t = 0$ and 48–72 were not significantly different between groups. Since LOS was higher in patients with a positive blood culture LOS was introduced into linear regression analysis as dependent variable. Independent variables gender, age, CRP, WBC, Ang-1, Ang-2, Ang2/Ang-1 ratio and sCAMs ($t = 0$) were introduced into the model with the least significant contributing predictors removed sequentially. No significant predictors of LOS were found.

DISCUSSION

We studied 51 pediatric patients admitted and treated for severe infection in a middle-income country. We observed no differences in serum levels of markers of endothelial activation between patients with and without a positive blood culture. If the levels of these markers reflect the degree of endothelial activation, these findings indicate that having a positive blood culture may not be the only driving factor of this degree. We suggest several reasons for this.

First, the serum levels of the Angiopoietins are associated with the severity and outcome (e.g. organ failure score, shock and mortality) of sepsis in neonates, children and adults. Especially, higher levels of Ang-2 and An2/Ang-1 ratio were associated with increased severity, defined as shock, organ failure score and mortality [5, 7, 9, 11, 18]. In our study,

Table 2. Levels of markers of infection and endothelial cell activation.

Laboratory data	Time point	*N*	Negative blood culture ($n = 41$)	*n*	Positive blood culture ($n = 10$)	*p*-value
WBC count (*10^9/l)	$t = 0$	35	18.2 (13.5)	10	20 (11.5)	0.989
	$t = 48–72$	10	12.5 (4.5)	3	15.8 (9.8)	0.692
Neutrophil count (*10^9/l)	$t = 0$	23	8.2 (9.4)	8	14.7 (10.3)	0.206
	$t = 48–72$	5	6.7 (4.8)	2	8.4 (6)	0.857
CRP mg/dl	$t = 0$	34	6.9 (14.8)	10	20.5 (12.6)	0.013
	$t = 48–72$	8	5.4 (16.4)	2	13.1 (0.8)	0.711
Ang-1 ng/ml	$t = 0$	38	53.5 (38.2)	8	60.8 (28)	0.889
	$t = 48–72$	16	36.8 (43.5)	4	30.5. (41)	0.92
Ang-2 ng/ml	$t = 0$	41	5 (2)	10	5 (5)	0.687
	$t = 48–72$	16	4.9 (3.9)	4	4.9 (4.5)	1.000
Ang-2/Ang-1 ratio	$t = 0$	38	0.10 (0.07)	10	0.11 (0.17)	0.910
	$t = 48–72$	16	0.11 (0.16)	4	0.16 (13.79)	1.000
P-Selectin ng/ml	$t = 0$	39	105.2 (84.1)	8	114.2 (73.5)	0.835
	$t = 48–72$	16	79.8 (40.9)	3	48.4 (46.2)	0.359
E-Selectin ng/ml	$t = 0$	39	272 (327.2)	8	316.9 (334.1)	0.749
	$t = 48–72$	16	273.3 (245.2)	3	95.3 (175)	0.254
ICAM-1 ng/ml	$t = 0$	39	128.8 (70.5)	8	126.1 (143.8)	0.966
	$t = 48–72$	16	105.4 (73.5)	2	129.4 (41.3)	1.000
VCAM-1 ng/ml	$t = 0$	39	611.1 (279.3)	8	531.4 (337)	0.771
	$t = 48–72$	16	514.7 (176.6)	3	1049.2 (1819.1)	0.303
PECAM-1 ng/ml	$t = 0$	39	23.9 (5.6)	8	24.5 (5.7)	0.810
	$t = 48–72$	16	24.4 (10.3)	2	25.2 (2.2)	1.000

patients in both the negative and positive blood culture groups had equal disease severity except for longer LOS. Thus, this equal disease severity may be expressed in equal serum levels of markers of endothelial activation. Although severe bacterial sepsis is contingent on presence of a positive blood culture, it is the immunological response that drives severity and clinical outcome of sepsis. This is reflected in a study by Giuliano *et al.* [10], in which only the septic shock outcome group had significantly higher levels of Ang-2 and higher Ang-2/lAng-1 ratios compared with healthy subjects, children with systemic inflammatory reaction syndrome, and sepsis in a pediatric intensive care unit (PICU) setting. Our study was not powered for analysis on severe clinical endpoints such as death and inotropic support. Interestingly, CRP levels were higher in the blood culture positive group at $t = 0$, which is not true for the levels of the Angiopoietins and sCAMs. This is indicative of more pronounced (intravascular) immunological responses in children with systemic infection, which is not paralleled by a higher degree of endothelial activation.

Although the study lacked a control group, the overall Ang-1, Ang-2 and sCAM serum levels in our study were elevated compared with healthy controls of other studies, indicating endothelial activation [10, 19]. Several studies have shown that markers of endothelial activation are associated with severe disease caused by non-bacterial pathogens such as dengue virus and malaria in low- and middle-income countries [14, 20–23]. With dengue being endemic in our region we suspect that some of the patients categorized as having fever of uncertain cause might have had a dengue viremia. We also suspect that endothelial activation might play an important role in severity of viral bronchiolitis [24]. In our study, patients with bronchiolitis were represented only in

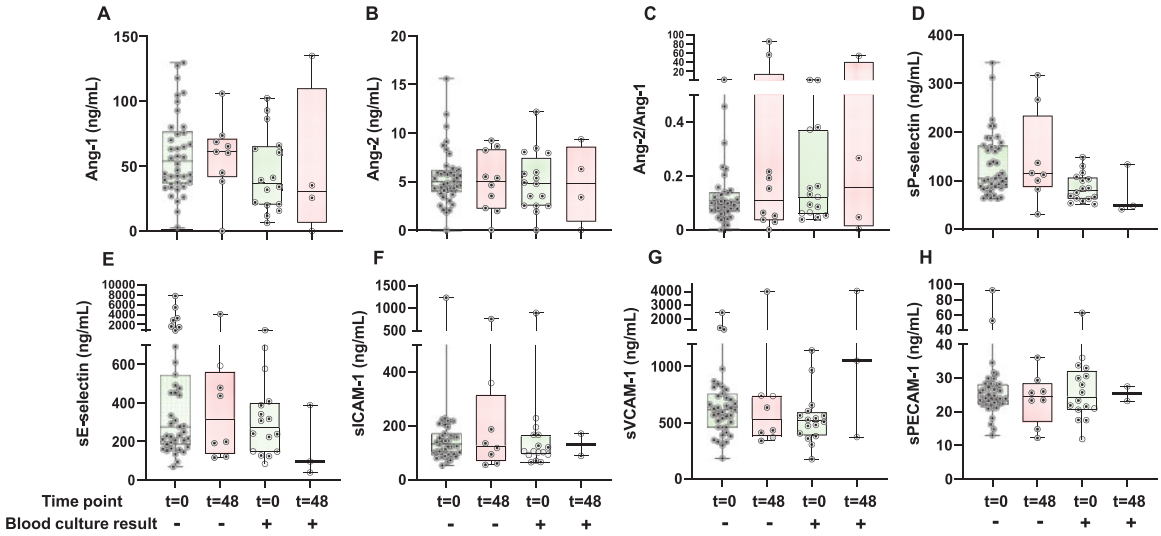

Fig. 2. Levels of markers of endothelial cell activation grouped by blood culture result sampled at admission ($t = 0$) and after 48–72 h ($t = 48$). Box and whisker plots showing IQR, median and total data range. Statistical analysis was done with Mann–Whitney U-test, with no statistical significant differences found between groups. (**A**) Ang-1. (**B**) Ang-2. (**C**) Ang-1 and Ang-2 ratios. (**D**) soluble P-Selectin. (**E**) Soluble E-Selectin. (**F**) sICAM-1. (**G**) sVCAM-1. (**H**) PECAM-1.

the blood culture negative group. Furthermore, endothelial activation and markers thereof are associated with severity in lower respiratory infections and ARDS irrespective of the cause [25–28]. Thus, markers of endothelial activation seem to be associated with clinical outcome of infections irrespective of the nature of the causative pathogen.

The first strength of this article is that it seeks to answer the question of whether markers of endothelial activation are associated with a positive bacterial blood culture in children admitted to hospital in a developing country like Suriname. Second, the markers were measured at two time points during admission. The main limitations are the relatively small number of patients, lack of a control group, the limited number of samples at $t = 48$–72 h, and the heterogeneity of the patients. Low presence of severe outcomes (i.e. ICU admission, inotropic support or death), made it impossible to explore whether Angiopoietins and sCAMs are associated with such clinical outcome variables.

In summary, serum values of the Angiopoietins and sCAMs were not different with a positive blood culture from those without in suspected severe infection. Diversity of causes and patients complicate interpretation of these levels and inhibit their use to predict presence of a positive blood culture and as prognostic biomarkers in a pediatric emergency department population. Future studies should focus on specific disease entities.

ACKNOWLEDGMENTS

The authors acknowledge the efforts of all employees of the Clinical Laboratory of the Academic Hospital Paramaribo and the Central Laboratory of Suriname, Paramaribo, Suriname, for assistance with sample storage, handling and transport.

FUNDING

This work was supported by a grant from the Thrasher Research Fund [TRF13064] and Tergooi Hospitals, Blaricum, The Netherlands received by Rens Zonneveld.

REFERENCES

1. van Meurs M, Kümpers P, Ligtenberg JJM, *et al.* Bench-to-bedside review: angiopoietin signalling in critical illness - a future target? Crit Care 2009;13:207.
2. Wang K, Bhandari V, Giuliano JS, *et al.* Angiopoietin-1, angiopoietin-2 and bicarbonate as diagnostic biomarkers in children with severe sepsis. PLoS One 2014;9:e108461.
3. Lymperopoulou K, Velissaris D, Kotsaki A, *et al.* Angiopoietin-2 associations with the underlying infection and sepsis severity. Cytokine 2015;73:163–8.

4. Ricciuto DR, dos Santos CC, Hawkes M, *et al.* Angiopoietin-1 and angiopoietin-2 as clinically informative prognostic biomarkers of morbidity and mortality in severe sepsis. Crit Care Med 2011;39:702–10.
5. Fang Y, Li C, Shao R, *et al.* Prognostic significance of the angiopoietin-2/angiopoietin-1 and angiopoietin-1/Tie-2 ratios for early sepsis in an emergency department. Crit Care 2015;19:367.
6. Parikh SM. Dysregulation of the angiopoietin-Tie-2 axis in sepsis and ARDS. Virulence 2013;4:517–24.
7. Zonneveld R, Martinelli R, Shapiro NI, *et al.* Soluble adhesion molecules as markers for sepsis and the potential pathophysiological discrepancy in neonates, children and adults. Crit Care 2014;18:204.
8. Garton KJ, Gough PJ, Raines EW. Emerging roles for ectodomain shedding in the regulation of inflammatory responses. J Leukoc Biol 2006;79:1105–16.
9. Giuliano JS, Tran K, Li F-Y, *et al.* The temporal kinetics of circulating angiopoietin levels in children with sepsis. Pediatr Crit Care Med 2014;15:e1–8.
10. Giuliano JS, Lahni PM, Harmon K, *et al.* Admission angiopoietin levels in children with septic shock. Shock 2007; 28:650–4.
11. Wright JK, Hayford K, Tran V, *et al.* Biomarkers of endothelial dysfunction predict sepsis mortality in young infants: a matched case-control study. BMC Pediatr 2018; 18:118.
12. Achten NB, van Meurs M, Jongman RM, *et al.* Markers of endothelial cell activation in suspected late onset neonatal sepsis in Surinamese newborns: a pilot study. Transl Pediatr 2019;8:412–8.
13. Mankhambo LA, Banda DL, Jeffers G, *et al.*; The IPD Study Group. The role of angiogenic factors in predicting clinical outcome in severe bacterial infection in Malawian children. Crit Care 2010;14:R91.
14. Lovegrove FE, Tangpukdee N, Opoka RO, *et al.* Serum angiopoietin-1 and -2 levels discriminate cerebral malaria from uncomplicated malaria and predict clinical outcome in African children. PLoS One 2009;4:e4912.
15. Glynn P, Coakley R, Kilgallen I, *et al.* Neutrophil CD11b and soluble ICAM-1 and E-selectin in community acquired pneumonia. Eur Respir J 1999;13:1380–5.
16. Florin TA, Ambroggio L, Brokamp C, *et al.* Biomarkers and disease severity in children with community-acquired pneumonia. Pediatrics 2020;145:e20193728.
17. Gutbier B, Neuhauß A-K, Reppe K, *et al.* Prognostic and pathogenic role of angiopoietin-1 and -2 in pneumonia. Am J Respir Crit Care Med 2018;198:220–31.
18. Mikacenic C, Hahn WO, Price BL, *et al.* Biomarkers of endothelial activation are associated with poor outcome in critical illness. PLoS One 2015;10:e0141251.
19. Jain V, Lucchi NW, Wilson NO, *et al.* Plasma levels of angiopoietin-1 and -2 predict cerebral malaria outcome in Central India. Malar J 2011;10:383.
20. Conroy AL, Phiri H, Hawkes M, *et al.* Endothelium-based biomarkers are associated with cerebral malaria in Malawian children: a retrospective case-control study. PLoS One 2010;5:e15291.
21. de Jong GM, Slager JJ, Verbon A, *et al.* Systematic review of the role of angiopoietin-1 and angiopoietin-2 in Plasmodium species infections: biomarkers or therapeutic targets? Malar J 2016;15:581.
22. van de Weg CAM, Pannuti CS, van den Ham H-J, *et al.* Serum angiopoietin-2 and soluble VEGF receptor 2 are surrogate markers for plasma leakage in patients with acute dengue virus infection. J Clin Virol 2014;60:328–35.
23. Yacoub S, Lam PK, Vu LHM, *et al.* Association of microvascular function and endothelial biomarkers with clinical outcome in dengue: an observational study. J Infect Dis 2016;214:697–706.
24. Juliana A, Zonneveld R, Plötz FB, *et al.* Neutrophil-endothelial interactions in respiratory syncytial virus bronchiolitis: an understudied aspect with a potential for prediction of severity of disease. J Clin Virol 2020;123: 104258.
25. Kumar NP, Velayutham B, Nair D, *et al.* Angiopoietins as biomarkers of disease severity and bacterial burden in pulmonary tuberculosis. Int J Tuberc Lung Dis 2017;21: 93–9.
26. Armstrong SM, Darwish I, Lee WL. Endothelial activation and dysfunction in the pathogenesis of influenza A virus infection. Virulence 2013;4:537–42.
27. Millar FR, Summers C, Griffiths MJ, *et al.* The pulmonary endothelium in acute respiratory distress syndrome: insights and therapeutic opportunities. Thorax 2016;71: 462–73.
28. Zinter MS, Spicer A, Orwoll BO, *et al.* Plasma angiopoietin-2 outperforms other markers of endothelial injury in prognosticating pediatric ARDS mortality. Am J Physiol Lung Cell Mol Physiol 2016;310:L224–231.